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Original Article

# Assessing the Antiurolithiatic Potentials of *Calamus floribundus* Griff. An *in vitro* and *in vivo* Approaches

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## ABSTRACT

**Background:** Urolithiasis, which is acquiring clinical relevance, is one of the most prevalent diseases seen in urology clinics. The most often reported uroliths in humans are oxalate, struvite, urate, brushite and cystine. The globally rising occurrence and severity of urolithiasis has made it an issue of demanding medical attention. Medicinal herbs and natural substances have been shown to be useful in the treatment and management of urolithiasis across various recent investigations. *Calamus floribundus* Griff. belongs to the Aracaceae family and its urolithiatic potential has yet to be established. **Objectives:** The objective of the study is to evaluate the antiurolithiatic potential of methanolic extract *Calamus floribundus* Griff. utilizing *in vitro* and *in vivo* methods. **Materials and Methods:** Nucleation assay and aggregation assay was done to evaluate the *in vitro* antiurolithiatic activity. In the *in vivo* studies ethylene glycol induced and glycolic acid induced model were used. MTT assay was done to evaluate the cytotoxic effect of the methanolic extract of *Calamus floribundus* Griff. **Results:** The $IC_{50}$ value of the extract was found to be 767.394±0.22 µg/mL and 759.435±0.29 µg/mL in the nucleation and aggregation assay respectively. In the *in vivo* study all the biochemical parameters were normalised in dose dependent manner. Methanolic extract exhibited cytotoxic effect on NRK-52E cell lines with a $IC_{50}$ value of 11.1 µg/mL. **Conclusion:** The obtained results indicates for the antiurolithiatic potential of *Calamus floribundus* Griff.

**Keywords:** *Calamus floribundus* Griff., Kidney stone, Nephrolithiasis, Urolithiasis.

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## INTRODUCTION

Urinary calculi are considered as the 3rd most common urinary system disorder.[1] It is a multifarious process that begins with the generation of microcrystals in the urine and typically ends with the development of matured renal calculi.[2] Studies suggest that, calcium oxalate and calcium phosphate accounts for 80% of renal stones, whereas struvite 10%, uric acid 9% and cystine accounting for 1%.[3] Age, sex, nutrition, geography, systemic and local medical problems, hereditary factors and urine composition are all risk factors for urolithiasis.[4] Although the processes behind stone formation are not fully understood, it is commonly believed that urinary lithiasis is multifactorial, involving supersaturation, crystal nucleation, precipitation, crystal growth, aggregation of crystals and crystal retention in renal tubular epithelial cells.[5] Though urine is usually supersaturated with stone-forming minerals, the crystallisation inhibitory capability of urine prevents urolithiasis in the majority of people, but this inherent restraint is absent in stone-forming individuals.[6]

If left untreated, urinary calculi can lead to major medical complications such as blockage, hydronephrosis, infection and bleeding in the urinary tract system.[7] Despite ground-breaking advances in the management of kidney stones in the form of improved surgical interventions, development of techniques such as extracorporeal shock wave lithotripsy, percutaneous nephrolithotomy and ureteroscopy, the costs and recurrence rate continue to be a concern. Furthermore, with these treatments, renal injury and impaired kidney function become the primary concerns. Diuretics, chelating drugs and nutrition treatment have all been used to treat urolithiasis.[8,9]

Most of the medications used in conventional medical practices were made using plants and have been shown to be beneficial in a range of medical circumstances. Notably, traditional herbal remedies are more effective and have fewer adverse effects than contemporary pharmaceuticals and they help minimise the recurrence rate of kidney stones. Many plants are said to be effective in the treatment of urinary stones in the extensive

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Ayurvedic literature; however, many plants have yet to be explored for their pharmacological activities.[10]

According to ethnomedicinal evidence, numerous plants in the Aracaceae family have urolithiatic potential. *Calamus floribundus* Griff. belongs to the Aracaceae family and its urolithiatic potential has yet to be established.[11] This plant's vernicular names are Lejai-bet in Assamese and Lee in Manipuri. In Northeast India, this plant is extensively consumed as a vegetable. This plant, on the other hand, has a strong ethnomedicinal usage. The shoot and root parts are used to cure pain, wounds, cuts and insect and dog bites. According to T. N. Manohara's 2013 study, the plant has a large number of proteins, dietary fibres and carbs. In dyspepsia, the ripe fruit pulp is consumed with a little common salt and honey.[12] People of Nongtalang village, West Jaintia Hills, Meghalaya, India, according to Myrchiang *et al* 2018, utilise root paste to treat fever and cough.[13] According to P Jiji (2015), the young shoot is used as medication for stomach problems by ethnic communities in the Sivasagar region of Assam, India.[14] Pal Gogoi *et al*. 2021 claimed that tribes in the Tinsukia district of Assam, India, use a paste of the shoot and roots to treat wounds.[15] The current study used *in vitro* and *in vivo* approaches to evaluate the antiurolithiatic activity of the shoots of *Calamus floribundus* Griff.

## MATERIALS AND METHODS

### Plant material and extraction

Plant parts of *Calamus floribundus* Griff. was collected from Mangaldai, (Darrang district) in Assam state, INDIA. The plant specimen was identified and authenticated by Dr. N. Odyuo, Scientist-D, Hoo, Botanical Survey of India, Eastern Regional Centre, Shillong 793003. A voucher specimen of the plant was deposited in the herbarium (DU/KD/2020/02). The shoot part of the plant was cut and the outer spiny leaf sheaths removed followed by drying under shade and then the dried parts were coarsely powdered. First Petroleum ether, then chloroform, then ethyl acetate and finally Methanol (MeCF) were used to remove the dried powdered materials. The solvents were used in order of increasing polarity. Each extract was concentrated by distilling off the solvent and evaporated to dryness in Petri dish on water bath. Before usage, crude extracts were kept in airtight containers at 4°C.

**Chemicals:** All the chemicals, solvents and reagents used were of analytical grade.

**Glasswares and instruments:** All glassware used were autoclaved for 15 min at 120°C.

**Animals:** The Institutional Animal Ethics Committee of Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh Assam authorized (IAEC/DU/199 Dated 19.05.2021) all of the experimental protocols and methods utilized in this experiment. The animals were kept in standardised conditions (temperature 27±1°C, humidity 60±4%, natural lighting) and were contained in polypropylene cages with regular supply of feed and water.

