



Lithotriptic Effects of *Phyllanthus fraternus* Methanol Leaf Extract on Ethylene Glycol-induced Kidney Calculi in Albino Rats

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Authors' contributions

This work was carried out in collaboration between both authors. Author IJO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author M SN managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

This study was designed to evaluate the Lithotriptic potentials of *Phyllanthus fraternus* methanol leaf extract on ethylene glycol-induced kidney calculi in albino rats. Ethylene glycol (1% v/v) was administered in their drinking water for a period of 28 days. The Treatment was done with the extract at 200 mg/kg, 400 mg/kg and 600 mg/kg body weights. Cystone® at 500 mg/kg body weight was also given for a period of 21 days to the standard control group. The serum parameters such as calcium, phosphates, magnesium and albumin were measured and evaluated. The results for the Lithotriptic activity, where the kidney homogenates were analyzed are described as thus, the phosphate concentrations when compared were significant ($p < 0.05$) between the groups' 600 mg/kg body weight (9.61 ± 1.17) and the normal control (5.67 ± 0.70). Significant differences ($p < 0.05$) for phosphates were also observed between 600 mg/kg (9.61 ± 1.17) and 200 mg/kg

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body weights (12.06 ± 0.51); 400 mg/kg (7.64 ± 0.44) and 200 mg/kg body weights (12.06 ± 0.51) and the 200 mg/kg and standard control groups Cystone® (7.96 ± 0.56) respectively. Significant differences ($p < 0.05$) were also observed for phosphates concentration, when the normal control (5.67 ± 0.70) was compared to the 400 mg/kg body weight (7.64 ± 0.44) and the standard control group Cystone® (7.96 ± 0.56). From this study, it can be deduced that, the presented data indicated that, the administration of *Phyllanthus fraternus* methanol leaf extract to rats with ethylene glycol-induced kidney calculi, reduced and prevented the growth of kidney calculi, supporting the folklore claim regarding its Lithotriptic activity.

Keywords: *Phyllanthus fraternus*; ethylene glycol; lithotriptic activity; kidney calculi.

1. INTRODUCTION

Kidney calculi are formed in a complex process that results from a succession of several physico-chemical events including supersaturation, nucleation, growth, aggregation and retention within the renal tubules [1]. Individual crystals may accumulate to form large crystals and they may bind to other specific sites in the renal epithelial cells. Nucleation, growth and aggregation of crystals are necessary steps in the formation of stones, but urine contains substances that inhibit and control these events. These substances often range from small molecules (citrate and pyrophosphates) to macromolecules (glycoproteins, glycosaminoglycans and proteoglycans). Genetic, metabolic, environmental and dietetic factors are involved in the pathogenesis of kidney calculi [2].

Epidemiological studies have revealed that, kidney calculi are more prone to men (12%) than women (6%) and is more prevailing with increase in age (20-40 in both men and women) [3]. The glomerular filtration rate (GFR) decreases when urine outflow is obstructed by stones in the urinary system. Thus, waste products, particularly nitrogenous substances such as creatinine, urea and uric acid gets accumulated [4].

In spite of considerable efforts to identify effective treatments for kidney calculi, this is a goal yet to be achieved. Therefore, it is imperative to search for alternative and cheaper sources to manage the disease.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

Fresh mature leaves of *Phyllanthus fraternus* plants were collected from a farm in Girei Local Government Area of Adamawa State ($9^{\circ}22'N$

$12^{\circ}33'E$). The plant specimen was identified and authenticated by the Department of Plant Sciences, Modibbo Adama University of Technology Yola Adamawa State.

2.2 Experimental Animals

Thirty five (35) apparently healthy male albino rats were procured from the National Veterinary Research Institute (NVRI), Vom Plateau State. These rats were allowed to acclimatize for one week prior to the commencement of the experiment. The animals were housed under standard experimental conditions and fed food and water *ad libitum*. The proper care and use of laboratory animals in research, testing, teaching, and production (*animal use*) require scientific and professional judgment based on the animals' needs and their intended use. An animal care and use program (hereafter referred to as the Program) comprises all activities conducted by and at an institution that have a direct impact on the well-being of animals, including animal and veterinary care [5].

