



## Antilithiatic Activity of *Trianthema portulacastrum* L. and *Gymnema sylvestre* R.Br against Ethylene Glycol induced Urolithiasis

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### ABSTRACT

Urolithiasis is the third most common disorder, which results from combined influence of dietary, geographical, biochemical & genetic risk factors. Hyperoxaluria was induced by administration of Ethylene glycol (EG) 0.75% and Ammonium chloride (AC) 1% in drinking water for 28 days. EG feeding results in hyperoxaluria induced oxidative stress as well as increased excretion of calcium, oxalate, and phosphate in serum levels. The present study was focused on evaluation of ethanolic extract of leaves of *Trianthema portulacastrum* Linn. (EETP) and *Gymnema sylvestre* R.Br (EEGS) on experimentally induced urolithiasis. Cystone, a polyherbal formulation is used as a reference standard. Parameters like urinary volume, urine analysis (calcium, oxalate, phosphate, magnesium, and phosphate), serum analysis (calcium, creatinine, uric acid, BUN) and antioxidant studies (SOD, CAT, MDA) were performed to access the activity. Treatment with ethanolic extract of *Trianthema portulacastrum* and *Gymnema sylvestre* at both the doses (200 mg/kg and 400 mg/kg b.wt) showed a significant restoration of urinary and serum parameters on EG&AC induction. The extracts at both doses (200 mg/kg and 400 mg/kg b.wt) showed significant increase in antioxidant enzymes activity & decrease in MDA levels. From all the protective findings of both extracts EEGS showed more potent antilithiatic activity when compared to EETP.

**Keywords:** Urolithiasis, Ethylene glycol, Ammonium chloride, Cystone, *Trianthema portulacastrum* Linn., *Gymnema sylvestre* R.Br.

### INTRODUCTION

Urolithiasis is the most common disorder estimated to occur in approximately 12% of the population.<sup>1</sup> Epidemiological studies revealed that urolithiasis is more common in men (12%) than in women (6%) and is more prevalent between the ages of 20-40 in both sex.<sup>2</sup> Some common causes are inadequate urinary drainage, foreign bodies in the urinary tract, microbial infections, diet with excess oxalates and calcium, vitamin abnormalities, viz. Vitamin A deficiencies, Vitamin D excess, metabolic diseases like hyperparathyroidism, cystinuria, gout and intestinal dysfunction.<sup>3</sup> Calcium oxalate is the predominant mineral in a majority of kidney stones. Urolithiasis is a complex process that results from several physicochemical events including crystal nucleation, aggregation and growth of insoluble particles in the kidney.<sup>4</sup> The management of urolithiasis involves surgical treatment and medical therapy. Extracorporeal shock-wave lithotripsy, percutaneous nephrolithotomy, ureteroscopy and nephrolithotomy are the surgical procedures used to eliminate the kidney stones. Among these treatments, ESWL become the standard procedure in eliminating kidney stones. However, ESWL may also cause acute renal injury, a decrease in renal function and also an increase in stone recurrence.<sup>5</sup> A numbers of non-steroidal, anti-inflammatory drugs are prescribed for the treatment of kidney stones, but these allopathic drugs for the prevention of stone formation have not been very effective and their adverse effects have put certain limits on their use. Therefore; it is worthwhile to look for an alternative to those conventional methods by using medicinal plants or phytotherapy. A large number of

Indian medicinal plants have been used in the treatment of urolithiasis and they have been reported to be effective with fewer side effects.<sup>6</sup> Diuretics have been introduced and medically been used as prophylactic agents for the treatment of urolithiasis due to their key role in regulating kidney function and alleviating the urinary risk factors for stone formation.<sup>7</sup>

*Trianthema portulacastrum* Linn commonly known as galijeru, desert Horse Purslane, belonging to Aizoaceae family. It is the most common weed in the major field crops such as mustard, maize, pigeon pea, mung bean, potato, onion, cotton, soybean, and sugarcane. The plant is bitter, analgesic, stomachic and laxative, Cures bronchitis, diseases of blood and heart and used in inflammation and rheumatism.<sup>8</sup> The leaves possess diuretic property and applied in the treatment of jaundice, strangury and dropsy.<sup>9,10</sup> The juice of the leaves dropped into the nostrils relieves migraine. The root is applied to the eye cures, corneal ulcers, itching, dimness of sight and night blindness (Ayurveda) mild purgative cathartic and abortion.<sup>9</sup> Various parts of the plant was reported as antioxidant,<sup>11</sup> analgesic,<sup>12</sup> hepato protective,<sup>13</sup> nephroprotective,<sup>14</sup> anti fertility,<sup>15</sup> anti hyperglycemic and hypolipidemic<sup>16</sup> and anti-cancer activity.<sup>17</sup>