### *In vitro* evaluation of antiurolithiatic potential

#### *Nucleation assay*

Calcium Chloride ($CaCl_2$) solution and Sodium Oxalate ($Na_2C_2O_4$) solution were made separately in a buffer comprising of Tris-HCl 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5, in a way that the final concentration of 5 mmol/L and 7.5 mmol/L were achieved for $CaCl_2$ and $Na_2C_2O_4$ respectively. 100 µL of MeCF and standard (cystone) at different concentrations (200,400,600,700 and 800 µg/mL) was added to 950 µL of $CaCl_2$ solution. 950 µL of $Na_2C_2O_4$ was added to initiate the crystallisation process. The temperature of the reaction mixture was kept constant at 37°C. After 30 min, the Optical Density (OD) of the reaction mixture was measured at 620 nm. Comparing the induction time in the presence of extract with that of control, nucleation rate was estimated.[16] The percentage inhibition of nucleation for the extract and Cystone was calculated using the formula:

$$\text{Percentage inhibition} = (1 - \text{OD of test/OD of control}) \times 100$$

### Aggregation assay

The methodology used in this test is similar to that reported by Atmani and Khan with some minor modification. $CaCl_2$ and $Na_2C_2O_4$ at 50 mmol/L were mixed to prepare the Calcium oxalate (CaOx) crystals (seed).[17] Both solutions were equilibrated in a water bath at 60°C for 1 hour before being cooled to 37°C overnight. Centrifugation was used to collect the crystals, which were subsequently evaporated at 37°C. CaOx crystals at a final concentration of 0.8 mg/mL, buffered with Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 were used. Experiments were performed at 37°C in the absence or presence of the MeCF and standard (Cystone) (200,400,600,700 and 800 µg/mL). By comparing the turbidity in the presence of the extract to that obtained in the control, the percentage aggregation Inhibition rate (Ir) was computed.[18] Formula used is as follows:

$$\text{Percentage inhibition} = (1 - \text{Turbidity of sample/Turbidity of control}) \times 100$$

### *In vivo* evaluation of antiurolithiatic potential

#### *Ethylene glycol and ammonium chloride induced urolithiasis*

A total of 25 animals (male wistar rats 180-220 g) were used for this experiment. Animals were allocated into five groups, each with 5 animals ($n$=5). Group 1 was fed with regular food and drinking water, it served as the normal control regimen. Group 2 to 5 received stone inducing treatment i.e., 1% ethylene glycol (w/v) and 1% ammonium chloride (w/v) for 4 days followed by 1% Ethylene Glycol (EG) (w/v) alone in water for 28 days. The

disease control group, Group 2 received just stone-inducing therapy for 28 days. From 15th till 28th day, Group 3 (standard control group) received Cystone (Standard antiurolithiatic drug) at a dose of 500 mg/kg body weight (b.w.). Group 4 and 5 were given 250 mg/kg b.w. and 500 mg/kg b.w. of the MeCF respectively from 15th day till 28th day.[19]

## Glycolic acid induced urolithiasis model

The animals in this model were also grouped into 5 groups. Group 1 was given solely a regular diet and drinking water and thus served as the normal control. For 42 days, Group 2-5 were fed with calculi inducing diet- Commercial diet combined with 3% glycolic acid. Group 3 received standard antiurolithiatic drug cystone at 500 mg/kg b.w. dose. Group 4 and 5 were given 250 mg/kg b.w. and 500 mg/kg b.w. of the MeCF respectively. All the extract dose, standard drug were given orally once daily for 42 days.[20]

## Parameters measured

All the parameters listed below were recorded/measured upon completion of, 28th day for Ethylene glycol and ammonium chloride induced urolithiasis model and 42nd day for Glycolic acid induced urolithiasis model.

## Body weight and water intake

The change in the body weight (in %) and water consumption (24 hr), measured for all the groups.

## Biochemical Parameters

### *Urine analysis*

Rats were kept in metabolic cages for 24 hr to collect urine. Through urine collection, animals had free accessibility to normal water for drinking. Quantity of important elements like phosphorus, calcium and magnesium oxalate, uric acid, urea and citrate were measured in urine samples.

## Serum analysis

Each group's rats were sacrificed and blood was collected. The serum was centrifugation at 6000 rpm for 15 min and then examined for phosphorus, calcium, urea, creatinine and uric acid.

## Kidney homogenate analysis

Kidneys were collected from the sacrificed animals and then perfused with Phosphate Buffer Saline (PBS). The left kidneys were minced very fine and a 20% homogenate was made in a Tris-HCl buffer (0.02 mol/L) (pH 7.4). Total kidney homogenate was utilised to compute the phosphorus, calcium and oxalate contents in the tissues.

## Kidney histopathology

Right kidney kept in 10% formalin. Thin slices of 5 µm thickness were cut with a Leica RM-2126 microtome and placed on slides after being stained with Haematoxylin and Eosin (H and E). The slides were examined using a light microscope to evaluate kidney architecture and CaOx crystal depositions.

## Statistical analysis

All of the data are shown as the mean±standard deviation of their values derived from triplicate testing. The significance ($p<0.05$) of the findings is determined by students t-test with the help of Graph Pad Prism 8.4.2 software.

## Cell-line and culture

Renal tubular epithelial cells generated from normal rat epithelium, also known as NRK-52E cells, were obtained from the National Centre of Cell Sciences in Pune, India. Dulbecco's Modified Eagle Medium with High Glucose (DMEM-HG), enhanced with 10% Foetal Bovine Serum (FBS), was used for growing and preserving the cells. The cell pellet was produced by centrifuging at 300xg after aspirating into a 5 mL tube. The cell count was adjusted, using Roswell Park Memorial Institute (RPMI) medium containing 10% FBS, such that 50 µL of suspension contains approximately 10,000 cells.

## Cytotoxicity Study

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) method was employed to evaluate the toxicity of MeCF towards NRK-52E cells. 50 µL of cell suspension was applied to each well of a 96-well microtitre plate and incubated for 24 hr at 37°C and 5% $CO_2$. After 24 hr, the spent medium was aspirated. To each well, 200 µL of various test concentrations (2X) of MeCF (6.25, 12.5, 25, 50 and 100 µg/mL) were applied. The plate was then incubated at 37°C and 5% $CO_2$ atmosphere for 24 hr. Subsequently, the plate was incubated for 24 hr at 37°C with 5% $CO_2$. Following the removal of the plate from the incubator, the medium carrying the drug was aspirated. To achieve a final concentration of 0.5 mg/mL, 200 µL of medium comprising 10% MTT reagent was thereafter introduced to each well. The plate was then left to incubate for 3 hr at 37°C with a 5% carbon dioxide atmosphere. Without causing any disruption to the crystals that had grown, the culture media was fully withdrawn. The produced formazan was then solubilized by adding 100 µL of solubilization solution (dimethyl sulfoxide, or DMSO) followed by carefully shaking the plate in a gyratory shaker. A microplate reader has been utilized to measure the absorbance at 570 nm and 630 nm wavelengths. After deducting the background as well as the blank, the percentage growth inhibition was computed and the dose-response curve for the cell line was used to determine the concentration of test medication required to inhibit cell growth by 50% ($IC_{50}$).[21]

## RESULTS

### *In vitro* evaluation of antiurolithiatic potential

#### *Nucleation assay*

The nucleation test used the solution's turbidity to measure the total amount of crystals produced. The recorded absorbance of the control was deducted from the absorbance obtained from the test. When incubated with sodium oxalate, there was a sharp decline in absorbance with a rise in the level of both the extract and cystone. The percent inhibition of cystone, MeCF were in the range of 33-64% and 13-53% respectively. On the other hand, Cysteine shown a 17-56% percent inhibition (Table 1). The percentage of nucleation inhibition by cystone and MeCF increased in a dose-dependent manner (Figure 1). The $IC_{50}$ value of the Cystone was found to be 555.908±0.12 µg/mL whereas for MeCF it was found to be 767.394±0.22 µg/mL (Table 1).