2.3 Induction of Kidney Calculi

Thirty (30) rats had received calculi inducing treatment for a period of 28 days using 1% v/v Ethylene glycol in their drinking water. Five (5) albino rats were sacrificed; Whole blood from which the serum was extracted and the serum parameters such as calcium, phosphates, magnesium and albumin were measured. Ten (10) kidneys were harvested; 5 kidneys were preserved in 10% formalin for histopathology while the other 5 kidneys were homogenized and the parameters mentioned earlier were also measured). After confirming the presence of kidney calculi, the remaining twenty five (25) albino rats were divided into Five (5) groups containing five (5) rats each (200 mg/kg b.w.; 400 mg/kg b.w.; 600 mg/kg b.w.; standard control (Cystone®) 500 mg/kg b.w. and the induced untreated control group). There were 5 albino rats in the normal control group.

2.3.1 Determining the lithotriptic properties of *Phyllanthus fraternus*

The methanol leaf extract of *Phyllanthus fraternus* was administered in graded doses (200 mg/kg, 400 mg/kg and 600 mg/kg groups) once daily for 21 days, the administration of the extract was done by the oral route. Other groups used in the experiment include; the normal control group, induced and untreated control group and standard control (Cystone®) group respectively. Each group had contained five (5) albino rats.

2.4 Statistical Analyses

All the parameters presented were measured and expressed as Mean \pm Standard error of mean (S.E.M.). Statistical analysis was done using the GraphPad Prism Six (GraphPad Software San Diego California, USA). Differences between and within the group Means were analyzed using the analysis of variance (ANOVA), which was eventually followed by the Bonferroni Post-hoc tests. $p < 0.05$ was considered as statistically significant.

3. RESULTS

3.1 Confirming the Presence of Kidney Calculi before Treatment with the Extract

Five (5) albino rats were selected after a 28 days induction period and humanely sacrificed. Their blood and kidneys (10) were collected. The serum was analyzed for the concentration of calculi forming constituents such as calcium, phosphates and magnesium. Albumin was also analyzed. Five (5) of the kidneys were each homogenized using a standard procedure and the homogenates analyzed for stone forming constituents. The remaining five (5) kidneys were preserved in 10% formalin, and prepared using a standard procedure; the kidneys were then stained with a Haematoxylin and Eosin stain and viewed under a Leica 750 light microscope.

3.2 Lithotriptic Effects of *Phyllanthus fraternus* Methanol Leaf Extract on the Serum Parameters

The study showed that albumin levels were significant ($p < 0.05$) between the normal control (47.90 ± 3.45) and the 400 mg/kg body weight (39.30 ± 1.12) groups. The values for albumin between the 600 mg/kg and 400 mg/kg body weight groups were also significant ($p < 0.05$). However, there were significant differences ($p < 0.05$) in the albumin levels measured

between the untreated group (43.12 ± 1.44) and the standard control (Cystone®) group (43.18 ± 1.01) when both groups were compared to the normal control (47.90 ± 3.45).

The differences between the albumin levels for the 400 mg/kg b.w. group (39.30 ± 1.12) and the standard control group (Cystone®) (43.18 ± 1.01) was considered significant ($p < 0.05$).

3.3 Lithotriptic Effects of *Phyllanthus fraternus* Methanol Leaf Extract on the Parameters Examined from the Kidney Homogenates

When the kidney homogenates were analyzed, the phosphate concentrations were significant ($p < 0.05$) between the groups; 600 mg/kg body weight (9.61 ± 1.17) and the normal control (5.67 ± 0.70). The phosphates levels for the groups; 200 mg/kg body weight (12.06 ± 0.51) and the untreated group (9.81 ± 1.05) were significant ($p < 0.05$) when both groups were compared to the normal control group (5.67 ± 0.70).

600 mg/kg (9.61 ± 1.17) and 200 mg/kg body weight groups (12.06 ± 0.51); 400 mg/kg (7.64 ± 0.44) and 200 mg/kg body weight groups (12.06 ± 0.51) and the 200 mg/kg and standard control groups (Cystone®) (7.96 ± 0.56) all showed significance at $p < 0.05$ when phosphate levels analyzed. The 400 mg/kg body weight group (7.64 ± 0.44) and the standard control group (Cystone®) (7.96 ± 0.56) also presented some level of significance at $p < 0.05$.

4. DISCUSSION

Ethylene glycol administration often results in increased renal excretion of calcium and phosphates. Rats treated with Ethylene Glycol had demonstrated significant crystal deposition in the tubular parts of the kidney. These intratubular, interstitial crystals were found mostly in the cortex with significant necrosis, and tubular dilatation [4].