*Gymnema sylvestre* R.Br is a climbing plant grows in open woods and bush lands, and is commonly known as madhunashini, belonging to Asclepiadaceae family. The plant is useful in inflammations, hepatosplenomegaly, dyspepsia, constipation, hemorrhoid, cough, asthma, bronchitis, cardiopathy, jaundice, intermittent fever, piles, amenorrhea, conjunctivitis, leucoderma, and



urinary disorders. Root is used to cure snake bite. According to ayurvedic literature leaves were used as cardio tonic, diuretic, laxative, stimulant, stomachic and uterine tonic and diuretic, anti-allergic, hypoglycemic, hypolipidemic, for the treatment of obesity and dental caries.<sup>18</sup> It is a potent anti-diabetic plant used in folk, ayurvedic and homeopathic systems of medicine.<sup>19</sup> Various parts of the plant was reported as anti diabetic,<sup>20</sup> anti oxidant,<sup>21</sup> anti obesity,<sup>22</sup> anti hyperlipidemic,<sup>23</sup> anticancer,<sup>24</sup> anti inflammatory<sup>25</sup> and anti biotic activity.<sup>26</sup>

The present study was undertaken to evaluate the ethanolic extract of leaves of *Trianthema portulacastrum* and *Gymnema sylvestre* on experimentally induced urolithiasis.

## MATERIALS AND METHODS

### Collection and Authentication of Plant

The leaves of the plants i.e *Trianthema portulacastrum* Linn and *Gymnema sylvestre* R.Br were collected from the surrounding areas of Tirupati and the plants were authenticated by Dr. K. Madhava Chetty, Assistant Professor, Department of Botany, S.V University, Tirupathi, Andhra Pradesh, India.

### Preparation of Extract

The leaves of the plants were separated, washed and dried under shade. The dried leaves were ground to powder. About 100 g of coarse powder of both plants was subjected to solvent extraction using 70% ethanol in the ratio of 1:4(drug: solvent) using soxhlet apparatus at a temperature of 50-60°C for 72 hours. The extracts thus obtained were dried under reduced pressure and temperature not exceeding 40°C to obtain a semi solid extract. The dried extract was then subjected to phytochemical screening.<sup>27</sup>

### Preliminary Phytochemical Screening

The extract was used for qualitative determination of phytoconstituents like alkaloids, glycosides, flavonoids, saponins, tannins, amino acids, carbohydrates, steroids and phenols.<sup>28, 29</sup>

### Experimental Animals

Male wistar rats weighing 100-150 gms were used for the study, the animals were purchased from Ragavendra enterprises, Bangalore, and acclimatized to a standard laboratory conditions (temperature 25±2°C and 12 h light/12 h light/dark cycles. All the animals were fed with standard pellets and allowed free access to water. The experimental protocol was approved by Institutional animal ethical committee (Registered No.1521/PO/a/11/CPCSEA)

### Acute Toxicity Studies

Acute toxicity studies for ethanolic extract of *Trianthema portulacastrum* L. (EETP) and *Gymnema sylvestre* R.Br (EEGS) were conducted as per OECD guidelines 423 using wistar rats. The pre defined doses of 2000mg/kg and

4000 mg/kg b.wt were administered by oral route and observed for gross behavioral changes and mortality up to 14 days. The number of deaths was recorded to calculate LD<sub>50</sub>.<sup>30</sup>

