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Anti-nephrolithiatic Evaluation of Partitioned Ethanol Extract of *Calotropis procera* Leaf in Wistar Rats

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Abstract

Nephrolithiasis is a common urinary disease that elicits excruciating pains and could be managed through alternative therapy. This investigation was planned to evaluate the action of partitioned fractions of ethanol *Calotropis procera* leaf extract in nephrolithiasis treatment. *In vitro* anti-nephrolithiatic and antioxidant tests were assayed using varying concentrations of the ethanol extract and its n-hexane and ethyl acetate fractions to determine which had the optimum activity. Thirty albino rats (101 ± 3 g) were randomly divided among six groups. (n=5). Group A (control) received drinking water and pellets daily. Groups B-F were made lithiatic by daily administration of 2% Ammonium Chloride and 0.75% ethylene glycol in drinking water and fed pellets. Groups C, D, E and F received 750 mg//kg body weight (b.w.) Cystone (reference drug), 25, 50 and 100 mg/kg b.w. n-hexane fraction of *Calotropis procera* ethanol leaf extract (HFCPL) respectively for 10 days. Urine samples were pooled on day eleven for electrolytes and uric acid estimation. Serum and tissue homogenates were assayed for antioxidant, liver and kidney function assessments. Organ tissues from the liver and kidney were fixed for histological analysis. The extract indicated saponin, flavonoid, alkaloid, tannins, terpenoids and phenolics as secondary metabolites. The HFCPL gave the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, a higher nucleation and aggregation activity and reduced uroliths *in vitro* better than the ethanol extract and the ethyl acetate fraction. HFCPL treated animals displayed decreased stone formation in the urine, a significantly ($p < 0.05$) increased urinary calcium and elevated magnesium levels. Significant decrease was recorded in lipid peroxidation activity with corresponding increase ($p < 0.05$) in superoxide dismutase, catalase and reduced glutathione levels. These findings indicated an effectiveness of HFCPL in the management of nephrolithiasis, and hence it may be a good alternative treatment regimen.

Keywords: Nephrolithiasis, *Calotropis procera*, calcium oxalate, nucleation, aggregation

1. Introduction

Nephrolithiasis is the third most common urinary disorder (Divakar *et al.*, 2010) and a complex process that occurs because of an imbalance between promoters and inhibitors in the kidneys (Jehti *et al.*, 1983). It is a succession of several physicochemical events including supersaturation, nucleation, growth, aggregation and retention within the kidneys (Yadav and Jain, 2011) occurring in both men and women but with higher risk in men (Selvem, 2002). The prevalence of nephrolithiasis has been increasing over time. In a survey demonstrated in adults aged 20-74 years, the outcome reflected 3.2% in 1976-1980 to 5.2% in 1988-1994 while overall prevalence increased to 8.8% by 2007-2010. A study estimates the prevalence of renal stones in the US as 7.1% in women and 10.6% in men (Ziemba and Matlaga, 2017). According to estimates, the patient's chance of developing secondary stones again is between 10 and 23 percent per year, 50 percent after five to ten years, and 75 percent within twenty years (Moe, 2006). One in eleven Americans in the country experience kidney stones, and it is believed that 600,000 Americans experience KSD each year (Scales *et al*., 2012). Urinary stones are predicted to affect about 13% of Indians, and half of them could eventually lose their ability to function (Joseph *et al.*, 2005). Calcium stones comprise about 80% of all urinary calculi (Coe and Worchester, 2005) and the principal component of calcium stone is brushite or hydroxyapatite. Factors that contribute to calcium stone formation include hypercystinuria, hyperoxaluria, hyperuricoxuria, hypomagnesuria and urinary pH of 5.0 - 6.5. Struvite or magnesium phosphate stones occur in around 10-15% of cases of chronic urinary tract infections (El Zoghby *et al*., 2012). These infections cause the production of urease (Barbasa *et al.*, 2002), which is required to break down urea into ammonia and CO_2 and raise the pH of the urine. A high-purine diet, particularly one heavy in animal protein like meat and fish, can result in hyperuricosuria, reduced urine volume, and low pH (Kumar *et al.*, 2012).

