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EVALUATIONS OF CURATIVE EFFICACY OF HYDROALCOHOLIC EXTRACT OF *APIUM GRAVEOLENS* IN EXPERIMENTALLY INDUCED NEPHROLITHIATIC WISTAR MALE RATS

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Abstract

Background: In Indian folk medicine, there is a claim that medicinal plants can treat urolithiasis although there is insufficient scientific evidence. The objective of this study was to evaluate the curative efficacy of hydro alcoholic extract of *Apium graveolens* in experimentally induced nephrolithiatic rats.

Methods: Urolithiasis was induced in male Wistar rats by feeding ethylene glycol in drinking water for 28 days. The curative effects were evaluated after oral administrations of 200 and 400 mg/kg of the extracts from 15 to 28 days. Urine samples were collected 1 day before sacrificing the rats. Blood and kidney samples were gathered under anaesthetic condition at day 28.

Results: Hydro alcoholic extract of *Apium graveolens* reduced significantly in the urinary level of protein, calcium, Uric acid, Creatinine, Oxalate and Phosphate ($P < 0.001$), whereas it was significantly elevated the levels of magnesium ($P < 0.01$) compared to lithiatic control. In the kidneys, CaOx crystal deposits were reduced significantly by hydro alcoholic extract of *Apium graveolens*.($P < 0.01$)

Conclusion: It has been noted that Hydro alcoholic extract of *Apium graveolens* was potent in treating urolithiasis. However, further study is required to assess the efficacy of the active compounds against urolithiasis.

Keywords: Hydro alcoholic extract of *Apium graveolens*, , Ethylene glycol, Hyperoxaluria, Micro crystals, 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 cause obstruction of the ureter (1) . Urolithiasis is a complex process which is a consequence of an imbalance between promoters and inhibitors in the kidneys (2).

An imbalance between urinary stone inhibitors and promoters has been suggested to be the cause of stone formation [3]. Promoters facilitate stone formation [4], but inhibitors decrease the initiation of supersaturation, nucleation, crystal growth and rate of aggregation.

Despite considerable improvements in medical therapy such as the utility of extracorporeal shock wave lithotripsy (ESWL), there is no satisfactory drug to treat renal calculi [5].

In India, most patients rely on traditional medicinal plants as an alternative therapy for various diseases including urolithiasis. Medicinal plants are affordable, accessible, effective, with less side effects and being better compatible with the human body [6], compared to conventional drugs [7]. In the present study, Hydro alcoholic extract of *Apium graveolens* were selected to examine their effects on experimentally induced urolithiasis. A thorough literature survey was carried out to ascertain that none of the selected medicinal plant parts have been studied so far on anti-urolithiatic activities. To date, there is also insufficiency of scientific evidence reported on the antiurolithiatic activity of this medicinal plant. Therefore, the objective of the study was to evaluate the curative efficacy of medicinal plants in experimentally induced nephrolithiatic male rats.

EXPERIMENTAL

Plant Material

The collection of the plant materials of *Apium graveolens* were done in the month of April-2021 at Alagar hills, Madurai- Tamilnadu. Early April will be the ideal time for the collection of medicinal plants since the plants will be enriched with phytoconstituents during that time. The identification and authentication of the plant was carried out by Dr. D.Stephan, Ph.D., Department of Botany, American College, Madurai-Tamil Nadu. Voucher specimen (No.KMCP/AG/120) was prepared and preserved in the Department of Pharmacognosy, K.M.College of Pharmacy, Madurai for future reference.

Preparation of extracts of *Apium graveolens* by hot continuous percolation method

About 500 gm of dried powder was properly packed in Whatmann filter paper (grade no.1) and kept in thimble and the soxhlet apparatus was set up. The extraction of powder was done with different solvents with solvents of increasing polarities like petroleum ether (60-80º C), chloroform, and hydro alcohol. Here temperature maintenance is based on the solvents used for extraction. The solvents were removed under reduced pressure using rotary evaporator and stored in desiccators. (8)

Animal selection

Male albino rats of Wistar strain, aged around 2 to 3 months and weighing 180-200 g were used. They were housed in standard conditions of temperature (25 ± 2°C), relative humidity of 45-55%, and maintained on 12–hour light: 12–hour dark cycle in animal house. Experiments were conducted in accordance with internationally accepted standard guidelines

for the use of animals. Animals were fed ad libitum on normal commercial chow and had free access to water.