### Aggregation assay

In aggregation experiment, cystone and MeCF showed a dose-dependent increase in inhibition (Figure 2). The percentage inhibition ranged from 28 to 63% in case of cystone which is the most potent. However, for MeCF it came out to be ranging from 21%-53%. The $IC_{50}$ (Table 2) was lowest for Cystone (646.01±0.32 µg/mL) and for MeCF it was 759.435±0.29 µg/mL.

### *In vivo* evaluation of antiurolithiatic potential

Tables 3 and 4 represents the effect of MeCF on the different blood, urine and kidney homogenate parameters in ethylene glycol induced and glycolic acid induced urolithiatic rats respectively. The results are discussed as follows:

### Measurement of changes in body weight and water intake

Before the trial began, each group's body weight and water consumption were the same. Tables 3 and 4 shows that in the disease control group there was loss in body weight, whereas other groups animal showed significant improvement in weight after the experiment. With the exception of the stone-induced group, where it was much higher than the typical control group, observations about water intake were nearly same in all of the groups.

### Urine, serum and Kidney homogenate analysis

Effect of methanolic extract of *Calamus floribundus* and standard cystone on the urine and serum parameters as well as kidney homogenate analysis are listed in Tables 3 and 4 for both ethylene glycol induced and glycolic acid induced urolithiatic models respectively.

**Table 1: $IC_{50}$ value for nucleation assay.**

| Nucleation inhibition | $IC_{50}$ (µg/mL) |
|---|---|
| Cystone | 555.908±0.12 |
| MeCF | 767.394±0.22 |

**Table 2: $IC_{50}$ value for aggregation assay.**

| Aggregation inhibition | $IC_{50}$ (µg/mL) |
|---|---|
| Cystone | 646.019± 0.32 |
| MeCF | 759.435± 0.29 |

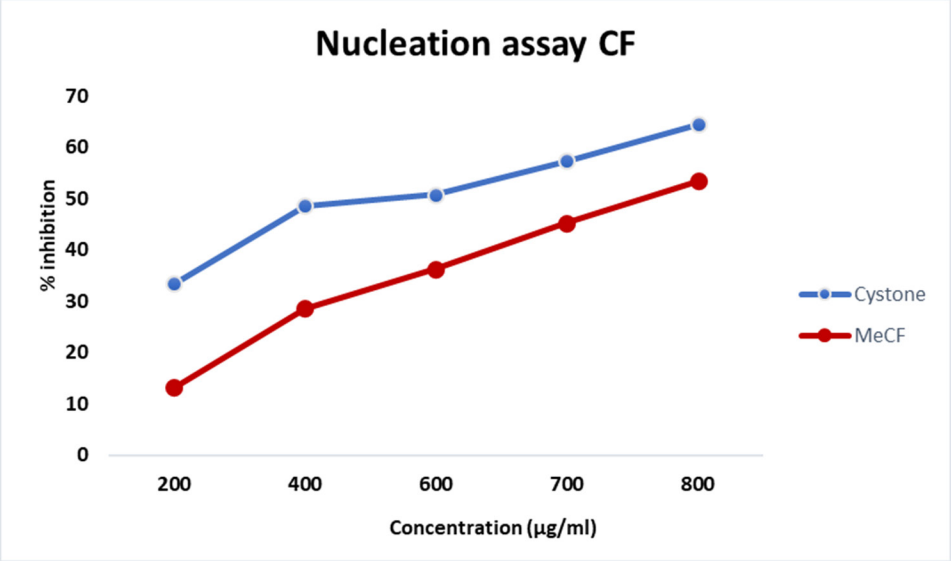


**Figure 1:** Percentage inhibition for nucleation assay. *All the values are expressed as %inhibition±SD and are significant when tested using Two-way ANOVA, Dunnett's multiple comparisons test, $p<0.0001$.

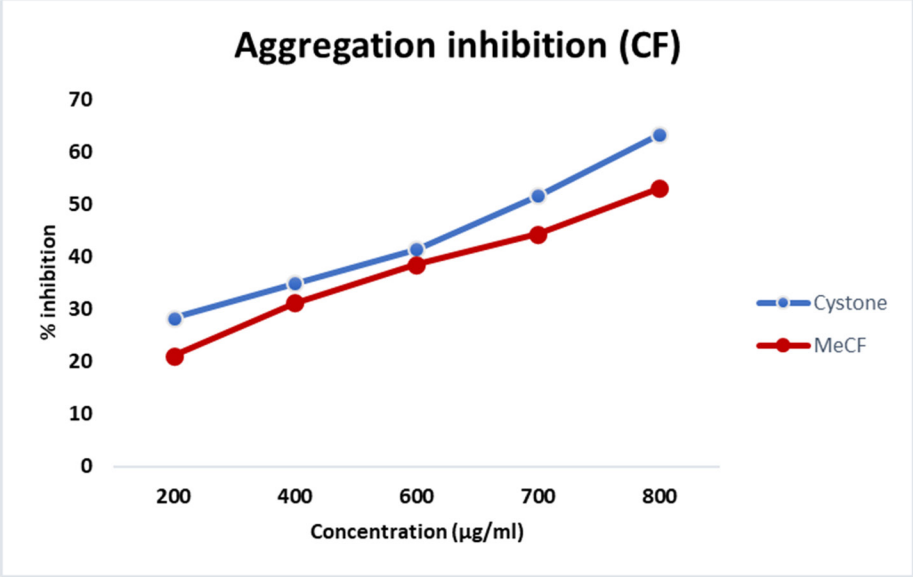


**Figure 2:** Percentage inhibition for aggregation assay. *All the values are expressed as %inhibition± SD and are significant when tested using Two-way ANOVA, Dunnett's multiple comparisons test, $p<0.0001$.