Similar to other disorders, the treatment of renal calculi has steadily advanced clinically, with techniques including classic open surgery, percutaneous nephrolithotomy, retrograde intra-renal surgery, and extracorporeal shockwave

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lithotripsy (Chung *et al.*, 2019). Nephrolithotomy is performed if large stones (up to 8 mm in width) lodge inside or adjacent to the kidneys using a telescopic tool (Aggarwal *et al.*, 2017). A sufficient amount of fluid intake lowers urine saturation and dilutes calcium oxalate crystallization promoters (Xu et al., 2013). A high sodium diet raises the risk of forming stones by lowering the reabsorption of calcium by the renal tubules and increasing the calcium excreted in the urine (Park and Pearle, 2007).

Current medical management methods have been shown to possess disadvantages that include being highly expensive and with side effects such as hemorrhage, hypertension, tubular necrosis, subsequent fibrosis of the kidney and an increase in stone reoccurrence. Standard pharmaceuticals used to treat and prevent urolithiasis are not always effective and have a number of negative side effects (Sathyaa *et al.*, 2011). Phytotherapy, however, is currently the subject of scientific research because it has been shown to be essential for preventing the recurrence of stones (Gilhorta and Christina, 2011) and act by either allowing spontaneous passage of small calculi in urine or by producing antioxidant, anti-microbial, analgesic and anti-inflammatory activities (Soundarajan *et al*., 2006).

Calotropis procera is an anthelmintic, abortifacient, and whooping cough, colic, headaches, lice removal, diarrhea, painful gums, toothaches, sterility, swellings, and ulcers are among the conditions for which it is used in folk medicine. Its stem is used in treating skin diseases, intestinal worms and leprosy (Esmail and Al Snafi, 2015). The plant is known for its antifungal (Lahsini *et al.*, 1997) and analgesic activity (Hassan *et al.* 2006). Its latex is scientifically reported for the management of diabetes (Mohsin *et al.*, 1989), anti-diarrheal activity (Kumar *et al.*, 2005), while the antioxidant and polyphenolic contents were shown to be effective in cancer studies (Kumar *et al.*, 2001; Prabha and Vasntha, 2011).The milky sap (latex) is well known for its traditional medical uses (Iqbal *et al*., 2005), as food, especially in West Africa as a coagulation ingredient for cheese manufacturing (O'Connor 1993) and a source of energy (Parsons and Cuthbertson, 2001). Given its antioxidant property and secondary metabolites-enriched nature, this investigation was intended to determine the anti-nephrolithiatic activity of *Calotropis procera* ethanol leaf extract and to investigate its toxicity on the kidney, liver and serum indices in Wistar rats.

2. Materials and Methods

2.1. Experimental Animals

Thirty Wistar rats (*Rattus novergicus*) were obtained from the Faculty of Veterinary Medicine, University of Ilorin, Ilorin, Nigeria. The animals were housed in standard cages and acclimatized for twelve days at the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. They were maintained under standard conditions and fed rat pellets (Grand Cereals, A subsidiary of UAC, Nigeria) and water *ad libitum*.

2.2. Drugs and Chemicals

Alkaline phosphatase, aspartate transaminase, alanine transaminase, creatinine, urea, uric acid and bilirubin reagent kits were products of Randox Laboratory Ltd., U.K. Cystone (Himalaya Drug Company, India), ammonium chloride (Loba Chemie Pvt. Ltd.) and ethylene glycol (CDH Ltd, New Delhi) were also procured. Other reagents used were of analytical grade prepared using distilled water.

2.3. Plant Material

Calotropis procera leaves were obtained at Gaa Akanbi, Ilorin, Kwara State, Nigeria in November 2017. It was identified at the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria, where a voucher specimen was deposited and a number (UILH/001/2019/1001) was assigned. The leaves were oven-dried at 50 °C for four days.

2.3.1. Preparation of Extract

The oven-dried leaves were pulverized using an electric blender. Exactly 200 g of the pulverized sample was infused in 800 ml ethanol for 24 hours in a conical flask. This was thereafter filtered with Whatman filter paper and the filtrate collected into a clean beaker. The process was repeated using ethanol for two more days to exhaustively obtain the extract. The total filtrate obtained was evaporated at 55 °C using a water bath (HH 420KW-1000DB) to obtain the crude extract. The crude ethanol extract was reconstituted in 50 ml distilled water, loaded in a separating funnel where 100 ml n-hexane was added. The n-hexane fraction was collected into a clean beaker and the solvent was allowed to evaporate. Ethyl acetate fraction was also collected after addition of 100 ml ethyl acetate to. The n-hexane and ethyl acetate fractions were stored in plain bottles and refrigerated. The percentage yield was then calculated. Secondary metabolites screening of the ethanol extract was carried out using standard procedures.