Acute toxicity studies

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co- operation and Development (OECD-423) The protocol of the animal experiments involved in this research work has been approved by IAEC/CPCSEA constituted for these purposes (No. IAEC/PREETHA PETER/AU/PhD/KMCP/48/2019.

Urolithiasis induction

Kidney stones were induced using ethylene glycol (EG) along with ammonium chloride (NH4Cl) administered in the rats' drinking water. In this hyperoxaluria model, 1% (w/v) NH4Cl was given with 0.75% (v/v) EG for the first 5 days to accelerate lithiasis, following this the water supply was switched to 0.75% EG alone for the next 25 days [9,10,11]. Exposure of these dose levels were sufficiently tolerable in animal studies [12]. EG administrations result in hyperoxaluria, which in turn leads to CaOx deposition in the kidneys [13]. The experimental rats assigned as stone curative groups were receiving stone inducing treatment for 28 days.

Curative effects of urolithiasis

In the curative treatment (dissolution), a total of 30 albino Wistar male rats were divided randomly into 5 groups comprising 6 individuals per group with matching body weights. In curative treatment, the disease was induced priory from day 1 to 14. Then, hydro alcoholic extract of *Apium graveolens* at a dose of 200 and 400mg/kg and cystone at a dose of 750mg/kg was administered orally from day 15 to 28 concurrently with disease induction (EG) protocols to determine curative effects [14,15].

At the end of 28th days, rats were sacrificed for biochemical and histopathological studies. The experimental design were assigned as Group I (Normal control), Group II (Lithiatic control), Group III (hydro alcoholic extract of *Apium graveolens* at a dose of 200mg/kg), Group IV (hydro alcoholic extract of *Apium graveolens* at a dose of 400mg/kg),Group V (Cystone at a dose of 750mg/kg). The dosing volume was 2 ml/100 g of body weight. The control group received distilled water once daily throughout the experiment. At the end of 28th days, rats were sacrificed for biochemical and histopathological studies.

Urine biochemical analysis

At the end of the respective treatment periods, the animals were individually housed in metabolic cages, and 24 h urine samples were collected and stored at 4 °C for 5 days. Then, these were centrifuged at 3000 rpm for 10 min (REMI, R24). Urine was analyzed for calcium, phosphate, magnesium, oxalate, uric acid and creatinine concentration were analyzed using commercially available diagnostic kits by the Automated clinical chemistry analyzer.

Serum collection and analysis

After the end of the experimental period (day 28th), rats were anesthetized using diethyl ether and 3 ml blood samples were collected from the retro-orbital vein by capillary puncturing. Serum was separated after centrifuged at 3000 rpm, 20 °C for 15 min. The collected serum was investigated for biochemical parameters like calcium, magnesium, oxalate, phosphate, creatinine and uric acid by Clinical Chemistry Autoanalyzer (Cobas 6000 analyzer, Germany) with the respective diagnostic kits.

Histopathological examinations

Histopathological examinations were done for kidney tissues of the experimental rats. All rats were sacrificed in a humane manner using diethyl ether anaesthesia at the end of the 28th day (urolithiasis curative studies). The tissue pieces were taken from kidneys and analysed for urolithiasis curative potentials of these extracts. The tissues were fixed by 10% buffered neutral formalin solution. The sections (5 µm thick) were cut using Rotary Microtome 4060E and stained with Hematoxylin and Eosin to study the histopathological changes (16)

Statistical Analysis

All the values are expressed as mean ± SEM. The data were statistically analyzed by One-way ANOVA followed Neumann Keuls multiple range tests. P values < 0.05 were considered significant.

Results

Curative efficacy of Hydro alcoholic extract of *Apium graveolens* (HAEAG)

In the curative (therapeutic) studies, the selected extracts dose was 200 mg/kg body weight of rats, which was one tenth of the maximum tolerated dose 2000 mg/kg b.w [17]. This was chosen based on prior acute and/or subacute toxicity studies revealing its safety up to dose 2000 mg/kg. The induction of kidney stones by the administration of 0.75% EG combined with ammonium chloride (1%) in drinking water was confirmed in Wistar male rats.