## Assessment of Antiurolithiatic Activity

### Experimental Design

- Group I** : Vehicle control received distilled water *p.o* for 28 days
- Group II** : Animals received Ethylene glycol 0.75% and ammonium chloride 1% In drinking water for 28 days
- Group III** : Animals received cystone at dose 750 mg/kg/b.wt + Ethylene glycol 0.75% and 1%Ammonium chloride
- Group IV** : Animals received EETP at dose level of 200 mg/kg/b.wt + Ethylene glycol 0.75% and 1%Ammonium chloride for 28 days
- Group V** : Animals received EETP at dose level of 400 mg/kg/b.wt+ Ethylene glycol 0.75% and 1% Ammonium chloride for 28 days
- Group VI** : Animals received EEGS at dose level of 200 mg/kg/b.wt+ Ethylene glycol 0.75% and 1% Ammonium chloride for 28 days
- Group VII** : Animals received EEGS at dose level of 400 mg/kg/b.wt+ Ethylene glycol 0.75% and 1% Ammonium chloride for 28 days

Antilithiatic activity of the both extracts was studied by determining urinary volume, urinary parameters and serum parameters.

### Urine Analysis

Urine samples of 24 hr were collected on 28<sup>th</sup> day by keeping the animals in metabolic cages. Animals had free access to drinking water during urine collection. The volume of urine from each group of animals was measured. A drop of concentrated Hydrochloric acid was added to the collected urine before stored at 4 C. Urine was analyzed for calcium, oxalate, phosphate, and magnesium using standard procedures.<sup>31</sup>

### Serum Analysis

After the experimental period, blood was collected from retro orbital puncture under anesthesia and serum was separated by centrifugation at 1000 rpm for 15 min and analyzed for calcium, creatinine, uric acid, BUN.<sup>31</sup>

### Kidney Homogenate Analysis

Rats were sacrificed by cervical decapitation and kidneys were separated from each rat. Isolated kidneys were cleaned off extraneous tissue and preserved in 10% neutral formalin. The kidneys are dried at 80 C in hot air oven. A sample of 100 mg of the dried kidney was boiled in 10 ml of 1 N hydrochloric acid for 30 min and homogenized. The homogenate was centrifuged at 2000 rpm for 10 min and supernatant liquid was collected and estimated for anti-oxidant parameters.<sup>32</sup>



**RESULTS**

**Preliminary Phytochemical Screening**

Phytochemical screening of EETP revealed the presence of alkaloids, flavonoids, phenolic compound, tannins and terpenoids, steroids and the EEGS showed the presence of alkaloids, carbohydrates, phenols, flavonoids, glycosides, tannins, and saponins.

**Acute Toxicity Studies**

The procedure was followed according to OECD 423 guidelines in wistar rats. There were no signs of toxicity

observed up to a dose level of 2000 and 4000 mg/kg b.wt. The mortality rate was found nil and the herbal extracts of EETP & EEGS were found safe up to a dose level of 4000 mg/kg body weight.

**Effect of EETP and EEGS on Urinary Parameters**

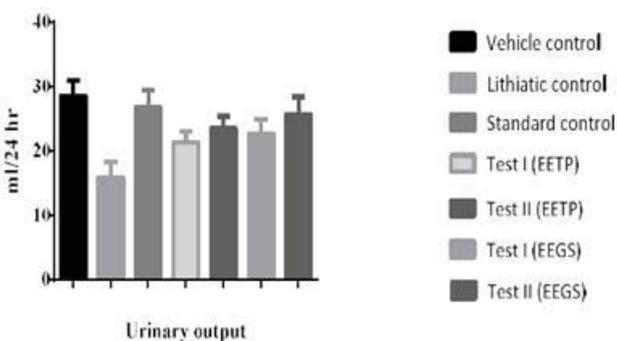
Table 1 showed a significant decrease (P < 0.001) in urinary output and magnesium levels with an increase in calcium, oxalate, and phosphate levels in EG & AC treated animals. Treatment with EETP and EEGS at both dose levels (200 and 400 mg/kg b.wt) altered the urinary parameters compared to lithiatic control group.