2.4. In vitro Anti-nephrolithiatic Activity

Nucleation and aggregation assays were performed according to the method described by Hess *et al.* (2000). A freshly prepared solution of 10 mM calcium chloride di-hydrate and 1.0 mM sodium oxalate, containing 200 mM NaCl and 10 mM sodium acetate trihydrate was adjusted to pH 5.7. All experiments were performed at 37 °C using a water bath (HH-420 KW-1000DB).

25 ml of sodium oxalate solution was transferred into beakers placed in the water bath and stirred continuously using a stirrer. 1 ml of distilled water, cystone and varying concentrations (62.5, 125, 250, 500 and 1000 µg/ml) of the ethanol extract and its fractions respectively were added and finally, 25 ml calcium chloride was added. The optical density was measured using spectrophotometer (UV 721, Axiom Medicals U.K.) at 620 nm at every 15 sec over 5 min and then every 1 min over 10 min. The percentage inhibition was calculated as:

$$(1- (Tsi/Tsc)) \times 100$$

Where Tsc is the optical density of control and Tsi, the optical density in presence of inhibitor.

The final solutions were viewed under a light microscope (B-Bran Olympus) to analyze the density of formed crystals in the solution.

2.5. DPPH Scavenging Activity

This assay was carried out using the method described by Molyneux (2004). Briefly, 1 ml varying concentrations (2, 4, 6, 8 and 10 mg/ml in methanol) of the extract and its n-hexane and ethyl acetate fractions and ascorbic acid were added to 4 ml 0.1 mM methanol solution of DPPH. A blank probe was obtained by mixing 4 ml 0.1 mM methanol DPPH solution and 200 µl of distilled water. After 30 min of incubation in the dark, the absorbance of samples and blank were taken at 517 nm (Visible Spectrophotometer 721, Axiom Medical, U. K.). Inhibition of free radicals by DPPH in percentage was calculated using the expression:

$$\% \text{ Inhibitioin} = 100 - ((ABS_{sample} - ABS_{blank})/ABS_{control}) \times 100$$

2.6. Acute Toxicity Testing (LD_{50})

The OECD (2002) guidelines for the Testing of Chemicals No. 420 were followed for conducting an acute toxicity investigation. After three rats were made to fast for 24 hours, the HFCPL was administered to them in steps at fixed doses of 5, 50, 300, and 2000 mg/kg body weight respectively, via gavage with a cannula in a single dosage. The rats were observed for physical changes on their bodies, hair loss, ease of movement, breathing pattern, etc.

2.7. In vivo Studies

Thirty Wistar rats were randomly divided into six groups (A-F) of five and subjected to different treatments. Animals in groups B-F were administered 2.0% ammonium chloride and 0.75% ethylene glycol in drinking water *ad libitum* for 10 days. Animals in group A (control) took only drinking water, those in group B were untreated (negative control group). Group C was administered 750 mg/kg cystone (reference drug), while groups D-F were administered 25, 50 and 100 mg/kg body weight HFCPL respectively. 0.5 ml of 750 mg/kg cystone and HFCPL doses and were administered to the respective groups using a cannula for 10 days.

Diethyl ether was used to anesthetize the rats, blood was collected using jugular puncture into plain bottles and centrifuged to get the serum. Serum was drawn out with a Pasteur pipette, which was then placed in plain sample vials and refrigerated. Portions of the kidney and liver were homogenized in 0.25M sucrose solution and refrigerated, while the rest were preserved in 10% formalin for histological studies.

2.7.1. Urinary Microscopy

As a way to validate the onset and resolution of kidney stone disorder following the injection of ethylene glycol, ammonium chloride, and the HFCPL treatment, respectively, urinary microscopy was performed. Animal urine was collected on slides and analyzed under a microscope (B-Bran Olympus) at a magnification of 400.

2.7.2. Biochemical Indices

The serum was used to estimate the ALP, AST and ALT activities. The concentrations of the serum functional parameters (albumin, creatinine, urea, bilirubin and globulin) were then determined using methods described in the respective kits. Histological studies (H & E staining's) were done on the liver, kidney and pancreas of the rats and viewed under the light microscope.