Effect of HAEAG on urinary & serum parameters in curative study.

The curative study was designed to evaluate the effect of these extracts in lithiasis, after it has been induced as treatment is normally instituted in humans only after the incidence of renal stones. So in this study treatment was instituted from 15th day after treatment with ethylene glycol for 14 days. After treatment with extracts for 2 weeks starting from 15th day the above mentioned parameters were studied (table no: 1,2). The values show that all parameters like protein, calcium, creatinine, oxalate, uric acid and phosphate were increased significantly in ethylene glycol treated group (G2), whereas magnesium levels were decreased significantly. However after treatment with hydro alcoholic extract of *Apium graveolens* at a dose of 200 and 400 mg/kg and cystone treatment,all above mentioned parameters reduced significantly and magnesium levels restore to near normal limits.

Histopathological studies

Histopathological studies of kidneys clearly revealed that the tissue section of Group I rats showing normal size tubules with single epithelial lining along the margin. Whereas the Group II rats showed dilated tubules and degeneration of epithelial lining with presence of crystals. But the kidney specimen from standard and extract treated groups showed characters similar normal control group (Figure-1 to 5).

Table 1 : EFFECT OF HAEAG ON URINARY BIOCHEMICAL PARAMETERS IN CURATIVE TREATMENT OF ANIMALS

GP	Magnesium (mg/dl)	Calcium (mg/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)	Oxalate (mg/dl)	Phosphate (mg/dl)
G1	4.85 ±0.78	6.54 ±0.72	3.90 ±0.75	14.94 ±2.24	19.14 ±1.75	35.25 ±2.34
G2	1.60 ±0.24$^{**(a)}$	18.78 ±2.15$^{**(a)}$	15.18 ±2.57$^{**(a)}$	54.85 ±4.20$^{**(a)}$	50.88 ±3.45$^{**(a)}$	81.48 ±4.85$^{**(a)}$

G3	3.24 ±0.45**(b)	11.85 ±1.27**(b)	9.55 ±1.36**(b)	36.80 ±3.24**(b)	26.75 ±2.45**(b)	43.30 ±3.22**(b)
G4	3.78 ±0.52**(b)	10.78 ±1.22**(b)	9.20 ±1.25**(b)	34.50 ±3.15**(b)	24.12 ±2.24**(b)	41.65 ±2.80**(b)
G5	4.14 ±0.60**(b)	10.22 ±1.05**(b)	8.29 ±1.09**(b)	31.40 ±2.85**(b)	21.25 ±1.93**(b)	37.45 ±2.40**(b)

G1- Normal control; **G2**- Lithiatic Control;
G3- *HAEAG* 200MG/KG
G4- HAEAG 400MG/KG
G5- CYSTONE 750MG/KG

Values are expressed as Mean± SEM

- Values were find out by using ONE WAY ANOVA Followed by Newman keul's multiple range test
- **(a)values were significantly different from normal control G1 at P< 0.01

**(b) values were significantly different from Lithiatic control G2 at P<0.01

Table 2 : EFFECT OF HAEAG ON SERUM PARAMETERS IN CURATIVE TREATMENT OF ANIMALS

GP	Magnesium (mg/dl)	Calcium (mg/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)	Oxalate (mg/dl)	Phosphate (mg/dl)
G1	5.35 ±0.86	10.30 ±1.25	4.40 ±0.85	0.92 ±0.25	8.85 ±0.80	15.90 ±2.14
G2	1.75 ±0.52**(a)	23.20 ±2.20**(a)	12.50 ±2.34**(a)	2.80 ±0.67**(a)	17.40 ±1.27**(a)	29.80 ±3.18**(a)
G3	4.25 ±0.78**(b)	20.40 ±1.65**(b)	7.25 ±1.15**(b)	1.57 ±0.50**(b)	11.14 ±1.07**(b)	21.70 ±2.55**(b)
G4	4.36 ±0.92**(b)	19.45 ±1.25**(b)	7.20 ±0.94**(b)	1.48 ±0.40**(b)	10.48 ±0.88**(b)	18.95 ±2.29**(b)
G5	4.80 ±0.96**(b)	18.20 ±1.10**(b)	6.70 ±0.78**(b)	1.28 ±0.29**(b)	9.55 ±0.83**(b)	17.12 ±2.11**(b)