Increased urinary calcium is a factor that favors the nucleation and precipitation of calcium oxalate or calcium phosphate from the urine and supports subsequent crystal growth. An increase in renal phosphates is also observed in calculi induced rats. Increased urinary phosphates excretion provides an environment suitable for stone formation, by forming calcium phosphate crystals, which in turn induces calcium oxalate deposition. The reduced excretion of oxalate may be due to inhibition of the formation of oxalates by the plant extract.

Table 1. Calculi-forming constituents measured from the serum and kidney homogenates before and after induction of kidney calculi using ethylene glycol (1% v/v) on albino rats

Parameters	Serum		Kidney Homogenates	
	Before induction	After induction	Before induction	After induction
Calcium (mmol/L)	2.69 ± 0.14	2.66 ± 0.15	0.24 ± 0.06	0.41 ± 0.06
Phosphates (mmol/L)	3.04 ± 0.52	2.84 ± 0.67	2.75 ± 0.15	4.19 ± 1.60
Magnesium (mmol/L)	1.11 ± 0.29	1.28 ± 0.07	0.67 ± 0.06	0.91 ± 0.21
Albumin (g/L)	29.33 ± 2.52	37.7 ± 2.25	3.18 ± 0.85	2.57 ± 0.45

Values are Mean ± SEM; n=5

Table 2. Lithotriptic effects of methanol leaf extract of *Phyllanthus fraternus* on the serum parameters

Treatment	Calcium (mmol/L)	Phosphates (mmol/L)	Magnesium (mmol/L)	Albumin (g/L)
Normal control	2.69 ± 0.17	7.88 ± 0.35	1.56 ± 0.07	47.90 ± 3.45 ^{ac}
Untreated group	2.13 ± 0.09	7.39 ± 0.29	1.22 ± 0.09	43.12 ± 1.44 ^c
Standard control (Cystone® 500mg/kg bw)	2.13 ± 0.07	7.77 ± 0.42	1.21 ± 0.23	43.18 ± 1.01 ^{cd}
ME 200mg/kg b.w.	2.16 ± 0.11	5.78 ± 0.14	0.99 ± 0.12	43.82 ± 1.74
ME 400mg/kg b.w.	2.06 ± 0.11	7.23 ± 0.21	1.04 ± 0.09	39.30 ± 1.12 ^{abd}
ME 600mg/kg b.w.	2.04 ± 0.09	7.27 ± 0.26	1.25 ± 0.11	45.66 ± 1.20 ^b

Values are Mean ± SEM; n=5. The same letter superscript along the same column means there is significant difference at $p < 0.05$. ME = Methanol extract, BW = Body Weight

Table 3. Lithotriptic effects of methanol leaf extract of *Phyllanthus fraternus* on the parameters measured from the kidney homogenates

Treatment	Calcium (mmol/L)	Phosphates (mmol/L)	Magnesium (mmol/L)	Albumin (g/L)
Normal control	0.20 ± 0.00	5.67 ± 0.70 ^{ab}	0.79 ± 0.08	2.04 ± 0.04
Untreated group	0.20 ± 0.00	9.81 ± 1.05 ^b	1.12 ± 0.12	3.52 ± 0.39
Standard control (Cystone® 500mg/kg bw)	0.23 ± 0.03	7.96 ± 0.56 ^d	1.15 ± 0.06	2.38 ± 0.09
ME 200mg/kg b.w.	0.21 ± 0.00	12.06 ± 0.51 ^{bc}	1.37 ± 0.14	3.58 ± 0.15
ME 400mg/kg b.w.	0.28 ± 0.42	7.64 ± 0.44 ^d	0.99 ± 0.09	2.86 ± 0.24
ME 600mg/kg b.w.	0.20 ± 0.00	9.61 ± 1.17 ^{ac}	0.87 ± 0.16	3.30 ± 0.45

Values are Mean ± SEM; n=5. The same letter superscript along the same column means there is significant difference at $p < 0.05$. ME = Methanol Extract, BW = Body Weight

Citrate is one of the major inhibitors of calcium phosphate crystal growth and aggregation. Impairment in the tubular re-absorption of citrate in Ethylene Glycol treated rats reduces the citrate-calcium complex formation and frees calcium ion activity [6].