## Kidney histopathology

The kidney histopathological results were similar in both the animal models (Figures 3 and 4). Normal control animals' kidneys had normal glomeruli and tubular epithelial cell organization. The glomeruli, blood vessels and renal tubules did not exhibit any histological alterations. The histological analysis of the urolithiatic control group revealed a minor multifocal influx of lymphocytes within the interstitium alongside dilated collecting tubules, tubular cystic dilatation and significant crystal deposition in the proximal convoluted tubules. The kidneys of the group receiving standard treatment exhibited reasonable glomerulus congestion. The tubules and blood arteries remained unchanged. Rats treated with MeCF (250 mg/kg) differed significantly from urolithiatic control rats. There was insignificant interstitial congestion, mild inflammation and slight tubular dilatation. The mice treated with MeCF (500 mg/kg) did not exhibit any alterations in blood vessels or tubules and only minor glomerular congestion was seen.

## Cytotoxicity Study

The effects of various concentration of MeCF on the viability of NRK-52E cells are shown in Figure 5. MeCF showed high cytotoxicity at the tested concentration. The $IC_{50}$ value was determined to be 11.1 µg/mL. The photomicrographic images of the cytotoxic effect of MeCF on NRK-52E is shown in Figure 6.

# DISCUSSION

We often consume dietary oxalate in our daily diets, but barely any quantities of it are absorbed. In the digestive system, ingested oxalate combines with calcium when it becomes available, or it may be broken down by gut-dwelling oxalate-degrading bacteria.[22] Stone is formed when one of the elements that constitute it goes beyond its limit. Because plants have been used as medicine since ancient times and since pharmaceutical goods are more expensive than traditional cures, phytotherapy is an important component of medical care in India. Additionally, phytotherapy appears to be a successful treatment for a wide range of disorders. Numerous plant-based products are already available on the market for use as preventive or therapeutic measures. Given that kidney stone illness is multifactorial as well as has a distinct chemical makeup, the development of conventional medicines has several drawbacks.[23] Medical experts have focused on the process of calcium oxalate kidney calculi development due to its extensive clinical incidence and difficulties of therapy.

Given the significance of nucleation in the formation of aggregates followed by the subsequent growth of crystals, an *in vitro* crystallization investigation was conducted. The nucleation assay validated the presence of substances in the extract that prevented nucleation. The mechanisms that influence crystal growth could pose as the limiting variables in the formation of stones considering the particles may grow to such a size that they obstruct the urinary system and induce stone formation.[24] The plant extracts may include compounds that prevent the formation of CaOx crystals. This characteristic of plants could be crucial in stopping kidney stones from developing. Stone formation may be significantly influenced by aggregation. Urine from normal individuals primarily consists of single crystals, while urine from recurrent calcium stone patients excretes clusters of crystals due to aggregation, also known as agglomeration.[25] Increasing the level of MeCF resulted in a greater degree of inhibition of crystal aggregation.

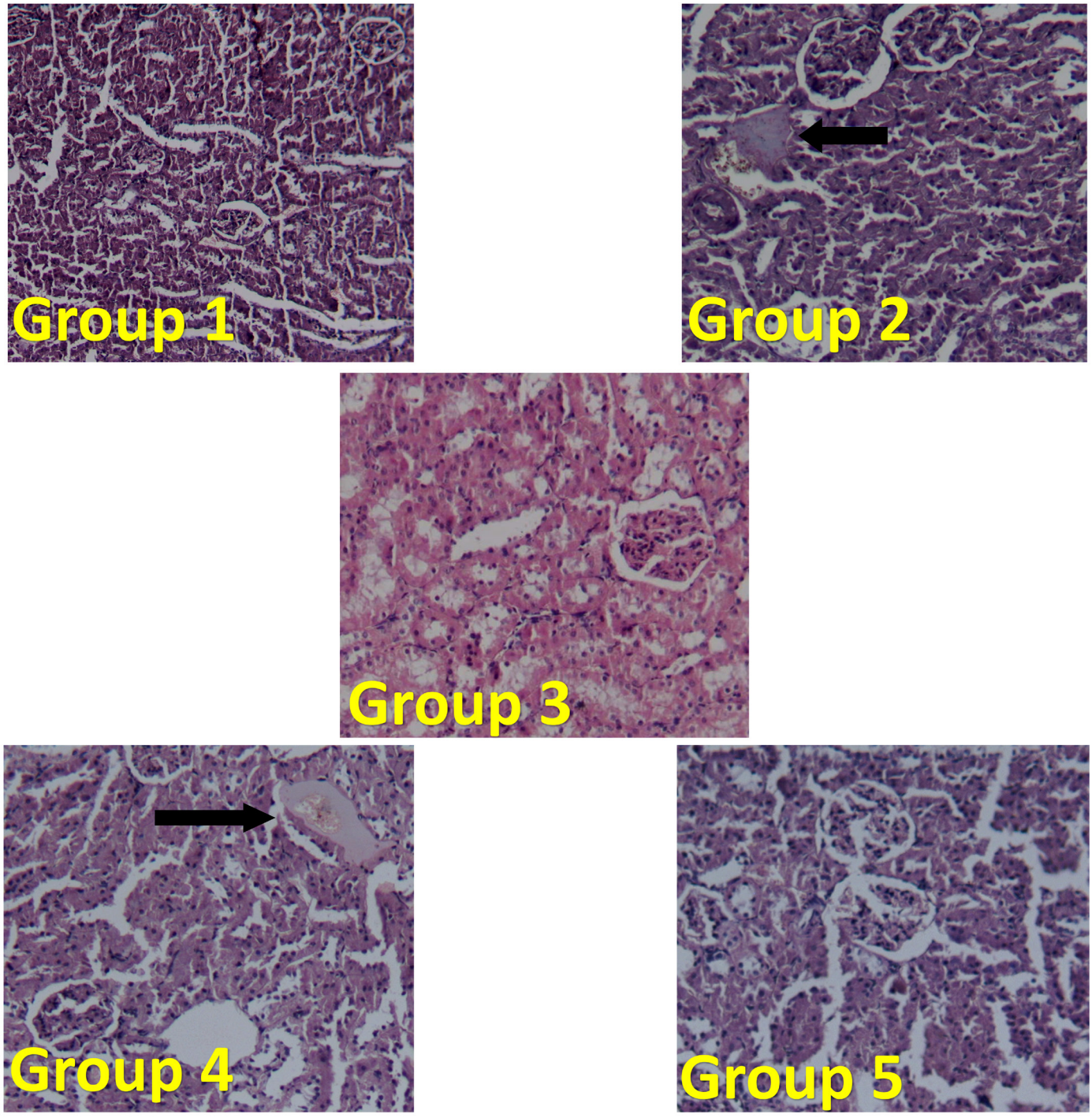


**Figure 3:** Kidney histopathological study of Ethylene glycol induced urolithiasis model. Black arrow shows crystal deposits.