2.8. Gas Chromatography-Mass Spectrometer (GC-MS) Analysis

GC-MS (model QP2010SE, Shimadzu, Japan) was employed to conduct a GC-MS analysis of the HFCPL in accordance with the instructions from the manufacturer. For the analysis, 10 ml of n-hexane were used to dissolve 1g of the extract. The injector and column oven temperatures were set at 60 ℃ and 250 ℃, respectively. The split ratio was set at 10:1 and the pressure was kept at 144.4 kPa while the flow control mode was maintained at a linear velocity of 46.3 cm/sec. The interface temperature was 250 ℃, while 230 ℃ was the temperature of the ion source. The mass to charge ratio (m/z) was 35 at the beginning and 700 at the conclusion (35-700 m/z). The retention time, % height and peaks were obtained and integrated after the analysis.

2.9. Statistical Analysis

The information was presented as the mean of five determinations ± SEM. One-way ANOVA and the Tukey Post-Hoc Test were used in the statistical study. At $p < 0.05$, all data were taken as statistically significant.

3. Results

3.1. Percentage yield

A yield of 8.42 g of ethanol extract was obtained from the leaf. On partitioning, the n-hexane fraction gave a percentage yield of 1.025 % while the ethyl acetate fraction yielded 0.885 %.

3.2. 3.2 Quantitative Secondary Metabolite Constituents

The analysis of secondary metabolite constituents of the ethanol extract of *C. procera* leaf revealed the presence of saponins, flavonoids, alkaloids, tannins, glycoside and phenolics (Table 1).

Table 1: Concentration of detected Secondary Metabolite Constituents of ethanol extract C. *procera* leaf

Secondary Metabolite	Concentration (mg/100 g)
Saponins	38.62 ± 0.02
Flavonoids	361.07 ± 17.69
Alkaloids	48.24 ± 0.11
Tannins	19.75 ± 0.37
Terpenoids	57.26 ± 0.09
Phenolics	34.46 ± 0.86

3.3. DPPH Scavenging Activity

There was a progressive increase as concentrations increase in the inhibition of DPPH with the highest values recorded in the n-hexane fraction (IC_{50} 10.2 ± 0.8) which compared favorably with the ascorbic acid standard (IC_{50} 8.01 ± 0.2) (Table 2).

Table2: *In vitro* DPPH Scavenging Activity of C. *procera* leaf ethanol extract and its n-hexane and ethyl acetate fractions.

Concentration (mg/ml)	% Inhibition Ethanol	% Inhibition Ethyl acetate	% Inhibition n-Hexane	% Inhibition Ascorbic acid
2	47.47 ± 0.07	65.88 ± 0.22	66.88 ± 0.28	66.91 ± 0.02
4	62.92 ± 0.26	72.50 ± 0.13	79.25 ± 0.20	79.39 ± 0.00
6	69.81 ± 0.19	76.37 ± 0.09	80.54 ± 0.25	80.42 ± 0.05
8	76.46 ± 0.11	80.36 ± 0.18	84.19 ± 0.17	85.12 ± 0.00
10	82.68 ± 0.10	82.00 ± 0.30	86.18 ± 0.32	91.75 ± 0.01

3.4. In vitro Anti-nephrolithiatic Activity

The results of nucleation (Fig. 1) gave an indication that the n-hexane fraction displayed better inhibitory potentials compared to the ethanol extract of *C. procera* and the ethyl acetate fraction at varying concentrations. Upon aggregation (Fig. 2), the findings demonstrated that, at various doses, the n-hexane fraction of the *C. procera* extract had the strongest inhibitory potentials in comparison to the ethanol and ethyl acetate extracts. The microscopy of the solution gave a progressive decrease in the number of stones *in vitro* (Fig. 3).

With results from the *in vitro* studies, the n-hexane fraction had the best activity across the parameters investigated and hence; it was further used to carry out *in vivo* studies.

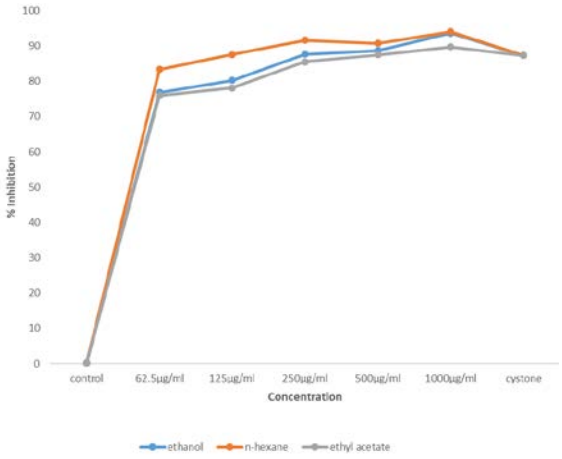

Figure 1: *In vitro* nucleation assay of *C. procera* leaf ethanol extract and its n-hexane and ethyl acetate partitioned fractions.