**Table 1: Effect Of EETP and EEGS on Urinary Parameters in EG & AC Induced Urolithiasis**

Groups	Urinary output	Calcium	Oxalate	Phosphate	Magnesium
Vehicle	28.50±2.429	1.555±0.434	2.747±0.660	3.628±0.551	1.320±0.026
Lithiatic (EG/AC)	15.83±2.483	4.383±0.491	5.049±0.607	7.542±0.514	0.571±0.023
Standard (Cystone) 750 mg/kg	26.83±2.631***	1.732±0.253***	1.925±0.544***	3.712±0.550***	1.297±0.028***
EETP (200 mg/kg)	21.33±1.633**	3.590±0.502**	3.750±0.441***	5.685±0.688***	0.805±0.018***
EETP (400 mg/kg)	23.50±1.871***	2.102±0.402***	3.050±0.187***	4.663±0.562***	1.043±0.032***
EEGS (200 mg/kg)	22.67±2.251***	2.775±0.324***	3.583±0.231***	4.790±0.487***	0.973±0.027***
EEGS (400 mg/kg)	25.67±2.733***	1.828±0.247***	2.650±0.389***	3.810±0.446***	1.162±0.028***

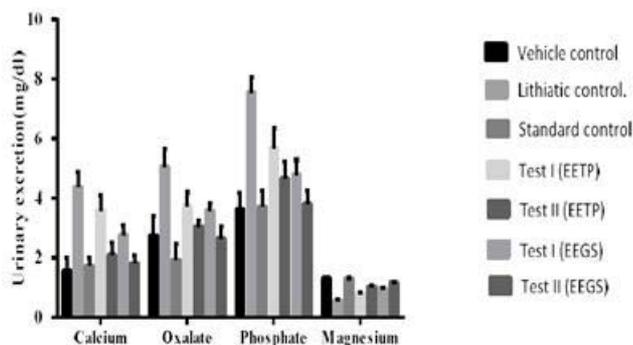
Data represents the Mean ± SD values (n=6). Statistical significance: \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 with respect to lithiatic control on 28<sup>th</sup> day (One way Anova followed by Dunnetts: Compare all columns vs. Lithiatic control).

**Figure 1: Effect of EETP and EEGS on Urinary output in EG&AC induced urolithiasis**



Values are expressed as Mean ± SD Values (N=6). Statistical Significance is done by One Way Anova followed by Dunnetts: Compare All Columns vs. Lithiatic Control

**Figure 2: Effect of EETP and EEGS on Urinary parameters in EG&AC induced urolithiasis**

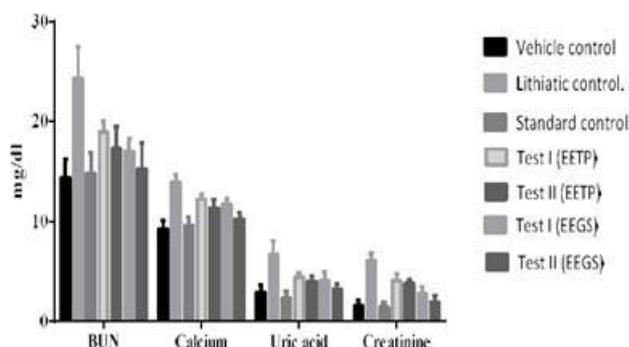


Values are expressed as Mean ± SD Values (N=6). Statistical Significance is done by One Way ANOVA followed by Dunnetts: Compare All Columns vs. Lithiatic Control

**Effect of EETP and EEGS on Serum Parameters**

Table 2 shows a significant increase (P < 0.001) in serum parameters i.e. Calcium, creatinine, uric acid, BUN levels in EG & AC treated animals. Treatment with EETP & EEGS at both the doses (200 & 400 mg/kg b.wt) restored the serum parameters when compared to lithiatic control group.

**Figure 3: Effect of EETP and EEGS on serum parameters in EG&AC induced urolithiasis**



Values are expressed as Mean ± SD Values (N=6). Statistical Significance is done by One Way Anova followed by Dunnetts: Compare All Columns vs. Lithiatic Control.

**Effect of EETP and EEGS on Tissue parameters**

Table no 3 showed significant decrease (P < 0.001) in antioxidant enzymes activity (SOD and CAT) and increased MDA levels in lithiatic control. The pretreatment with EETP and EEGS showed induced activities of SOD and CAT with a prominent decrease in MDA.