G1- Normal control; **G2**- Lithiatic Control;
G3- *HAEAG* 200MG/KG
G4- HAEAG 400MG/KG
G5- CYSTONE 750MG/KG

Values are expressed as Mean± SEM

- Values were find out by using ONE WAY ANOVA Followed by Newman keul's multiple range test
- **(a)values were significantly different from normal control G1 at P< 0.01

**(b) values were significantly different from Lithiatic control G2 at P<0.01

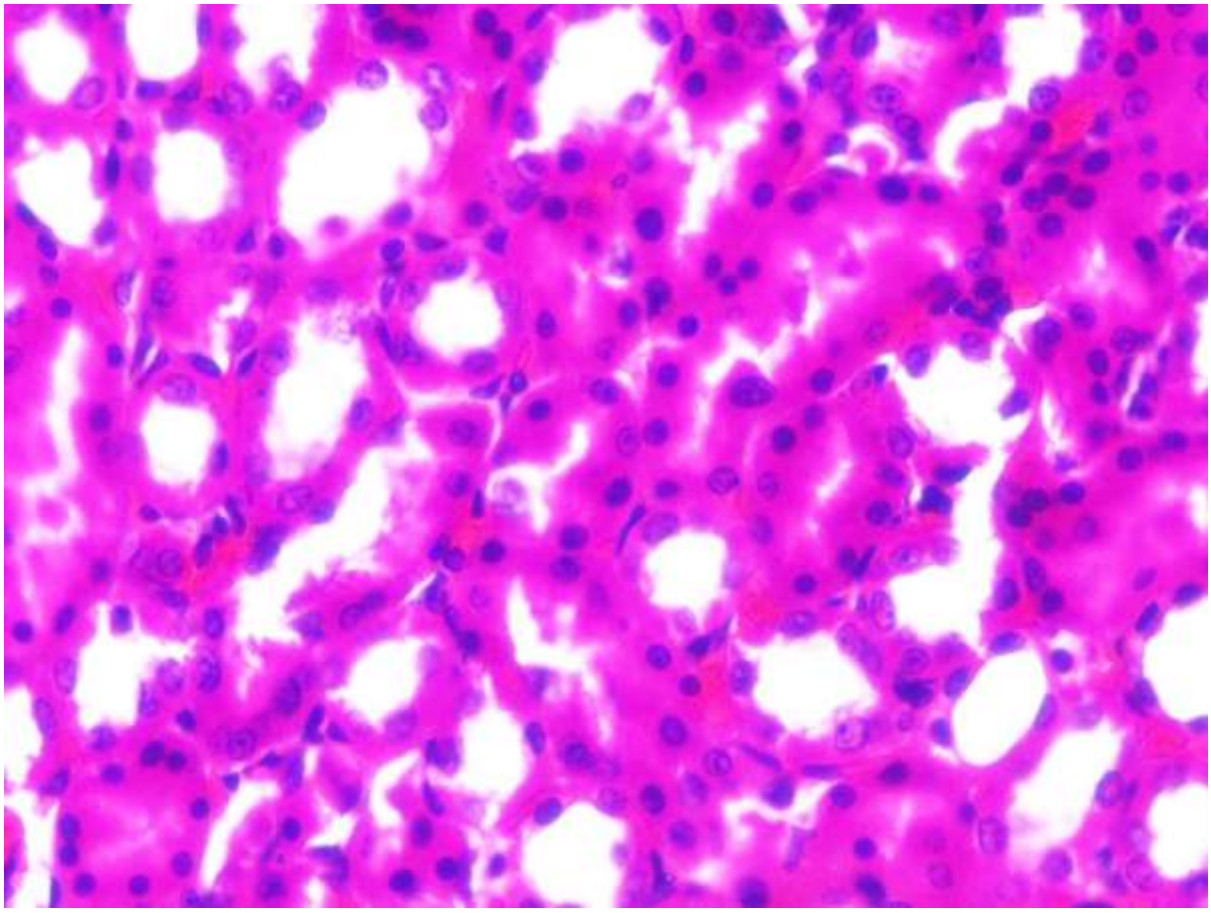

FIG.NO:1 Normal control

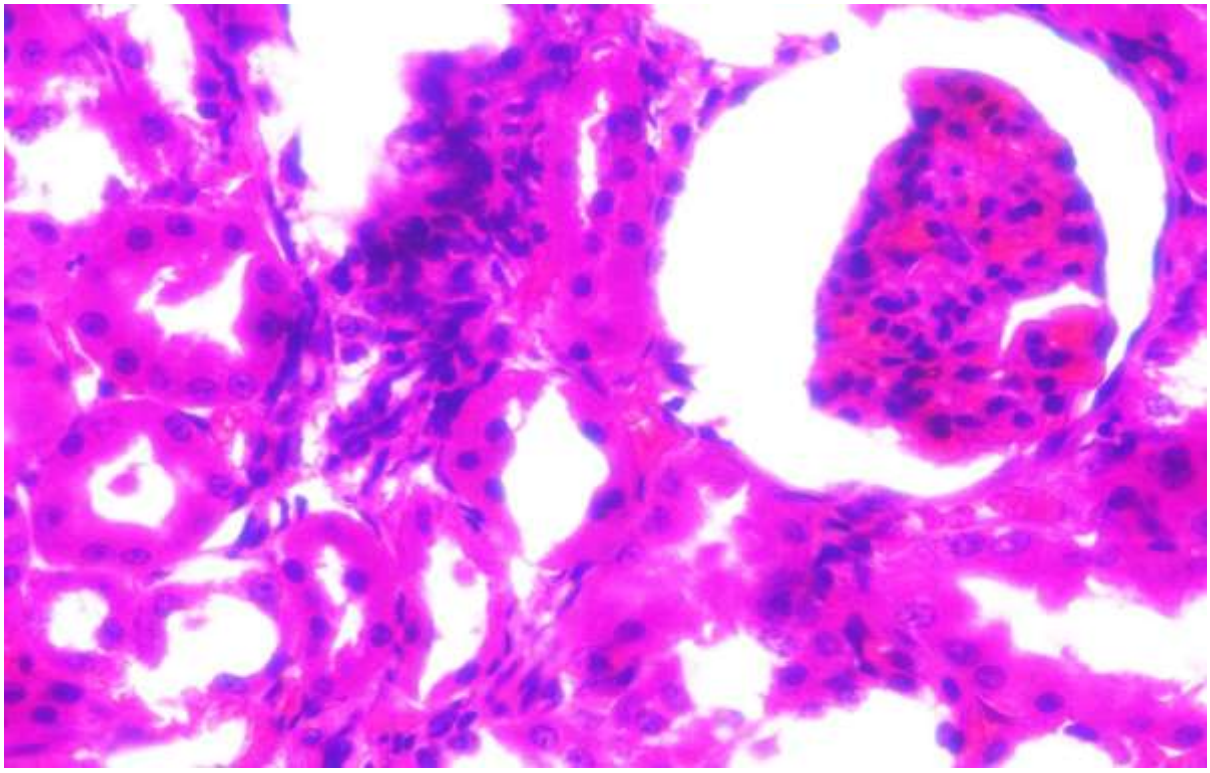

FIG.NO:2 Lithiatic control

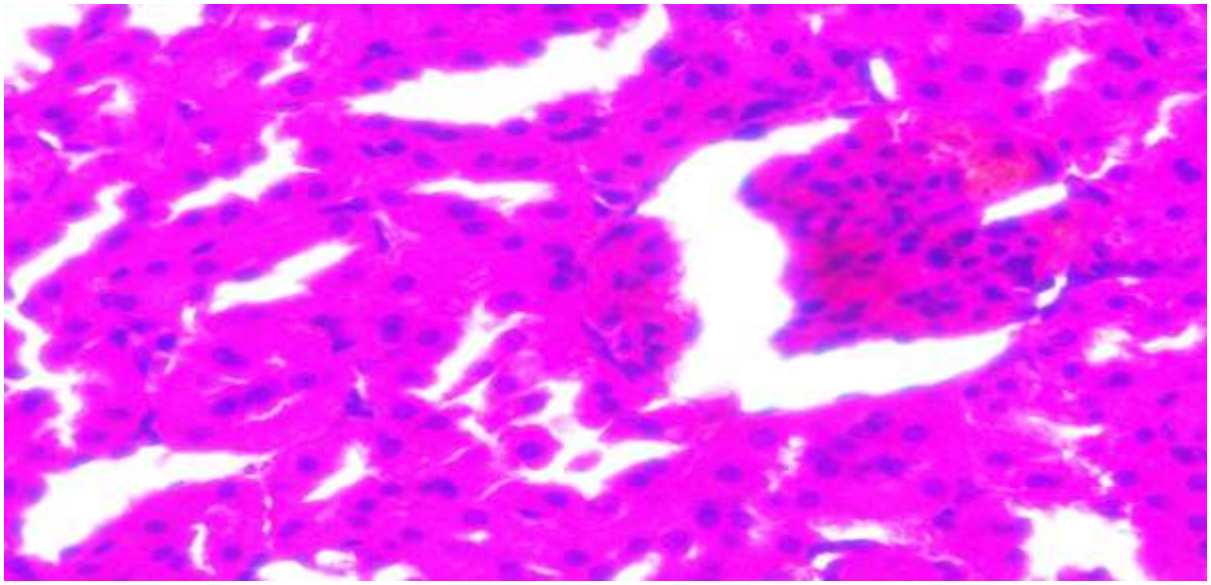

FIG.NO:3 Treatment control HAEAG 200mg/kg