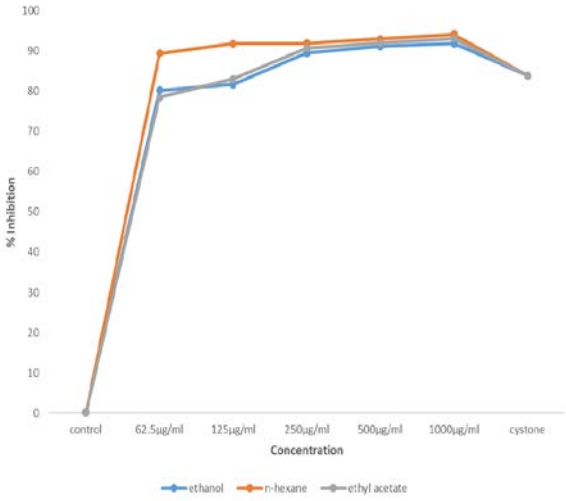

Figure 2: *In vitro* aggregation assay of *C. procera* leaf ethanol extract and its n-hexane and ethyl acetate partitioned fractions.

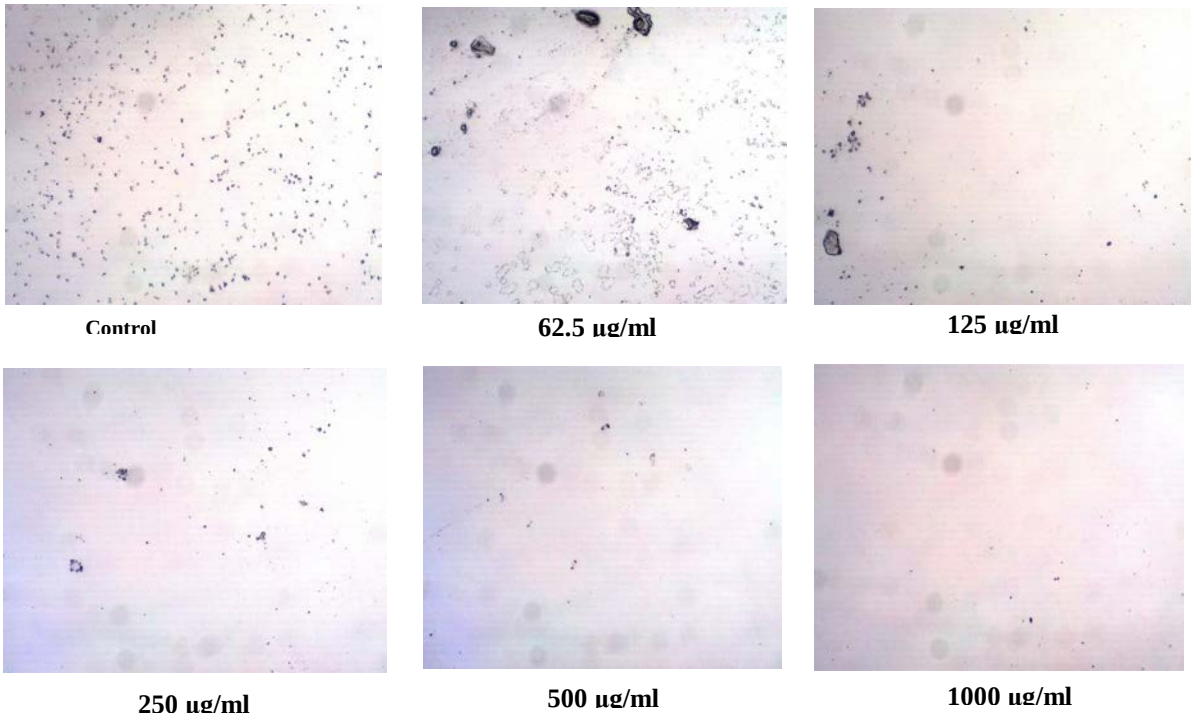

Figure 3: Photomicrograph of inhibition of urolith by various doses of HFCPL ethanol extract *in vitro*.