According to Gupta *et al.* 2011, urolithiasis is a progressive pathological process that begins with urine that is supersaturated and progresses via nucleation, crystal development, aggregation and crystal retention.[26] The most appropriate and often utilized laboratory animal regarding calcium oxalate-induced urolithiasis is the rat. Rats with experimentally induced urolithiasis and humans have numerous parallels in the development of renal stones.[27]

The two models utilized for the *in vivo* assessment were the glycolic acid- and ethylene glycol-induced urolithiasis models. The formation of CaOx calculi in rats was exhibited by both models. Oxalate's metabolic precursor is EG. Following administration, it causes crystalluria and hyperoxaluria in rats, as well as the sporadic buildup of crystals of calcium oxalate in the kidney. When rats are given AC, they develop metabolic acidosis. Therefore, ethylene glycol is frequently employed to investigate the pathophysiology of kidney crystal deposition, either by

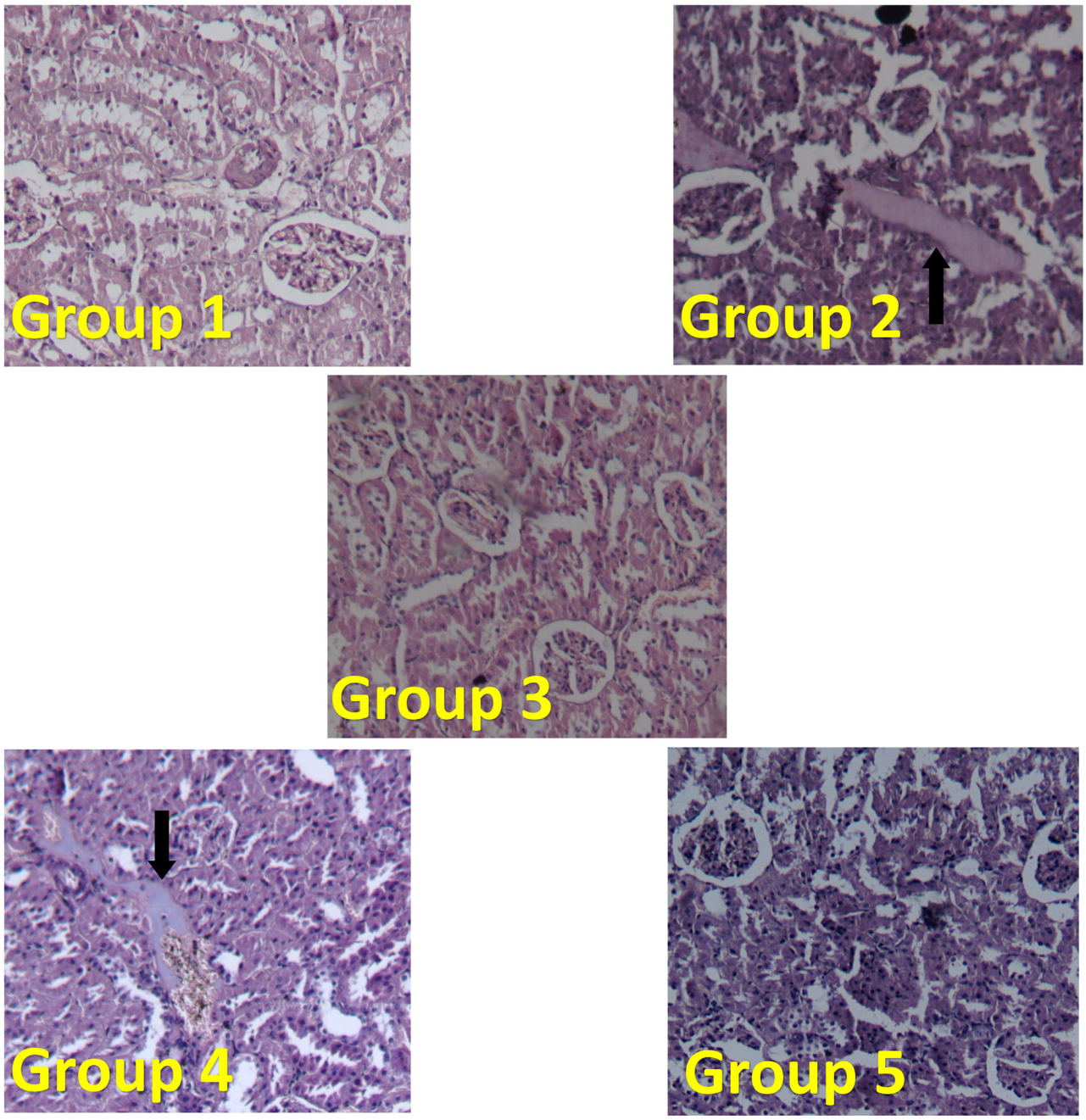


**Figure 4:** Kidney histopathology in Glycolic acid induced urolithiasis model. Black arrow shows crystal deposits.

itself or in conjunction with other medications like ammonium chloride.[27] The liver's glycolate oxidase enzyme readily converts glycolic acid to oxalate, which is why the second model uses 3% glycolic acid to induce hyperoxaluria.[20]

It was observed that the experimental rats' body weight dropped and they drank less water as a result of the urolithiasis induction. However, after receiving both the standard and test medication, these values returned to their usual levels. Urinary chemistry is an important factor in determining the crystal type and the macromolecules found on its surface. Consequently, an accurate assessment of the degree of stone formation can be obtained through the investigation of urinary chemistry in relation to the minerals that form calculi.

According to certain reports, hypercalciuria encourages the formation of calcium oxalate in urine.[28] Consequently, calcium oxalate crystals are more likely to develop in urine with high oxalate plus calcium ion concentrations. Previous studies have indicated that the production of urinary stones is influenced

by inorganic phosphate excretion in addition to calcium and oxalate.[20] The excretion of phosphate, calcium and oxalate that was seen in the disease control group of rats in this study is probably the result of calcium phosphate crystal formation. MeCF reduces oxalate, calcium and phosphate excretion, which prevents calculi development.

Significant reductions in phosphorus and calcium deposition were observed in the kidney, as evidenced by the amounts of these elements in blood, urine and kidney homogenates. It is generally known that hyperoxaluria is thought to be the primary cause of urolithiasis. The oxalates and phosphates in the renal homogenate were significantly elevated in stone induced. These speeds up the production of stones. In urolithiasis, elevated oxalate level is more important than calcium. Increased urine calcium leads to aggregation and crystal development, which enhances stone formation. In MeCF-treated mice, decreasing oxalate, calcium and phosphate concentrations in urine slowed stone formation.