In the present study, only male rats were used because of the similarities with the urinary system of humans. Previous reports have shown that the male sex hormone testosterone promotes formation of kidney calculi while estrogen tends to inhibit its formation [4]. Treatment with the methanolic leaf extract of *Phyllanthus fraternus* restored phosphate levels, thus reducing the risk of stone formation [6].

Magnesium is known to decrease the risk of stone formation by reducing absorption of oxalate or by forming soluble complexes with oxalate in urine. A reduction in urinary magnesium could be consistent with a state of mild magnesium depletion, possibly related to the rapid decrease in ATP levels that occurs in the liver during fructose metabolism. A decrease in urinary magnesium could also predispose to kidney stones [7].

Low renal magnesium content is a common feature in stone forming individuals. A similar condition was observed in the groups treated with *Phyllanthus fraternus* extract. They were observed to have elevated urinary magnesium levels. Thus, reducing their ability to crystallize, thereby creating an ambience unfavorable for the precipitation of calculi [6]. Cystone treatment may have led to increase in citrate concentration, which might have reduced crystallization of calcium oxalate [8].

Decreased levels of albumin, observed in the untreated group might be due to decrease in protein synthesis or increased renal loss [9]. The reason for decreased serum albumin is usually renal loss. Diseases that cause damage to the glomerular membrane, increases its permeability to all proteins. However, its permeability to albumin may be particularly affected if the negatively charged groups on the membrane surface are neutralized [10].

Results evaluated from this study have been concurred by the works of [11]. They had evaluated the effects of oral administration, using the aqueous and alcoholic extracts of *Moringa oleifera* root-wood on calcium oxalate crystals in male albino rats.

[2] Reported similar findings with *Phyllanthus niruri*. This plant has been shown to interfere with

the many stages of stone formation; reducing crystals aggregation, modifying their structure and composition as well as altering the interaction of the crystals with tubular cells leading to reduced endocytosis.

The findings of [12] were in agreement to those evaluated in this study. The extract of some plant leaves suppressed increases in intracellular calcium. The exact reason for this effect is not clear, however it might be due to the increased bioavailability of NO (nitric oxide) which in turn activates cGMP (3,5 cyclic guanosine monophosphate) that controls the increase in intracellular calcium levels.

Nitric oxide donors have the capacity to control the intracellular rise in calcium levels. Plant extracts could effectively control the levels of both salts by mechanisms such as inhibiting the oxalate or increasing the bioavailability of Nitric oxide to sequester calcium through the cGMP pathway [13].

Oxidative stress is responsible for biomolecular damage. During these processes, reactive oxygen species are produced and cause tissue damage [14]. However, *Phyllanthus fraternus* possess some scavenging activity and reducing power associated with the presence of phenols and flavonoids in the plant which have been previously reported to be responsible for various antioxidant activities [15].

The detection of phenols in the *Phyllanthus fraternus* extract is evidence of its possible antioxidant activity. Polyphenols have the ability to undergo electron donation reactions with oxidizing agents producing stable species [16]. Phenol antioxidants are potent free radical terminators and their presence is a good marker of potential antioxidant activity. *Phyllanthus fraternus* showed an ability to inhibit lipid peroxidation.

Examination of Figures of the kidney sections after Haematoxylin and Eosin staining were done to detect crystal deposits and tubulointerstitial damages in the kidneys. These were visible as transparent crystals in the renal tubules. Microscopic examination of kidney sections derived from ethylene glycol induced rats showed polymorphic irregular crystal deposits which were considerably reduced or absent after treatment with the extract. Tubulointerstitial changes such as tubular atrophy, dilatation and tubular cell necrosis and interstitial inflammation were observed in the renal tissues.