**Table 2:** Effect of EETP and EEGS on Serum parameters in EG&AC induced urolithiasis

Groups	Calcium	Creatinine	Uric acid	BUN
Vehicle	9.240±0.832	1.530±0.545	2.878±0.748	14.34±1.827
Lithiatic (EG/AC)	13.96±0.687	6.070±0.687	6.665±1.394	24.26±3.215
Standard (Cystone) 750 mg/kg	9.565±0.887***	1.400±0.461***	2.328±0.627***	14.82±2.038***
EETP (200 mg/kg)	12.20±0.495**	4.007±0.695***	4.405±0.432***	18.94±1.063***
EETP (400 mg/kg)	11.32± 0.868***	3.805±0.404***	3.925±0.593***	17.27±2.234***
EEGS (200 mg/kg)	11.66±0.567***	2.757±0.688***	4.067±0.848***	17.01±1.244***
EEGS (400 mg/kg)	10.18± 0.706***	1.938±0.634***	3.215±0.486***	15.26±2.591***

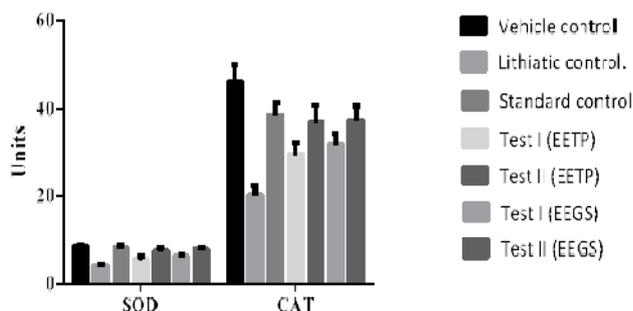
Data represents the Mean ± SD values (n=6). Statistical significance: \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 with respect to lithiatic control on 28<sup>th</sup> day (One way Anova followed by Dunnetts: Compare all columns vs. Lithiatic control).

**Table 3:** Effect Of EETP and EEGS on Tissue Parameters in EG&AC Induced Urolithiasis

Groups	SOD	CAT	MDA
Vehicle	8.116±0.185	46.03±3.821	167.5±3.090
Lithiatic (EG/AC)	4.039±0.337	20.15±2.008	321.0±9.824
Standard (Cystone) 750 mg/kg	7.843±0.518***	38.56±2.999***	146.6±4.037***
EETP (200 mg/kg)	5.396±0.639***	29.69±2.641***	252.3±5.853***
EETP (400 mg/kg)	7.166±0.737***	36.97±3.876***	182.3±4.504***
EEGS (200 mg/kg)	6.068±0.334***	31.96±2.498***	203.4±6.260***
EEGS (400 mg/kg)	7.467±0.549***	37.4±3.413***	179.1±3.655***

Data represents the Mean ± SD values (n=6). Statistical significance: \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 with respect to lithiatic control on 28<sup>th</sup> day (One way Anova followed by Dunnetts: Compare all columns vs. lithiatic control).

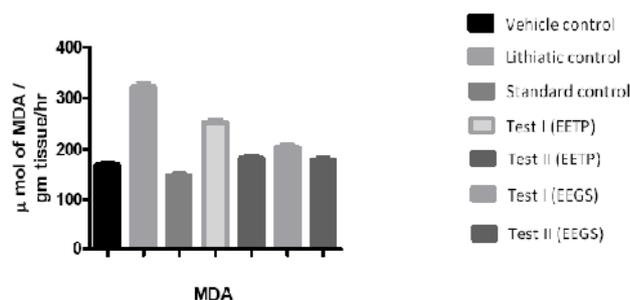
**Figure 4:** Effect of EETP and EEGS on tissue parameters in EG&AC induced urolithiasis



SOD- units/mg protein; CAT- μ moles of H<sub>2</sub>O<sub>2</sub> utilized/mg protein/min

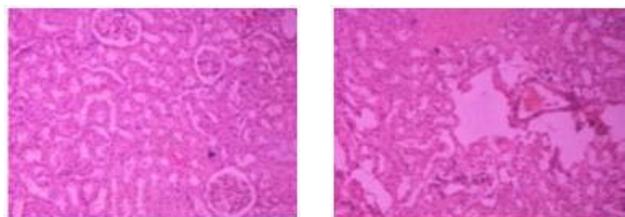
Values are expressed as Mean ± SD Values (N=6). Statistical Significance is done by One Way Anova followed by Dunnetts: Compare All Columns vs. Lithiatic Control.

**Figure 5:** Effect of EETP and EEGS on MDA level in EG&AC induced urolithiasis



Values are expressed as Mean ± SD Values (N=6). Statistical Significance is done by One Way Anova followed by Dunnetts: Compare All Columns vs. Lithiatic Control.