3.5. Electrolyte Parameters

The magnesium level was higher in the urine samples in comparison to the serum (Table 3). However, at a dose of 25 mg/kg there was a substantial drop ($p < 0.05$) in the blood magnesium level compared to the control, as well as a significant lower level ($p < 0.05$) in the urine magnesium. At doses of 25 and 50 mg/kg, there was a notable decline ($p < 0.05$) in urinary calcium levels compared to the control; however, there was no discernible alteration in serum calcium levels.

Table 3: Effect of hexane fraction of *Calotropis procera* leaf (HFCPL) ethanol extract on some electrolyte parameters of nephrolithiatic rats.

Group	Serum Calcium (mmol/l)	Serum Magnesium (mg/dl)	Urine Calcium (mmol/l)	Urine Magnesium (mg/dl)
Control + dw	2.03 ± 0.10 [a]	1.79 ± 0.29 [a]	2.26 ± 0.25 [a]	2.70 ± 0.04 [a]
Lithiatic + dw	2.50 ± 0.48 [a]	1.26 ± 0.01 [b]	1.01 ± 0.13 [b]	2.52 ± 0.02 [b]
Lithiatic + 750 mg/kg bw cystone	1.80 ± 0.09[b]	1.51 ± 0.24 [a]	2.65 ± 0.30 [a]	2.83 ± 0.01[c]
Lithiatic + 25 mg/kg bw HFCPL	2.24 ± 0.34 [a]	1.32 ± 0.20[b]	1.69 ± 0.17[c]	2.58 ± 0.07 [b]
Lithiatic + 50 mg/kg bw HFCPL	1.97 ± 0.06[a]	1.61 ± 0.18[a]	1.81 ± 0.08 [c]	2.60 ± 0.11 [a]
Lithiatic + 100 mg/kg bw HFCPL	2.59 ± 0.59 [a]	1.65 ± 0.16 [a]	2.41 ± 0.15 [a]	2.71 ± 0.15 [a]

Values are expressed as Means ± SEM of 5 replicates. Values with superscripts different from the control are significantly different ($p < 0.05$).

3.6. Kidney FunctionTests

All HFCPL-treated groups experienced a substantial rise in creatinine concentration ($p < 0.05$) in contrast to the control (Table 4). Among all treated and untreated groups, there was no discernible difference in the amounts of either urea or uric acid ($p < 0.05$). The globulin concentration was discovered to be considerably less ($p < 0.05$) in the cystone and 25 mg/kg body weight groups compared to the control. At 25 mg/kg, the bilirubin level significantly increased ($p < 0.05$) compared to the control, whereas at 50 mg/kg, it significantly reduced ($p<0.05$). Only at a dose of 25 mg/kg compared to the other groups did albumin level show a significant value ($p < 0.05$).

Table 4: Effect of hexane fraction of *Calotropis procera* leaf (HFCPL) ethanol extract on kidney function parameters of nephrolithiatic rats

Group	Albumin (g/dl)	Globulin (mg/dl)	Bilirubin(mg/dl)	Creatinine(mg/dl)	Urea (mg/dl)	Uric Acid(mg/dl)
Control + dw	4.47 ± 0.90[a]	10.41 ± 1.69[a]	1.08 ± 0.00[a]	0.71 ± 0.00[a]	16.42 ± 0.28[a]	5.07 ± 0.46[a]
Lithiatic + dw	3.99 ± 0.18[b]	10.34 ± 1.66[b]	0.75 ± 0.05[b]	0.77 ± 0.01[b]	14.51 ± 0.85[b]	5.14 ± 0.66[a]
Lithiatic + 750 mg/kg bw cystone	4.04 ± 0.53[a]	8.37 ± 1.02[c]	0.93 ± 0.16[a]	0.73 ± 0.06[ab]	16.54 ± 0.31[a]	5.13 ± 0.39[a]
Lithiatic + 25 mg/kg bw HFCPL	2.21 ± 1.46[c]	8.13 ± 1.88[c]	1.93 ± 0.39[c]	0.95 ± 0.01[c]	17.58 ± 1.84[a]	5.99 ± 0.79[a]
Lithiatic + 50 mg/kg bw HFCPL	3.44 ± 0.91[ab]	10.12 ± 1.12[ab]	0.56 ± 0.11[d]	0.74 ± 0.01[d]	16.23 ± 1.38[a]	6.23 ± 0.83[a]
Lithiatic + 100 mg/kg bw HFCPL	4.24 ± 0.58[a]	9.52 ± 1.03[ab]	1.20 ± 0.25[a]	0.85 ± 0.04[e]	16.20 ± 1.43[a]	5.79 ± 0.85[a]

Values are expressed as Means ± SEM of 5 replicates. Values with superscripts different from the control are significantly different ($p < 0.05$).