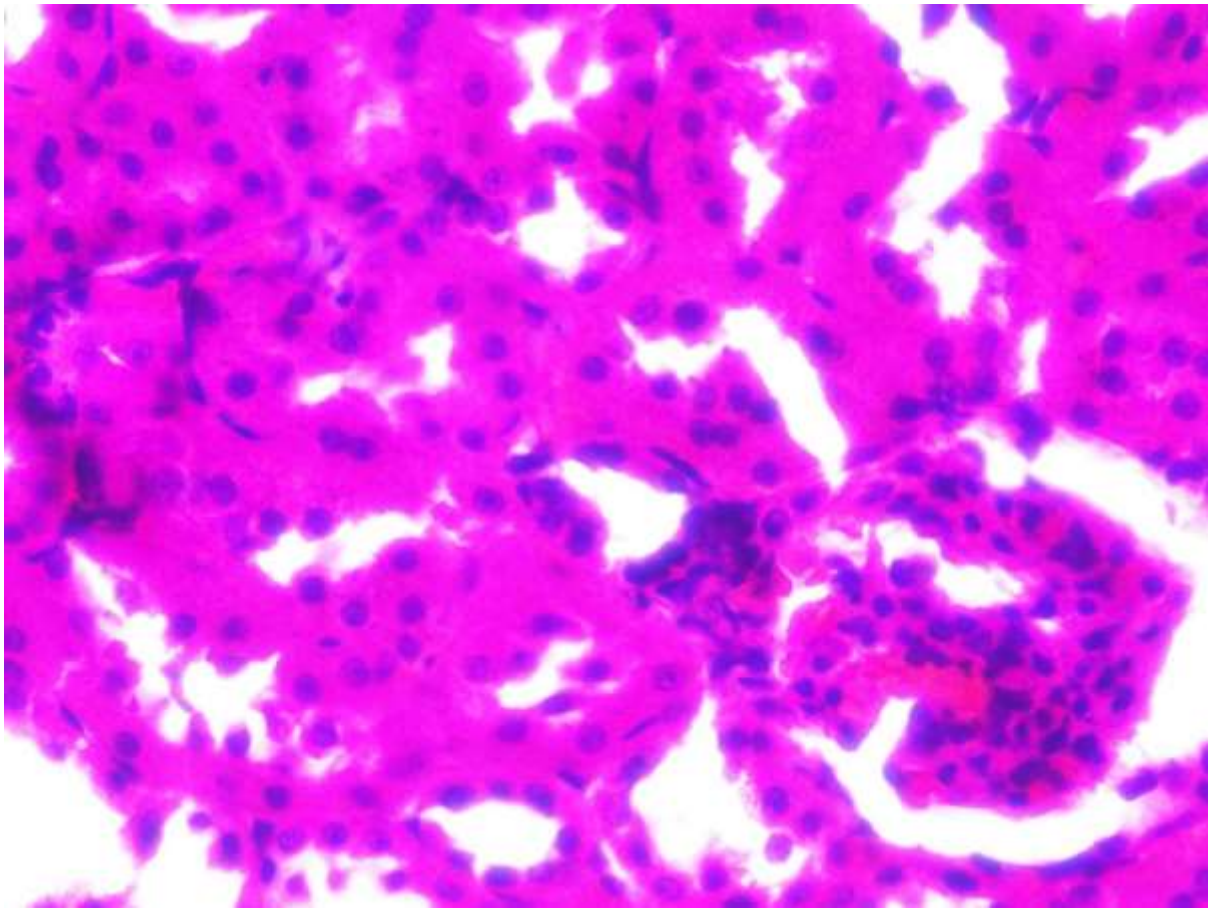

FIG.NO:4 Treatment control HAEAG 400mg/kg

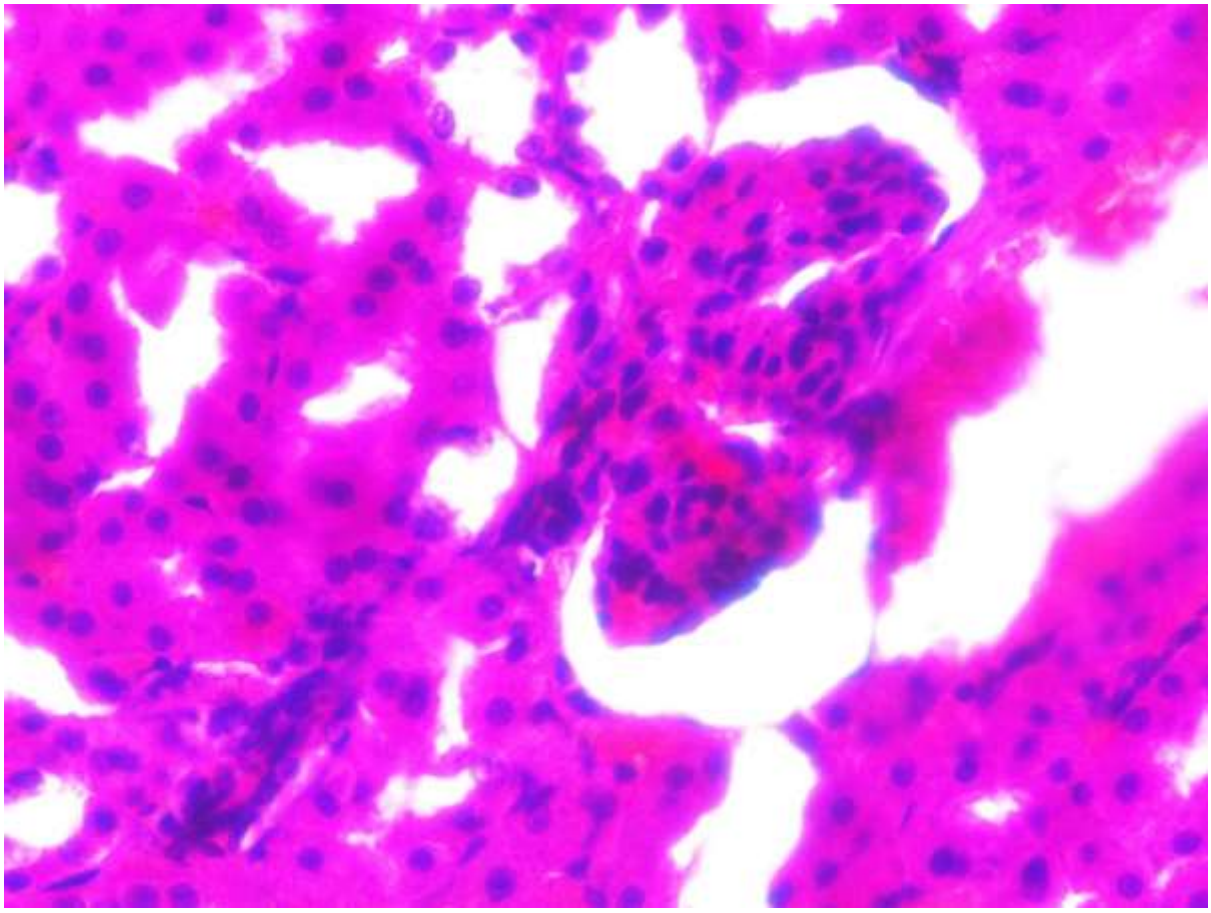

FIG.NO:4 Treatment control Cystone 750mg/kg

DISCUSSION

Rats are the most frequently used animals in models of CaOx deposition in the kidneys, a process that mimics the etiology of kidney stone formation in humans. Rat models of CaOx urolithiasis induced by either EG alone or in combination with other drugs such as AC, are often used to study the pathogenesis of kidney crystal deposition(18). Using the accelerated model, in the present study rats were treated with 0.75% EG and 1% Ammonium chloride for 14 days.