**Histopathological Studies**



Kidney with normal glomeruli and tubules

Lithiatic control showing degeneration of epithelial cells in glomeruli and tubules



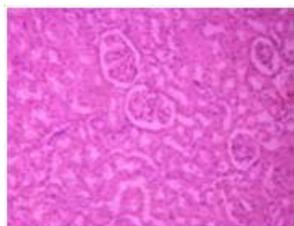
Standard control showing normal architecture of glomeruli and tubules



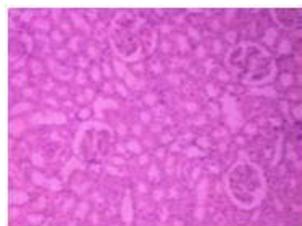
EETP (200 mg/kg b.wt) shows regenerative changes in glomeruli and renal tubule



EETP (400 mg/kg b.wt) shows regenerative changes in glomeruli and renal tubule



EEGS (200 mg/kg b.wt) shows regenerative changes in glomeruli and renal tubule



EEGS (400 mg/kg b.wt) shows regenerative changes in glomeruli and renal tubule

## DISCUSSION

The present study was aimed at evaluating the antilithiatic effect of EETP and EEGS against EG & AC induced urolithiasis in wistar rats. Different chemicals used to induce lithiasis in experimental animals include ethylene glycol (EG), glycolic acid and ammonium oxalate.<sup>33</sup> To achieve a uniformity high rate of kidney crystal deposition, other drugs such as ammonium chloride, vitamin D, and gentamycin, or a magnesium deficient diet has been used in combination with EG.<sup>34</sup> The most commonly employed method is EG and AC induced urolithiasis model. The mechanism of this process may be due to an increase in the urinary concentration of oxalates. Stone formation in EG is caused by hyperoxaluria, which causes increased renal retention and excretion of oxalate. In the present study, ammonium chloride is used to promote urinary acidification and corresponding decrease in urinary citrate excretion, which is a potent inhibitor in stone formation.<sup>35</sup> So in the present study, ammonium chloride has been used to accelerate lithiasis.

In the present study male wistar rats were selected to induce urolithiasis because the urinary system of male rats resembles to that of humans,<sup>36</sup> where as earlier studies have shown that the amount of stone deposition in female rats was significantly less due to inhibitory effect of female sex hormone on kidney stone formation.<sup>37</sup> Therefore in the present study ethylene glycol (0.75%) and Ammonium chloride (1%) for 28 days was given to the male wistar rats to induce urolithiasis.

Cystone (750 mg/kg b.wt) was used as a standard drug in inhibiting urinary stones, due to its potent diuretic, spasmolytic and saluretic effect, which is beneficial in relieving crystalluria.<sup>38</sup>

The pathology of urolithiasis was assessed by measuring the urinary parameters include urinary volume, calcium, magnesium, phosphate, oxalates and serum parameters include calcium, BUN, creatinine, and uric acid.

In the present study, the urinary volume was significantly decreased in ethylene glycol treated animals due to obstruction of stones in the bladder.<sup>39</sup> Cystone treated animals showed a significant increase in urinary volume due to its potent diuretic activity.<sup>40</sup> Pretreatment with EETP (Group IV-V) showed dose dependent increase in urinary volume, compared to lithiatic group. Treatments with EEGS at both the doses showed more significant

increase in urinary output when compared with EETP, given in table no 1 & figure no 1. This reinforces the plant extract i.e. EEGS shows potent diuretic activity such an effect may be advantage in lithiatic condition. As increased urine output is recommended to reduce the possibility of stone formation.<sup>41</sup>

EG&AC administration in the control rats, enhanced excretion and deposition of calcium, oxalate and phosphates levels indicate supersaturation of urine with CaOx. Increase in urinary phosphate excretion along with oxalates provides an environment appropriate for stone formation by forming calcium phosphate crystals in lithiatic control animals.<sup>42</sup> Cystone treatment significantly lowered the calcium, oxalate values probably by its inhibitory action on glycolate oxidase.<sup>43</sup> Pretreatment with EETP (200 and 400 mg/kg), showed prominent decrease in these elevated levels, when compared with lithiatic group, whereas treatment with EEGS shows more significant decrease in dose dependent manner, when compared with EETP group animals shown in table no 1 & figure no 2.