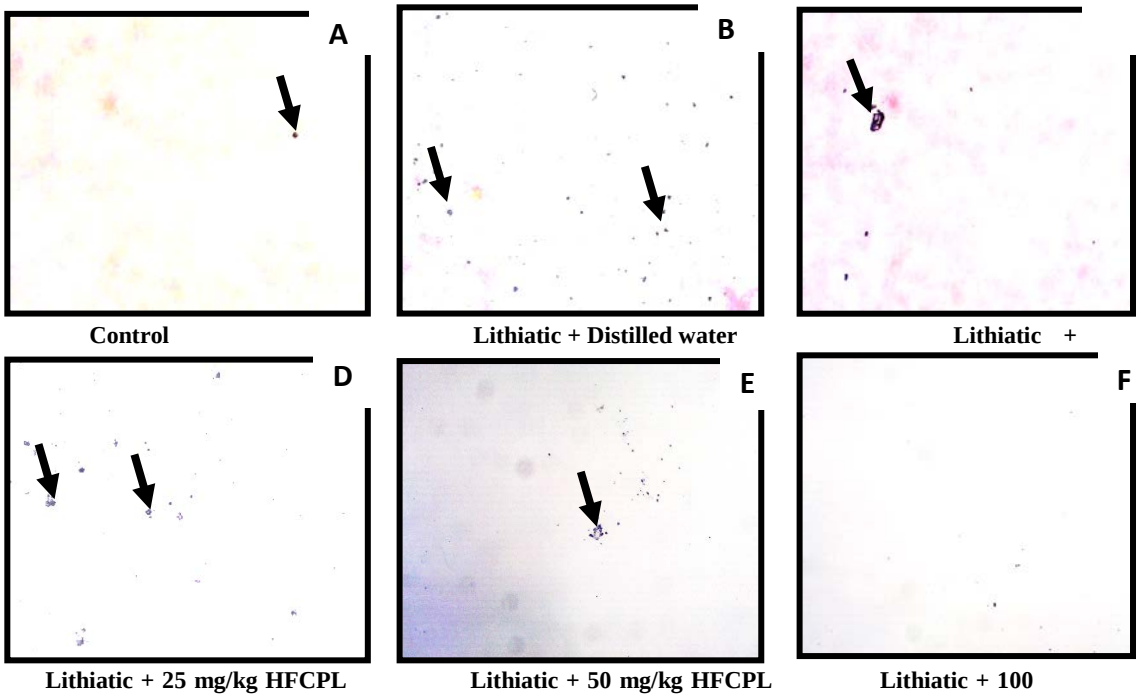

Figure 4: Photomicrograph of urine samples of lithiatic rat treated with HFCPL ethanol extract showing kidney stones (arrows)

3.7. Enzyme Activity

All doses and the cystone group saw a substantial rise ($p< 0.05$) in the actions of the enzymes alanine transaminase and aspartate transaminase; however, only the 25 and 100 mg/kg body weight groups saw a similar increase ($p <0.05$) in alkaline phosphatase activity (Table 5).

Table 5: Serum enzyme activity in nephrolithiatic rats treated with hexane fraction of *Calotropis procera* leaf (HFCPL) ethanol extract

Group	ALT (U/I)	AST (U/I)	ALP (U/I)
Control + dw	6.49 ± 0.18[a]	24.31 ± 0.26[a]	0.62 ± 0.41[a]
Lithiatic + dw	7.34 ± 0.21[b]	26.59 ± 0.24[b]	2.09 ± 0.29[b]
Lithiatic + 750 mg/kg bw cystone	9.12 ± 0.47[c]	35.72 ± 0.44[c]	0.32 ± 0.16[a]
Lithiatic + 25 mg/kg bwHFCPL	11.12 ± 0.46[d]	41.05 ± 3.22[d]	1.69 ± 0.35[b]
Lithiatic + 50 mg/kg bw HFCPL	8.32 ± 0.37[c]	26.51 ± 0.32[b]	0.37 ± 0.11[a]
Lithiatic + 100 mg/kg bw HFCPL	10.80 ± 0.11[d]	36.06 ± 1.61[d]	1.52 ± 0.18[b]

Values are expressed as Means ± SEM of 5 replicates. Values with superscripts different from the control are significantly different ($p < 0.05$).