Male rats are selected to induce urolithiasis because testosterone plays a significant role in oxalate production as increased serum testosterone level in male rats results in increased endogenous production of oxalate by liver and further ethylene glycol solutions of low concentration induces calcium oxalate nephrolithiasis in male rats does not produce similar results in females that is why male rats are more susceptible to develop CaOx stone than female rats.(19,20) Therefore, in the present study male wistar rats were treated with ethylene glycol (0.75% V/V) and ammonium chloride (1% W/V) for 14 days. It has been reported that EG is oxidized to oxalic acid by non-specific dehydrogenase which leads to hyperoxalaurea key factor for urolithiasis. EG metabolizes into CaOx monohydrate and produces renal mitochondrial toxicity similar to clinical CaOx renal calculi.(21)

The analysis of urine with respect to stone forming agents is an apt indicator for the risk of stone formation. Previous studies reported that EG causes hypercalciuria, hyperphosphaturia

and hyperoxaluria. The increased urinary calcium is a reason for favoring the nucleation and precipitation of calcium oxalate from urine and subsequently crystal growth.(22)

In the present study, urinary calcium,oxalate and phosphate increased significantly ($p < 0.001$) in lithiatic animals. The test extract in both the doses and cystone showed significant reduction in urinary calcium,oxalate and phosphate. Reduction of calcium level in urine provides less calcium to bind with oxalate which results in reduction of calcium oxalate crystals. Further reduction in urinary calcium also reduces super saturation which is the major risk factor for stone formation.(23) Though the serum calcium level was not significantly reduced in treated groups; it was much lesser than the disease control. On the contrary, phosphorus level remained unaffected. The reason behind this is that the phosphorus level in blood is reliant on the calcium level and activity of parathyroid hormone thus at times it is difficult to regulate the blood.

In the present study, we estimated the effect of the EG and AC- treated group on intracellular levels of magnesium, which revealed that EG and AC-treatment significantly decreases the levels of magnesium as compared to the normal group. Whereas, in another set of the group it was postulated that both does of HAEAG and Cystone significantly restore the intracellular magnesium levels.

Creatinine and uric acid are the indicators of kidney and tubular damage. Glomerular filtration rate (GFR) decreases in kidney tissue injury; may be due to the presence of stone in urinary tract, which obstructs urine flow and the waste products, particularly nitrogenous substances thus an increase in their level is noted.(24) Urinary creatinine was found to be increased in disease control groups, may be due to decrease in GFR. Treatment with the test extract showed significant decrease in serum creatinine and uric acid in test groups. Improvement in GFR after the treatment with test drug may be due to reduction in inflammation and injury of kidney tissues. *Apium graveolens* has been reported for antioxidant, anti-inflammatory, antispasmodic, antibacterial, antifungal, anticancer, diuretic and sedative activities,(25) thus the efficacy of the test drug is justified by these properties.

The histological features also supported the above findings. In lithiatic animals, there were cellular derangement, hyper cellularity and injured glomerulus remarkably observed. However, on treatment with both doses of HAEAG and cystone, notable improvements were observed. As reported in earlier studies that the antioxidant effect of flavonoid found in green tea (26) and Orthosiphon grandioxorum(27) decreases oxidative injury in renal tubular cells and calcium oxalate deposition in rat kidney. The chemical constituents of *Apium graveolens* might exert their effect through anti-oxidant activity to make expulsion of renal stone easy.

Conclusion

The current experimental outcomes inferred that administration of hydro alcoholic extract of *Apium graveolens* can prevent the urolithiasis and protect the kidneys via inhibition of injuries caused by the crystal deposition and neutralization of reactive oxygen species. This may prove to develop cost-effective alternatives for the cure of urolithiasis.

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