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Anti-Urolithic Activity Of Ethanolic Extract Of Rhus Mysorensis Against Calcicum Oxalate Induced Urolithiasis In Wistar Albino Rats

Gnrlt Sowjanya[1], Pasumarthi Phaneendra[2*]
Department Of Pharmacology, Jntu Kakinada, Andhrapradesh, India
Email: gnrltsowjanya@gmail.com

Abstract: This study was undertaken to evaluate the antiurolithiac effect of ethanolic extract of *RHUS MYSORENSIS* (ERM) against calcium oxalate urolithiasis using male wistar rats. Urolithasis was induced by administration of 0.75% V/V ethylene glycol with 1%w/V ammonium chloride in drinking water for 3 days followed by only 0.75% v/v ethylene glycol for next 25 days. Antiurolithiatic activity was evaluated at four doses of ERM (viz.,250, 350,450,550mg/kg) in curative and preventive regimen by estimating histological changes in kidney tissue and biochemical changes in urine, serum, and kidney tissue homogenate. Ethylene glycol-ammonium chloride feeding caused alteration in volume and levels of calcium, oxalate, phosphate, uric acid in the urine. The ERM treatment also increased urine volume but less than calculi induced animals. The ERM supplementation significantly prevented biochemical changes in the urine dose dependently. Supplementation with ERM prevented the elevation serum creatinine, uric acid and calcium, phosphate levels. The increased calcium, oxalate and phosphate levels in the kidney tissue of lithiatic rats were significantly reduced by the ERM treatment. The ERM supplementation also caused significant decrease in accumulation of calcium oxalate deposits, histological changes in the kidney tissue. These results indicate that administration *RHUS MYSORENSIS* extract reduced and prevented the growth of urinary stones. The possible mechanism underlined this effect is mediated collectively through diuretic properties and lowering the concentration of urinary stone forming constituents.

Keywords: *RHUSMYSORENSIS*, Ethyleneglycol, Urolithiasis

INTRODUCTION

Urolithiasis is the third prevalent disorder of the urinary system which is approximately 2- 3% in the general population. Urinary calculi may cause serious medical consequences such as extreme obstruction, hydronephrosis, infection and hemorrhage in the urinary tract system [1]. Surgical operation, lithotripsy and local calculus disruption using high-power laser are commonly used techniques to remove the calculi. However, these procedures may cause serious complications such as acute renal injury, decrease in renal function and an increase in stone recurrence [2]. Medicinal plants are always remained important source of drugs. Some medicinal plants and proprietary composite herbal preparations are reported to be effective in the treatment as well as prevention of recurrence of renal calculi. With minimal side effects [4]. In Indian indigenous system of medicine, several plants including *RHUS MYSORENSIS* are claimed to be useful for the renal calculi [5]. *RHUS MYSORENSIS* (family: Anacardiaceae) is a medicinal plant, found common in foot hills, Northwest India to the peninsular India. The objective of this study was to evaluate the Antiurolithiatic activity of *RHUS MYSORENSIS* extract against calcium oxalate urolithiasis and its possible underlying mechanisms using male Wistar albino rats.

EXPERIMENTALSECTION

Animals:

Male Wistar albino rats weighing between150-200g were used for this study. They were procured from National Institute of Biosciences, Pune, India. The animals were acclimatized for ten days under standard laboratory conditions (Temperature:25±2℃,Relative humidity 65± 10% under 12 hlight/dark cycles) in the animal house of Sigma Institute of Clinical Research and Administration Pvt. Ltd, Hyderabad, which is approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India. The animals fed with standard diet supplied by Nutrivet Life Sciences, Pune, India. The study protocol was approved from the Institutional Animal Ethics Committee (Ref.769/2011/CPCSEA) of the institute.

Chemicals and apparatus:

Ethylene glycol (Qualigens Fine Chemicals, India; B.No.75316806-2), Ammonium chloride (Ana lab Fine Chemicals, India; B.No. RKC221713) of analytical grade, procured from approved vendors were used for the study. Apparatus such as the metabolic cages (New Neeta Chemicals, India), cold centrifuge (BioEra, India) and UV-spectrophotometer (Lab India, India) were used for this study.