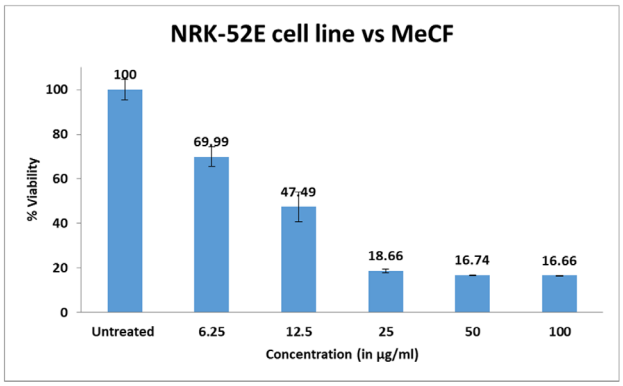


**Figure 5:** % viability of NRK-52E cell line against MeCF.

Magnesium is one of many chemical and inorganic crystallization inhibitors found in normal urine. Magnesium attaches to oxalate and turns soluble, reducing the availability of oxalate for binding with calcium, reducing CaOx production and decreasing supersaturation.[29] In addition to stone-forming rats, low magnesium levels are also found in stone formers. When taking medication, the magnesium levels recover to normal.

Stone-induced rats showed a lower amount of urine citrate. The primary metabolic aberration in individuals with renal stones is hypocitraturia. The effectiveness of citrate in preventing urinary stones has been the subject of substantial investigation. Studies have demonstrated that citrate is particularly efficient in

**Table 3: Effect of *Calamus floribundus* on the parameters in Ehylene glycol induced urolithiatic rats.**

| Parameters | Group 1 NC | Group 2 DC | Group 3 std | Group 4 MeCF D1 | Group 5 MeCF D2 |
|---|---|---|---|---|---|
| Change in body weight | 25.12±0.91$^{D▲}$ | -5.91±1.2$^{N▲}$ | 21.99 ±0.82$^{N▲D▲}$ | 14.86±0.83$^{N▲D▲}$ | 15.68±1.13$^{N▲D▲}$ |
| Water intake (ml/24 hr) | 8.9±0.23$^{D▲}$ | 14.92±0.29$^{N▲}$ | 10.22±0.17$^{N■D▲}$ | 11.23±0.28$^{N▲D▲}$ | 10.3±0.19$^{N■D▲}$ |
| **Urine (mg/dL)** | | | | | |
| Calcium U | 7.23±0.41$^{D▲}$ | 13.98±0.49$^{N▲}$ | 9.11±0.27$^{N▲D▲}$ | 12.12±0.43$^{N▲D▲}$ | 11.24±0.32$^{N▲D▲}$ |
| Phosphorus U | 4.12±0.53 $^{D▲}$ | 13.61±0.38$^{N▲}$ | 7.24±0.67$^{N▲D▲}$ | 11.34±0.54$^{N▲D▲}$ | 9.32±0.03$^{N▲D▲}$ |
| Magnesium U | 3.88±0.98 $^{D▲}$ | 0.74±0.54$^{N▲}$ | 2.51±0.29$^{N■D▲}$ | 2.01±0.19$^{N▲D■}$ | 2.35±0.22$^{N▲D▲}$ |
| Oxalate U | 1.15±0.78 $^{D▲}$ | 4.59±0.29$^{N▲}$ | 2.52±0.61$^{N■D▲}$ | 2.99±0.23$^{N▲D▲}$ | 2.78±0.43$^{N▲D▲}$ |
| Uric acid U | 3.37±0.42 $^{D▲}$ | 7.01±0.23$^{N▲}$ | 4.81±0.52$^{N▲D▲}$ | 5.78±0.38$^{N▲D•}$ | 4.99±0.62$^{N▲D▲}$ |
| Urea U | 11.91±0.03 $^{D▲}$ | 19.56±0.19$^{N▲}$ | 13.32±0.11$^{N■D▲}$ | 16.78±0.11$^{N▲D▲}$ | 14.92±0.09$^{N▲D▲}$ |
| Citrate U | 20.15±0.09 $^{D▲}$ | 12.72±0.12$^{N▲}$ | 18.56±0.20$^{N▲D▲}$ | 15.78 ±0.09$^{N▲D▲}$ | 14.24±0.03$^{N▲D▲}$ |
| **Serum (mg/dL)** | | | | | |
| Calcium S | 6.01±0.45 $^{D▲}$ | 13.19±0.23$^{N▲}$ | 7.43±0.21$^{N▲D▲}$ | 11.21±0.23$^{N▲D▲}$ | 9.9±0.19$^{N▲D▲}$ |
| Phosphorus S | 2.31±0.21 $^{D▲}$ | 8.72±0.29$^{N▲}$ | 5.41±0.26$^{N▲D▲}$ | 7.36±0.31$^{N▲D■}$ | 6.91±0.25$^{N▲D▲}$ |
| Urea S | 22.62±0.67 $^{D▲}$ | 57.9±0.56$^{N▲}$ | 25.63±0.59$^{N▲D▲}$ | 46.51±0.49$^{N▲D▲}$ | 38.63±0.61$^{N▲D▲}$ |
| Creatinine S | 0.32±0.12 $^{D▲}$ | 2.73±0.28$^{N▲}$ | 1.34±0.53$^{N•D■}$ | 1.67±0.36$^{N•D■}$ | 1.39±0.16$^{N•D■}$ |
| Uric acid S | 1.26±0.19 $^{D▲}$ | 5.38±0.32$^{N▲}$ | 2.25±0.41$^{N•D▲}$ | 4.16±0.42$^{N▲D•}$ | 3.42±0.19$^{N▲D▲}$ |
| **Kidney Homogenate (mg/dL)** | | | | | |
| Calcium KH | 5.35±0.64 $^{D▲}$ | 11.05±0.31$^{N▲}$ | 6.54±0.73$^{N•D▲}$ | 9.33±0.36$^{N▲D▲}$ | 8.41±0.43$^{N▲D▲}$ |
| Phosphorus KH | 2.47±0.21 $^{D▲}$ | 6.45±0.54$^{N▲}$ | 3.65±0.12$^{N•D▲}$ | 4.65±0.37$^{N▲D▲}$ | 3.99±0.25$^{N▲D▲}$ |
| Oxalate KH | 2.09±0.32$^{D▲}$ | 6.73±0.89$^{N▲}$ | 3.12±0.43$^{N•D▲}$ | 4.16±0.19$^{N▲D▲}$ | 3.57±0.82$^{N▲D▲}$ |

*Values are expressed as Mean±SD ($n$=5), Twoway ANOVA followed by Dunnett's test, Statistical significance▲: $p$<0.0001, ■: $p$<0.001, •$p$<0.05, ♦: $p$>0.05 (not significant), N compared with normal control group, D compared with disease control group.