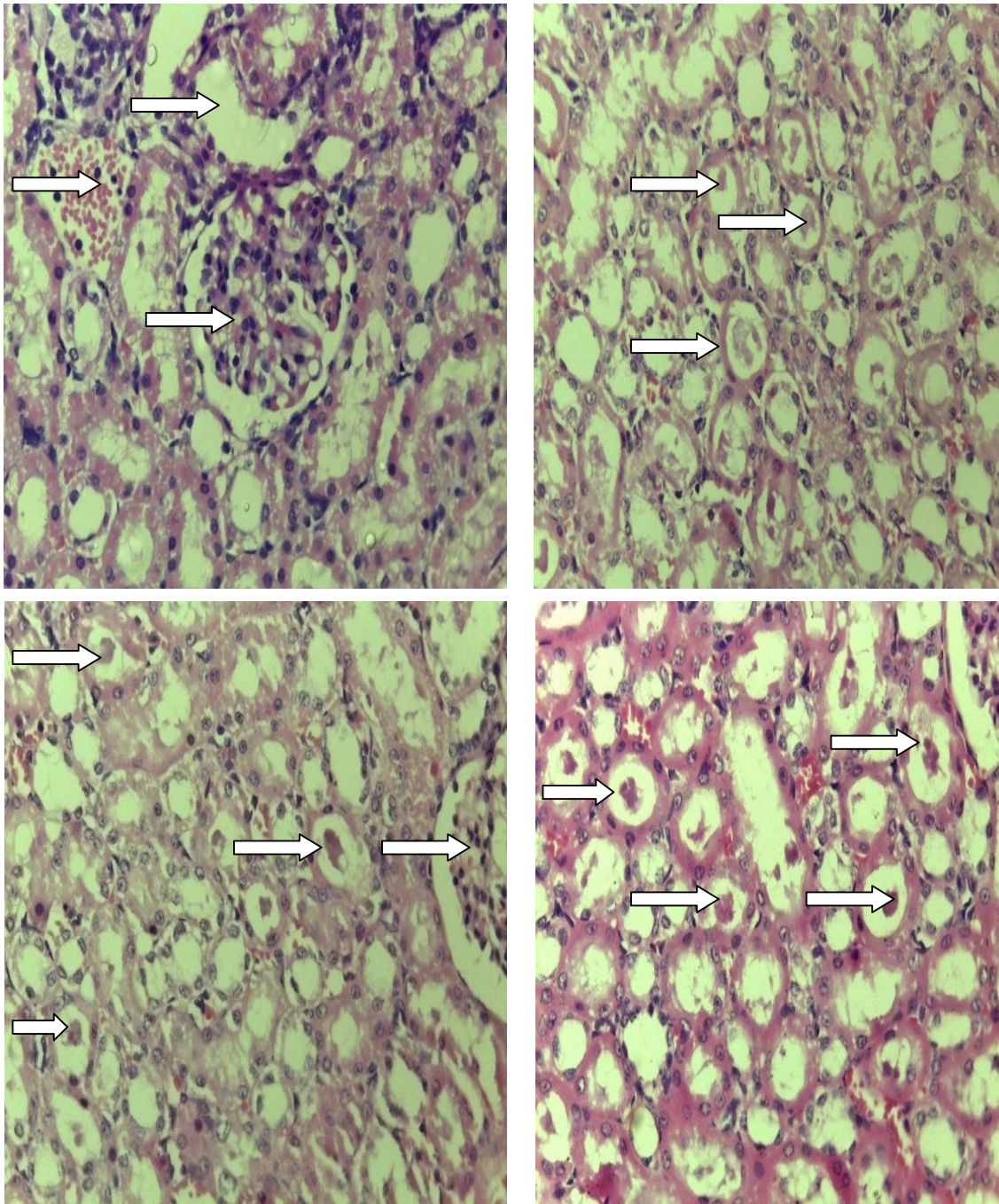


Fig. 1. Kidney Cortex showing a glomerulus, blood vessel and proximal convoluted tubules x400 magnification (Baseline)

Fig. 2. Kidney Cortex showing a glomerulus and proximal convoluted tubules containing calculi (200mg/kg b.w Induction of Calculi) x400 magnifications

Fig. 3. Kidney Cortex showing proximal convoluted tubules containing calculi (400mg/kg b.w Induction of Calculi) x400 magnifications

Fig. 4. Kidney Cortex showing proximal convoluted tubules containing calculi in different stages of formation (600mg/kg b.w Induction of Calculi) x400 magnifications