Collection and extraction of plant material:

The whole plant of RHUS *MYSORENSIS* were collected in the month of November from local region of hyderabad, telangana, India. It was authenticated by K. srinivas, department of pharmacognosy sri vasavi institute of pharmaceutical sciences, India. The whole plant was dried under shade for 10-12days. The dried plant of *RHUS MYSORENSIS* were coarsely powdered, packed into soxhlet column and extracted with 80%v/v ethanol in water at 65-70℃ for 22h. This ethanol extract (ERM) was then evaporated at 45℃ and then dried in oven. The dried extract was stored in airtight container.

Preliminary phytochemical analysis:

The ERM was subjected to qualitative analysis of the various phytoconstitutents using well established procedure [19].

Experimental Design:

Ethyleneglycol and ammonium chloride induced hyperoxaluria model were used to induce urolithiasis in rats [20]. The four dose levels of ERM (250,350,450,550mg/kg) were used for the evaluation of Antiurolithiatic effect [21]. Animals were randomly divided into seven groups containing six animals in each. Group I served as vehicle control %w/v gumacacia solution (5ml/kg, p. o.). All remaining groups received calculi inducing treatment for the period of 28days, comprised of 0.75%v/v ethylene glycol with1%w/v ammonium chloride in drinking water *ad libitum for* 0.75%v/v ethyleneglycol for next 25days.GroupII served as lithiatic control and received 5%w/v gum acacia as solution (5ml/kg, p. o.). Group III treated with cystone (750mg /kg/b.w.p.o),IV,V,VI and VII served as preventive treatment groups and received ERM at doses of 250,350,450,550 mg/kg respectively from 1^{st} day to 28^{th} day of calculi induction. The ERM was suspended in distilled water using5%w/v gum acacia and given once daily by oral route (5ml/kg bodyweight).

On 0,14 and 28^{t} thday of calculi induction, all the animals were kept in individual metabolic cages and 24h urine Samples were collected. The collected urine samples were analyzed for its volume, calcium, oxalate,

phosphate and uricacid contents. The urine calcium level was estimated using kit by Beacon Diagnostic Pvt. Ltd., India. The28thday urine samples were also subjected to crystal uria study to estimate presence of calcium oxalate crystals in 3h urine sample by viewing under light microscope.

Serum analysis:

After28 th day urine collection period, blood was collected from retro-orbital sinus under ether anesthesia. Serum was separated by centrifugation at10,000×gfor10min and analyzed for creatinine, uric acid, calcium and inorganic phosphate spectrophotometrically by using diagnostic kit from Beacon Diagnostic Pvt. Ltd., India.

Kidney histopathology and homogenate analysis:

After blood collection, all the animals were sacrificed by cervical dislocation. The abdomen was cut open to remove either kidney from each animal. Isolated kidneys were cleaned off extraneous tissue and preserved in 10% neutral formalin. One of the isolated kidneys was then embedded in paraffin using conventional methods and cut into 5µm thick sections and stained using hematoxylin–eosin dye and finally mounted in diphenyl xylene. Then the sections were observed under microscope for histopathological changes in kidney architecture and their photomicrographs were taken.

Statistical analysis:

Results were expressed as mean± standard error of mean (SEM). The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's comparison test and $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

The yield of ERM was found to be 23%w/w. Preliminary phytochemical analysis revealed the presence of carbohydrates, proteins and aminoacids, flavonoids, tannins, saponins and phenolic compounds in the ERM. A number of animal models using rats have been used to induce calcium oxalate urolithiasis is [32]. The most commonly employed method is to provide ethylene glycol and ammonium chloride in drinking water to rats. Therefore, we evaluated the Antiurolithiatic potential of *RHUS MYSORENSIS* on calcium oxalate urolithiasis using this model. The biochemical mechanism of ethyleneglycol and ammonium chloride-induced lithiasis is related to an increase in the urinary concentration of oxalate. Ethyleneglycol is readily absorbed along the intestine and is metabolized in the liver to oxalate that further leads to hyperoxaluria. The oxalate precipitates in the urine as calcium oxalate due to its poor solubility. High oxalate levels and calcium oxalate crystals especially in nephron damages epithelial cells, leading to heterogeneous nucleation followed by aggregation of crystals [33,34]. Furthermore, ammonium chloride has been reported to accelerate lithiasis [35].