**Table 4: Effect of *Calamus floribundus* on the parameters in Glycolic acid induced urolithiatic rats.**

| Parameters | Group 1 NC | Group 2 DC | Group 3 std | Group 4 MeCF D1 | Group 5 MeCF D2 |
|---|---|---|---|---|---|
| Change in body weight | 24.73±1.02$^{D▲}$ | -4.68±0.81$^{N▲}$ | 18.78±0.78$^{N▲D▲}$ | 15.99±0.92$^{N▲D▲}$ | 17.26±1.32$^{N▲D▲}$ |
| Water intake (ml/24 hr) | 9.3±0.19$^{D▲}$ | 13.67±0.21$^{N▲}$ | 10.03±0.23 $^{N♦D▲}$ | 12.29±0.19$^{N▲D●}$ | 11.54±0.43$^{N▲D▲}$ |
| **Urine (mg/dL)** | | | | | |
| Calcium U | 6.92 ±0.55$^{D▲}$ | 14.12 ±0.39$^{N▲}$ | 9.34±0.25$^{N▲D▲}$ | 12.56±0.37$^{N▲D■}$ | 11.1±0.44$^{N▲D▲}$ |
| Phosphorus U | 4.63±0.43$^{D▲}$ | 13.92±0.48$^{N▲}$ | 7.85±0.77$^{N▲D▲}$ | 11.49±0.66$^{N▲D▲}$ | 10.36±0.91$^{N▲D▲}$ |
| Magnesium U | 3.88±1.01$^{D▲}$ | 0.86±0.31$^{N▲}$ | 2.53±0.20$^{N●D▲}$ | 1.92±0.24$^{N▲D●}$ | 2.27±0.54$^{N▲D■}$ |
| Oxalate U | 1.27±0.76$^{D▲}$ | 4.26±0.37$^{N▲}$ | 2.29±0.54$^{N●D▲}$ | 2.62±0.40$^{N●D▲}$ | 2.34±0.77$^{N●D▲}$ |
| Uric acid U | 3.21±0.43$^{D▲}$ | 6.91±0.21$^{N▲}$ | 4.78±0.48$^{N■D▲}$ | 5.56±0.43$^{N▲D●}$ | 5.03±0.64$^{N▲D▲}$ |
| Urea U | 12.03±0.08$^{D▲}$ | 18.78±0.15$^{N▲}$ | 14.25±0.19$^{N▲D▲}$ | 17.43±0.29$^{N▲D●}$ | 15.19±0.75$^{N▲D▲}$ |
| Citrate U | 19.97±0.21$^{D▲}$ | 13.54±0.29$^{N▲}$ | 17.16±0.31$^{N▲D▲}$ | 16.82±0.98$^{N▲D▲}$ | 14.71±0.29$^{N▲D●}$ |
| **Serum (mg/dl)** | | | | | |
| Calcium S | 6.1 ±0.39$^{D▲}$ | 12.45 ±0.25$^{N▲}$ | 7.32 ±0.49$^{N●D▲}$ | 10.62 ±0.64$^{N▲D▲}$ | 9.13 ±0.11$^{N▲D▲}$ |
| Phosphorus S | 2.22 ±0.31$^{D▲}$ | 9.15 ±0.44$^{N▲}$ | 5.62 ±0.12$^{N▲D▲}$ | 8.13 ±0.23$^{N▲D●}$ | 6.78 ±0.37$^{N▲D▲}$ |
| Urea S | 21.59 ±0.76$^{D▲}$ | 55.09 ±1.01$^{N▲}$ | 24.92 ±0.98$^{N▲D▲}$ | 46.22 ±0.76$^{N▲D▲}$ | 37.32 ±0.98$^{N▲D▲}$ |
| Creatinine S | 0.49 ±0.09$^{D▲}$ | 2.93 ±0.21$^{N▲}$ | 1.4 ±0.45$^{N♦D■}$ | 1.78 ±0.29 $^{N●D●}$ | 1.49 ±0.28$^{N●D▲}$ |
| Uric acid S | 1.19 ±0.22$^{D▲}$ | 5.3 ±0.42$^{N▲}$ | 2.69 ±0.43$^{N■D▲}$ | 3.78 ±0.30$^{N▲D■}$ | 3.19 ±0.15$^{N▲D▲}$ |
| **Kidney Homogenate (mg/dl)** | | | | | |
| Calcium KH | 5.49 ±0.69$^{D▲}$ | 11.82 ±0.45$^{N▲}$ | 6.35 ±0.76$^{N♦D▲}$ | 8.95 ±0.63$^{N▲D▲}$ | 7.22 ±0.54$^{N▲D▲}$ |
| Phosphorus KH | 2.28 ±0.30$^{D▲}$ | 6.68 ±0.83$^{N▲}$ | 3.62 ±0.09$^{N●D▲}$ | 4.78 ±0.87$^{N▲D▲}$ | 3.87 ±0.21$^{N■D▲}$ |
| Oxalate KH | 2 ±0.27$^{D▲}$ | 6.59 ±0.54$^{N▲}$ | 3.28 ±0.65$^{N●D▲}$ | 4.92 ±0.32$^{N▲D▲}$ | 3.41 ±0.97$^{N■D▲}$ |

*Values are expressed as Mean±SD ($n$=5), Two-way ANOVA followed by Dunnett's test, Statistical significance ▲: $p$<0.0001, ■: $p$<0.001, ●: $p$<0.05, ♦: $p$>0.05 (not significant), N compared with normal control group, D compared with disease control group.

inhibiting calcium oxalate.[30] According to Kok *et al.* (1986), the most firmly-rooted action of citrate in urine involves its ability to complex with calcium, which lowers the overall level of CaOx and prevents CaOx agglomeration.[31] The current investigation discovered that the MeCF therapy raised the citrate level and reduced the chance of stone formation.

In the current study, urolithic rats had higher excretion of uric acid. According to Selvam *et al.* (2001), uric acid decreases the inhibitory action of glycosaminoglycans and tampers with the solubility of calcium oxalate.[32] The fact that uric acid binding proteins can bind to calcium oxalate and alter its crystallization and the preponderance of uric acid crystals within these stones indicate that uric acid appears to possess a significant role in the formation of calcium oxalate stones.[33]

The disease control group's serum and urine uric acid levels were notably elevated than those of normal controls. The levels of uric acid were significantly maintained in the groups who got both standard and MeCF therapy.

According to Thangarathanam *et al.* (2013), urolithiasis is a condition in which calculi within the urinary system block the flow of urine, lower Glomerular Filtration Rate (GFR) and eventually lead to a build-up of nitrogenous compounds in the blood, including urea, creatinine and uric acid.[27,34] The test drug's treatment prevented these alterations, decreased blood creatinine, urea and uric acid and increased GFR-a condition that would have otherwise encouraged the development of new kidney stones.