Magnesium in urine plays an inhibitory role in the growth and nucleation rates of calcium oxalate crystals by forming complexes with calcium and oxalates and decreases its excretion in urine.<sup>44</sup> Decreased levels of magnesium were observed in EG&AC induced rats. Pretreatment with EETP & EEGS at both dose levels, restored the magnesium levels in dose dependent manner and thus reduced the growth of calcium oxalate crystals. Among both, EEGS treated animals showed more significant increase in magnesium levels i.e. near to the normal range compared to lithiatic control, given in table no 1 & figure no 2.

In EG&AC induced rats, significant raise in calcium, uric acid, creatinine and BUN was observed in serum, because of decreased glomerular filtration rate due to obstruction in the urine flow in urinary system with the deposition of calcium oxalate in renal tubules.<sup>41</sup> Cystone treatment showed significant reduction of these elevated levels whereas pretreatment with EETP and EEGS at both dose levels showed a significant reduction in nitrogenous substances in serum. The EEGS showed more significant decrease in creatinine, uric acid and BUN levels compared to EETP, shown in table no 2 & figure 3.

It was reported that EG induced hyperoxaluria promotes OS.<sup>45</sup> Previous studies suggest that oxalate promotes LPO and resulting in renal tissue damage. The impaired antioxidant protection might be responsible for the accumulation and retention of oxalate and subsequent deposition of CaOx in the kidney.

SOD and CAT were the most important enzymes in the enzymatic anti-oxidant defense system. SOD scavenges the superoxide anion to form hydrogen peroxide and diminishes the effect caused by the free radical and CAT, which decomposes hydrogen peroxide and protects the tissue from the highly reactive hydroxyl radical. Depletion in the activities of these enzymes with EG&AC induced

rats was observed in lithiatic control rats.<sup>46</sup> Cystone treated animals showed significant increase in the enzyme activities of SOD and catalase. Pretreatment with EEGS shows significant increase in the enzyme activities when compared with EETP, given in table no 3& figure 4 .The antioxidant activities may be due to the presence of polyphenols such as flavonoids, tannins and phenols, which contribute to induce antioxidant potential activity.

Reactive oxygen species degrade polyunsaturated fatty acids, forming malondialdehyde as end product.<sup>47</sup> This reactive aldehyde cause oxidative stress in cells and form covalent protein adducts resulting in lipoxidation end products. The declined levels of lipid peroxidation were observed in cystone treated group animals. Pretreatment with EETP and EEGS at 200 & 400 mg/kg b.wt significantly reduced the MDA levels in a dose dependent manner. Among both EEGS shows more significant decline levels of lipid peroxidation, given in table no 3& figure no 5. This may be due to the presence of poly phenols such as flavonoids, tannins and saponins.

Histopathological observation of the kidney sections of ethylene glycol induced lithiatic rats showed presence of polymorphic irregular calcium oxalate crystals in Lumina of tubules accompanied by edema and cast formation which causes dilation of proximal tubules; this might be attributed to oxalate formation. On administration of EETP at different doses (200 and 400 mg/kg b.wt) moderate to few crystals were observed along the mild appearance of edema dilation in tubules and crystals are present focally indicating the ability of EETP to dissolve the preformed stones to some extent. Similarly on administration of EEGS significantly reduce the number and size of the crystals than the EETP, indicating the ability of EEGS to dissolve pre-formed stones to a greater extent.

## CONCLUSION

In the present study, administration of EG&AC in male wistar rats for 28 days period showed altered urinary, serum and tissue parameters. The pretreatment with EETP and EEGS at both dose levels showed a significant restoration of altered parameters near to normal level. Among both, EEGS showed a potent antilithiatic activity compared to EETP and cystone treated animals. It was concluded that the antilithiatic activity of EETP may be due the diuretic property and presence of phytochemicals like alkaloids, phenols, flavonoids, saponins, tannins and terpenes whereas the antilithiatic activity of EEGS may be due to the potent diuretic activity and presence of phytochemicals such as alkaloids, phenols, flavonoids, saponins. Further studies were required to isolate the chemical moiety which showing potent antilithiatic activity.

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