TableNo.1: Effect of ethanolic extract of *Rhus mysorensis* on various physical parameters in ethylene glycol induced urolithiasis preventive study

PARAMETRS	NORMAL	CONTROL	STANDARD	T1	T2	T3	T4
CHANGE IN BODY WEIGHT (gm)	253.33±4.96*	273.33±4.69	213.45±3.59#	245.33±2.02**	252±4.21*	271±2.11*	273±3.11*

VOLUME OF URINE IN (ml)	17.22±0.21	15.14±0.42 ##	27.11±0.42 *	23.42±0.70*	25.25±0.42 *	26.36±0.12 *	28.12±0.11 *
URINE pH	6.1±0.18	8.16±0.21#	7.13±0.21*	7.43±0.16	7.26±0.21* *	7.28±0.1**	7.31±0.2**
KIDNEY WEIGHT (gm)	0.80±0.03	1.35±0.08#	0.72±0.02*	0.74±0.02*	0.73±0.01*	0.74±0.01*	0.75±0.03*

N = 6; Significance: *** $P<0.001$, ** $P<0.01$, * $P<0.05$ from control
# Highly significant difference from normal $p \leq 0.05$
Significant difference from normal $p < 0.01$

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Significant difference from normal $p < 0.01$

TableNo.2: Changes in kidney retention of stone forming constituents in control and experimental animals in urine

PARAMETRS	NORMAL	CONTROL	STANDARD	T1	T2	T3	T4
CALCIUM (mg/dl)	5.2±0.24	7.09±0.53#	4.04±0.37**	5.04±0.23*	4.46±0.22*	4.28±0.33*	4.16±0.42*
OXALATE (mg/dl)	1.53±0.34	6.25±0.17#	0.52±0.05*	2.80±0.30*	2.64±0.20*	2.58±0.20*	2.45±0.10*
INORGANIC PHOSPHATE (IP) (mg/dl)	0.16±0.01	0.85±0.05#	0.14±0.01*	0.56±0.08**	0.51±0.07*	0.46±0.06* *	0.42±0.02*
URIC ACID (mg/dl)	10.01±0.51	25.52±0.76 #	9.17±0.43* *	20.71±0.84* *	18.22±0.80 *	16.71±0.74 **	14.12±0.60 *

N = 6; Significance: *** $P<0.001$, ** $P<0.01$, * $P<0.05$ from control
# Highly significant difference from normal $p \leq 0.05$
Significant difference from normal $p < 0.0$

TableNo.3: Changes in serum parameters in control and experimental animals

PARAMETES	NORMAL	CONTROL	STANDARD	T1	T2	T3	T4
CALCIUM (mg/dl)	4.24±0.15	5.32±0.21 ##	3.45±0.13 *	4.53±0.36	3.84±0.23**	3.75±0.16	3.65±0.03**
INORGANIC PHOSPHATE (I.P.)	0.85±0.01	1.19±0.09 ##	0.65±0.02 *	0.95±0.05	0.81±0.09*	0.7±0.05	0.68±0.09*

(mg/dl)							
URIC ACID (mg/dl)	2.44±0.19	4.06±0.25#	1.65±0.13*	2.57±0.26*	2.12±0.16*	2.07±0.26*	1.09±0.16*
Creatinine (mg/dl)	0.72±0.01	0.92±0.01	0.79±0.01***	0.86±0.01***	0.85±0.01***	0.83±0.01***	0.81±0.01***

N = 6; Significance: *** P<0.001, ** P<0.01, * P<0.05 from control
# Highly significant difference from normal p ≤ 0.05
Significant difference from normal p < 0.01

Discussion

Male rats were selected to induce urolithiasis because the urinary system of male rats has more resemblance to that of humans. In addition, earlier studies have reported that the amount of stone deposition in female rats was significantly less compared to male rats [36].

Consistent with previous reports [20,37], urine volume was significantly (p<0.001) increased by calculi-inducing treatment to rats (Table1). Treatment of animals with ERM in curative as well as preventive regimen also caused significant increase in urine volume in dose dependent manner. However, this increased urine volume in ERM-treated animals was significantly less than that of calculi-induced animals. This may be due to diuretic effect of ERM [7,8] which reduced calcium oxalate super saturation in the urine and there by stone formation.
As reported in some previous reports [20,37,38], calculi-inducing treatment caused significant increase (p<0.001) in oxalate excretion in the urine (Table1). This increased urinary oxalate level was significantly decreased by treatment with all doses of ERM in curative as well as preventive regimen in dose dependent manner.
Hyper oxaluria is a more significant risk factor in the pathogenesis of renal stone and reduced rate of alteration of urinary oxalate by ERM treatment indicates that ERM act by inhibiting some steps of oxalate synthesis.