Microscopic analysis of rat kidney sections from both models' disease control groups reveals the presence of interstitial inflammation, dilated renal tubules and calcium oxalate deposits.[35] In dose-dependent way, co-treatment with MeCF lowers calcium oxalate deposits, renal tubule dilatation and renal tubule damage.

As previously stated, hyperoxaluria is regarded as the key trigger for CaOx stones. It typically results in damage to the kidneys and alters the normal tubular epithelial cell architecture, which makes it easier for CaOx crystals to cling to and remain in the kidneys. Additionally, it modifies the integrity of the cell membrane, raises the generation of free radicals and lowers antioxidant levels, all of which promote cell death.[21] The goal of the experiment was to determine how MeCF protects cells from damage caused by

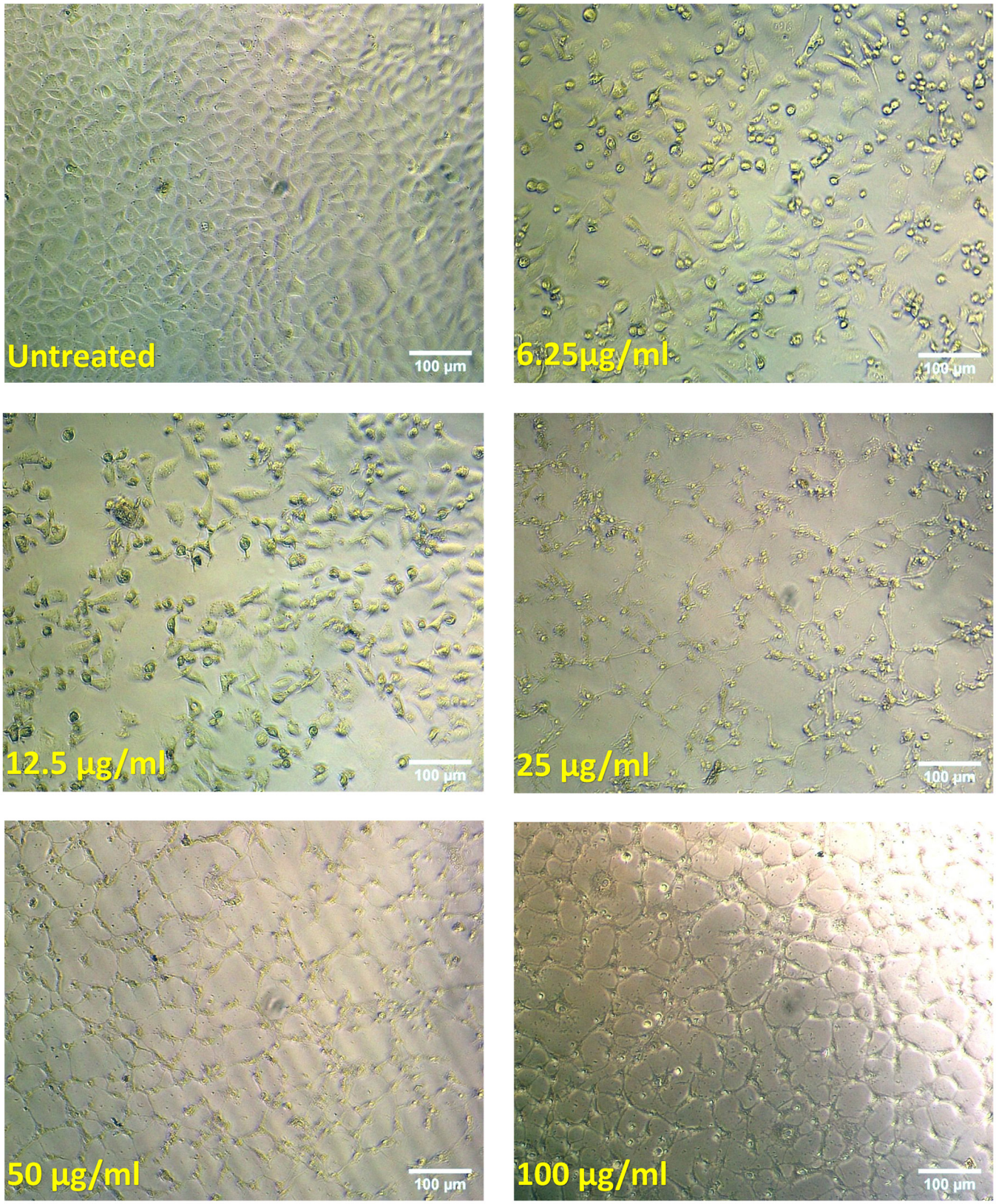


**Figure 6:** Photomicrographic images of cytotoxic effect of different concentration of MeCF on NRK-52E.

oxalate. Nevertheless, MeCF was used in the MTT test, which revealed cytotoxicity against NRK 52E with an $IC_{50}$ value of 11.1 µg/mL. MeCF's cytotoxic effects were identified, hence its cytoprotective effects were not examined further.

## CONCLUSION

The goal of this study was to assess *Calamus floribundus* Griff.'s anti-urolithiatic capability. *In vitro* nucleation as well as aggregation experiments, the methanolic extract exhibited inhibiting effect, suggesting that MeCF could act on the nucleation - aggregation stage of kidney stone development. The *in vivo* study results corroborated the *in vitro* findings.

The two models used namely the ethylene glycol induced and glycolic acid induced urolithiatic accounts for the formation of CaOx stones in the kidney. *Calamus floribundus* Griff's antiurolithiatic potential is explained by the decrease of stone promoters in MeCF-treated groups and the normalized levels of all inhibitors, which are validated by histological investigations. Nonetheless, MeCF's cytotoxicity toward NRK-52E was discovered. As a result, it may be decided to do more research to measure the extracts' active ingredients as well as assess their safety and effectiveness for usage in clinical settings.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**MeCF:** Methanolic extract of *Calamus floribundus*; Ca**Ox:** Calcium Oxalate; **OD:** Optical Density; **PBS:** Phosphate buffer saline; **FBS:** Foetal Bovine Serum; **DMEM-HG:** Dulbecco's Modified Eagle Medium with High Glucose; **NRK 52E:** Normal rat epithelium derived renal tubular epithelial cells; **RPMI:** Roswell Park Memorial Institute; **MTT:** (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide); **DMSO:** Dimethyl sulfoxide.

## SUMMARY

In this study the antiurolithiatic potential of Methanolic extract of *Calamus floribundus* was established experimentally. All the *in vitro* and *in vivo* results provide evidence of its antiurolithiatic activity. However, the cytotoxicity of the extract was detected which needs to be addressed before proceeding towards higher level of experiments.

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