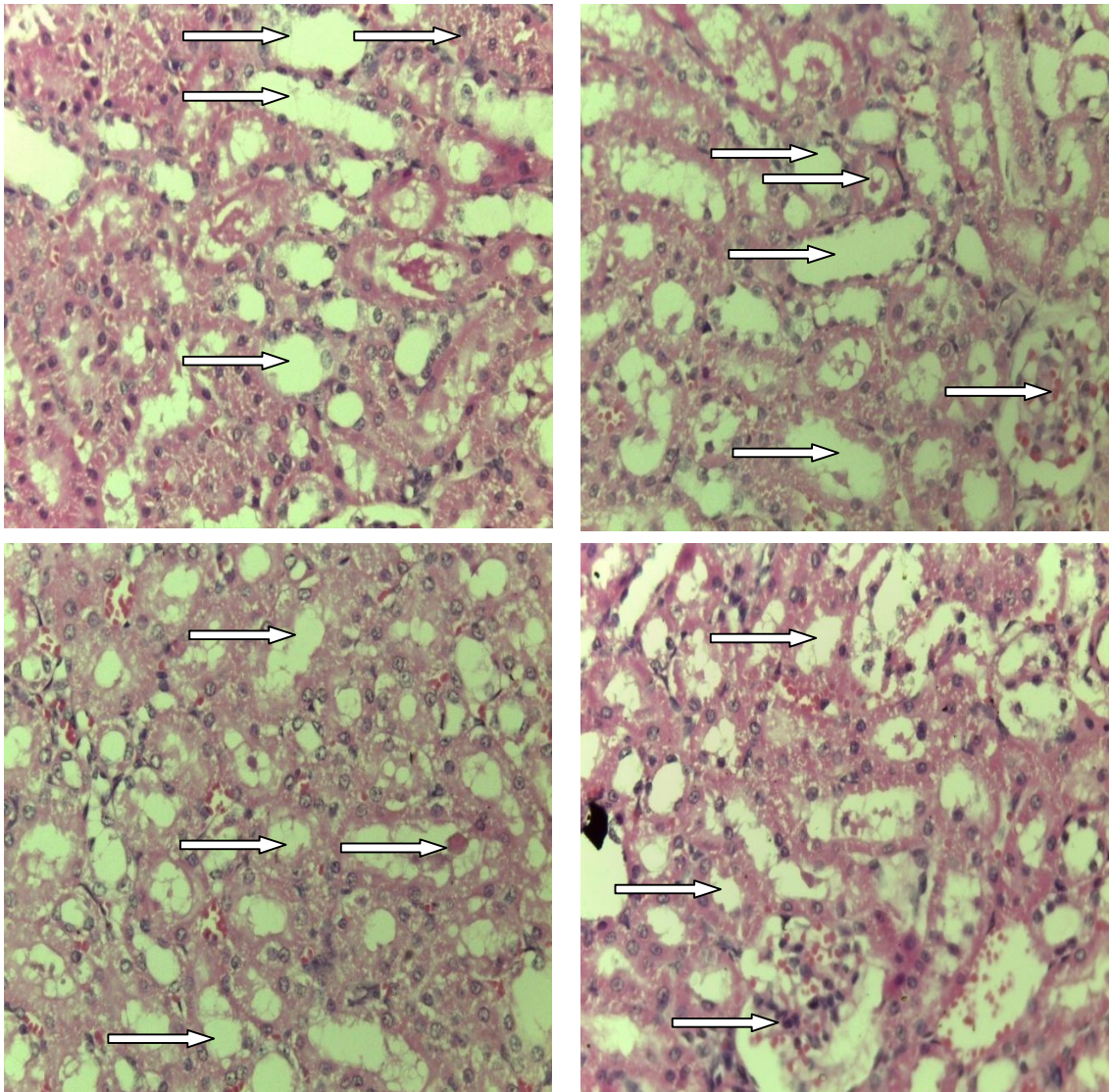


Fig. 5. Kidney Cortex showing Glomeruli and proximal convoluted tubules containing few calculi (200mg/kg b.w Extract Treatment) x400 magnifications
Fig. 6. Kidney Cortex showing a Glomerulus and proximal convoluted tubules containing few and regressing calculi (400mg/kg b.w Extract Treatment) x400 magnifications
Fig. 7. Kidney Cortex showing proximal convoluted tubules containing few and regressing calculi (600mg/kg b.w Extract Treatment) x400 magnifications
Fig. 8. Kidney Cortex showing a glomerulus and proximal convoluted tubules containing few and regressing calculi (Standard control Cystone® Treatment) x400 magnifications

5. CONCLUSION

From this study, it can be concluded that *Phyllanthus fraternus* methanolic leaf extract has reduced and regressed the growth of kidney calculi, supporting the traditional claim regarding its Lithotriptic activity. The mechanism underlying this effect is still unknown but is apparently related to diuresis and the lowering of urinary

concentrations of stone forming constituents. Its protective effect against calculi-induced lipid peroxidation may have been contributory to the recovery from renal damage.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our

area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It's not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study. Animal handling was performed in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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