Consistent with previous reports [20,36], urinary total protein excretion was significantly (p<0.001) increased in calculi-induced animals compared to vehicle treated animals (Table1). This increased urinary total protein excretion was significantly decreased by treatment with all doses of ERM in curative as well as preventive regimen in dose dependent manner. Super saturation of urinary colloids results in precipitation of crystal initiation particle which when trapped acts as an idus leading to subsequent crystal growth. This process is associated with proteinuria that reflects proximal tubular dysfunction [36]. Treatment of ERM showed significant reduction in the protein excretion and thus might have prevented then idus formation of crystal formation.

As reported in previous reports [20,37], urinary phosphate excretion was significantly (p<0.001) increased by calculi-induced treatment to rats (Table1). This increased urinary phosphate excretion was significantly decreased by ERM treatment in both curative as well as preventive regimen in dose dependent manner. Previous study reports showed that increased urinary phosphate excretion along with oxalate stress provides an environment suitable for stone formation by forming calcium phosphate crystals, which induces calcium oxalate deposition [36]. In the present study, the ERM treatment found to decrease the

rate of urinary phosphate excretion and there by reduced the risk of stone formation.

Consistent with previous reports [20,36], urinary uric acid excretion was significantly (p<0.001) increased in calculi-induced animals compared to vehicle treated animals (Table1). Increased uric acid excretion has been reported in kidney stone patients and hyperoxaluric rats. Uric acid reported to interferes with calcium oxalate solubility. It also binds and reduces the inhibitory activity of glycosaminoglycans [20,36]. The predominance of uricacid crystals in calcium oxalate stones and the observation that uric acid binding proteins are capable of binding to calcium oxalate and modulate its crystallization also suggests its primary role in stone formation [36]. In the present study, the ERM treatment caused significant decrease in the urinary uric acid excretion and thereby reduces the risk of stone formation.

As reported in previous reports [20, 37], urinary calcium excretion was significantly (p<0.001) decreased by calculi-inducing treatment to rats (Table2). Urinary calcium is utilized in calcium oxalate nucleation and crystal growth process and this may result in decreased urinary calcium excretion. This decreased urinary calcium excretion was significantly increased by ERM treatment suggesting that ERM interferes with nucleation and/or growth of calcium oxalate crystals.

Fig1: The calcium oxalate crystals, viewed under light microscope (400x), in 3hurine from animals of (A) normal, (B)calculi induced (untreated) (C) cystone treated (D)REM 250 mg/kg (E) REM 350 mg/kg (F) REM 450 mg/kg (G) REM 550 mg/kg

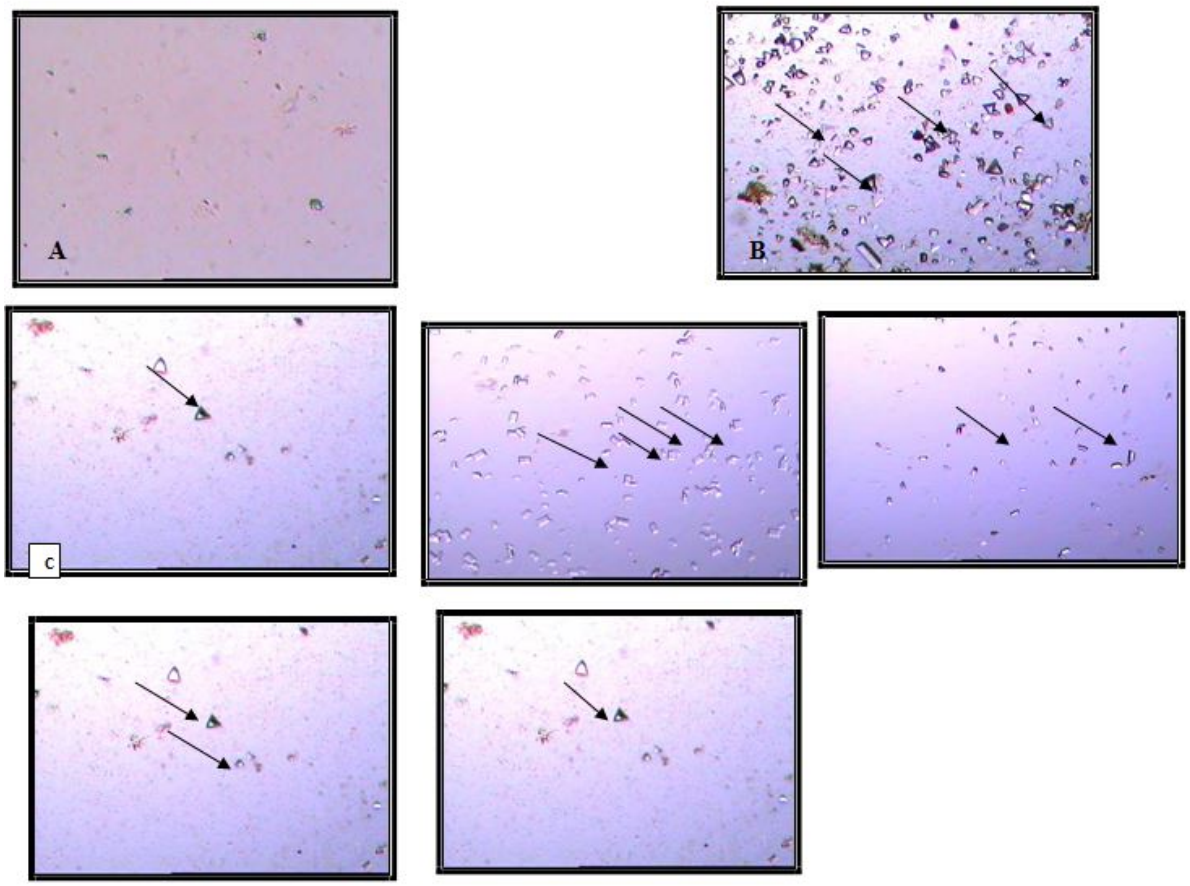
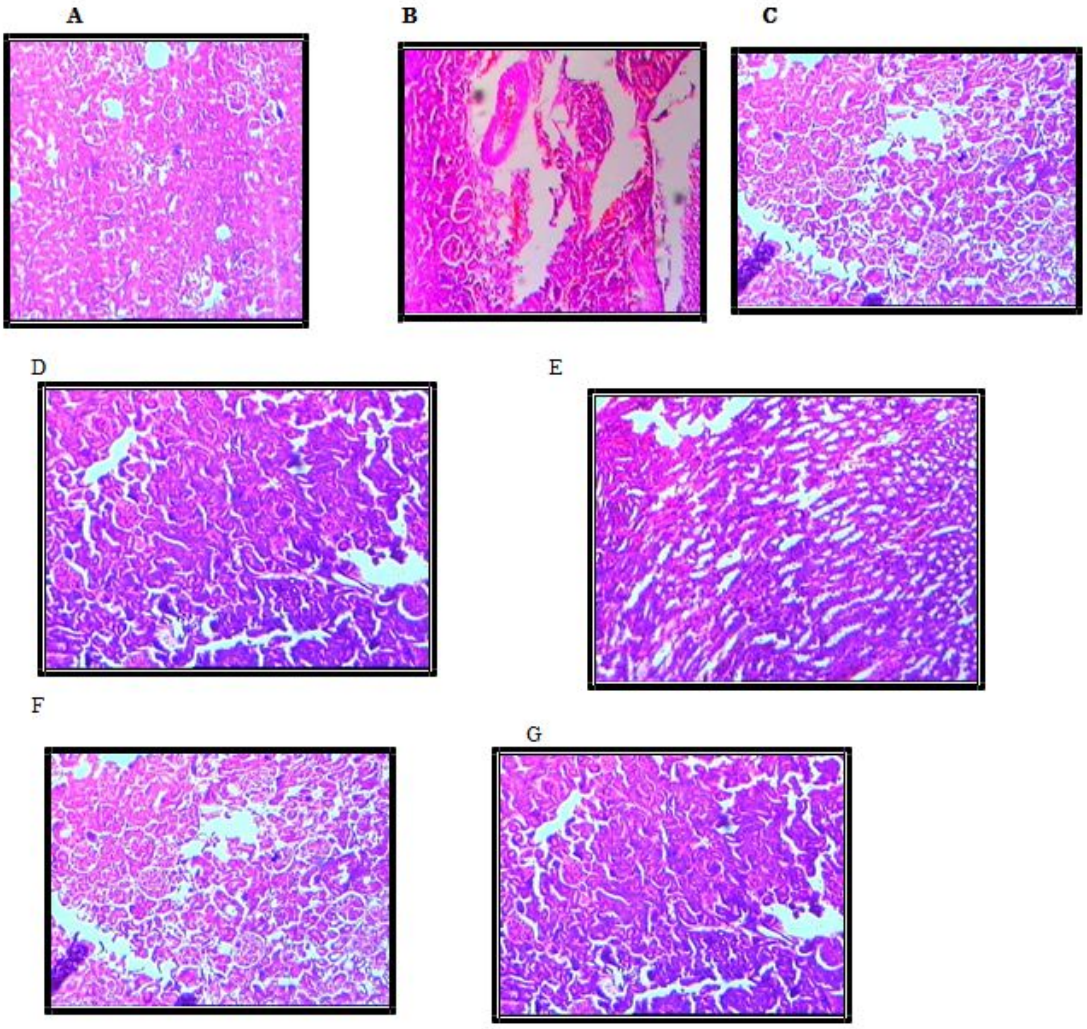


Fig2: Microscopic images of kidney sections under light microscope(100x) after Hematoxylin and Eosin

staining from animals of(A)normal,(B)calculi induced (untreated) (C) cystone treated (D)REM 250 mg/kg (E) REM 350 mg/kg (F) REM 450 mg/kg (G) REM 550 mg/kg



As reported in previous reports [20], urinary citrate level was significantly (p<0.001) decreased by calculi-inducing treatment to rats (Table2). Hypocitraturia is the major metabolic abnormality in patients with renal stones. In the present study, the ERM treatment caused significant increase in citrate excretion in both curative as well as preventive regimen in dose dependent manner. This indicates that ERM interferes with tubular citrate reabsorption which is reported as the main mechanism regulating citrate excretion in lithiatic patients [39].

In urine microscopy, no calcium oxalate crystals were seen in the urine of vehicle control animals (Fig.1). Calculi-inducing treatment resulted in appearance of bipyramid shaped calcium oxalate crystals in the urine. This calcium oxalate crystal Luria was decreased in the ERM-treated animals in dose-dependent manner.

Consistent with previous reports [20], serum levels of creatinine, uric acid and BUN were significantly (p<0.001) increased in the calculi-induced animals compared to vehicle treated animals (Table3). In addition, increased lipid Peroxidation was also observed in the kidneys of calculi-induced animals (Table3). Elevated urinary oxalate level has been reported to induce lipid peroxidation and cause renal damage by reacting with

polyunsaturated fatty acids in cell membrane [20,40]. This renal damage was indicated by the elevated serum levels of creatinine, uric acid which are markers of glomerular and tubular damage. In the present study, ERM treatment showed to prevent elevation of serum levels of these markers as well as lipid peroxidation of kidney tissue. This indicates that ERM act by inhibiting the lipid peroxidation and thereby reduces the extent of tubular dysfunction.

Consistent with previous reports [36,37], renal calcium, oxalate and phosphate levels were significantly (p<0.001) increased by calculi-inducing treatment to rats (Table3). These elevated levels of calcium, oxalate and phosphate in kidney tissue were significantly decreased by treatment with all doses of ERM in curative as well as preventive regimen in dose dependent manner. This may be due to protective effect of ERM which inhibits retention of stone in the renal tubules.

Kidneys of calculi induced animals showed marked histological changes such as accumulation of calcium oxalate crystals, dilatation of tubules, interstitial fibrosis and dense infiltration of mononuclear cells (Fig.2). This resulted insignificant (p<0.001) increase in the damage index of kidneys of calculi-induced animals as compared to vehicle-treated animals(Table3). All these histological changes and damage index were significantly (p<0.001) reduced in the ERM-treated animals of both curative and preventive regimen in dose dependent manner. The Pizzolato's staining method revealed the presence of calcium oxalate deposits (stained black) in tubules of all regions of kidney (cortex, medulla and papilla) of calculi-induced animals. However, such deposits were small and less abundant in kidneys of animals treated with ERM as compared to those in the calculi-induced kidneys (Fig.3). This maybe in part due to potent antioxidant capacity [9] of the plant.

CONCLUSION

The present investigation supports the use of *RHUS MYSORENSIS* in folk medicine against urolithiasis. It is concluded that administration of *RHUS MYSORENSIS* seed extract reduced and prevented the growth of urinary stones. It also seems that the preventive effect is more effective than its curative treatment. The possible mechanism underlying this effect is mediated collectively through diuretic, antioxidant, anti-inflammatory properties and lowering the concentration of urinary stone-forming constituents. The further phytochemical exploration is required to establish exact mechanism of action.

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