3.8. Antioxidant Enzyme Activity

All of the dosages significantly differed from the control in terms of liver glutathione activity ($p <0.05$). But both the cystone and 100 mg/kg groups showed a statistically significant increase ($p< 0.05$) in the kidney glutathione activity (Table 6). When compared to the control and standard medication, kidney MDA activity was significantly lower in all doses of HFCPL ($p< 0.05$). The only group in which the liver's MDA activity rose considerably ($p< 0.05$) in comparison to the control was the one that received cystone at a dose of 25 mg/kg. When equated to the control group, the kidney's catalase activity considerably decreased ($p <0.05$) in both groups. In contrast to the untreated groups, SOD activity considerably increased ($p <0.05$), reaching its peak at a dose of 50 mg/kg.

Table 6: Effect of hexane fraction of *Calotropis procera* leaf(HFCPL) ethanol extract on antioxidant parameters in nephrolithiatic rats (×10^4 mg/protein).

Group	Liver GSH	Kidney GSH	Liver MDA	Kidney MDA	Kidney SOD	Kidney Catalase
Control + dw	0.89 ± 0.01[a]	0.86 ± 0.02[a]	1.63 ± 0.15[a]	2.22 ± 0.15[a]	3.50 ± 0.81[a]	0.74 ± 0.25[a]
Lithiatic + dw	0.80 ± 0.02[b]	0.87 ± 0.01[a]	2.41 ± 0.13[b]	2.53 ± 0.06[b]	0.81 ± 0.33[b]	0.15 ± 0.02[b]
Lithiatic + cystone	0.90 ± 0.05[a]	0.91 ± 0.01[b]	2.03 ± 0.19[c]	2.25 ± 0.10[a]	1.87 ± 0.42[c]	0.49 ± 0.03[c]
Lithiatic + 25 mg/kg bwHFCPL	1.03 ± 0.07[c]	0.88 ± 0.00[a]	2.17 ± 0.14[b]	1.53 ± 0.15[c]	1.76 ± 0.21[c]	0.64 ± 0.09[a]
Lithiatic + 50 mg/kg bwHFCPL	1.00 ± 0.03[c]	0.88 ± 0.01[a]	1.43 ± 0.08[a]	1.87 ± 0.18[c]	4.46 ± 0.88[a]	0.23 ± 0.17[c]
Lithiatic + 100 mg/kg bw HFCPL	0.99 ± 0.02[c]	0.94 ± 0.02[b]	1.55 ± 0.16[a]	0.96 ± 0.01[d]	2.20 ± 0.40[a]	0.32 ± 0.13[c]

Values are expressed as Means ± SEM of 5 replicates. Values with superscripts different from the control are significantly different ($p < 0.05$)

3.9. GCMS Analysis

The GCMS analysis revealed the presence of five active principles in the n-hexane fraction of ethanol extract of *C. procera* leaf. The compound 9, 12, 15-Octadecatrien-1-ol (61.22 %) had the highest percentage abundance while 5-Methyldocosane (2.81 %) had the lowest (Table 7).

Table 7: Bioactive constituents of hexane fraction of *Calotropis procera* Leaf(HFCPL) ethanol extract

S/N	Name of Compounds	Retention Time	Peak area %
1.	n-Hexadecanoic acid	37.758	27.21
2.	Phytol	38.584	5.83
3.	9,12,15-Octadecatrien-1-ol	38.859	61.22
4.	Tetrabenzo[e,i,o,s][1,4,7,11,14,18] dithiatetraazacycloeicosine	39.410	2.94
5.	5-Methyldocosane	40.504	2.81

4. Discussion

Results from the analysis of the secondary metabolites revealed that ethanol extract of *C. procera* leaf contains saponins, alkaloids, flavonoids, tannins and terpenoids (Table 1). It has been reported that saponins (Touhami *et al.*, 2007; Rad *et al.*, 2011), flavonoids and alkaloids (Rad *et al.*, 2011) possess anti-nephrolithiatic activity, the concentrations of which might have contributed significantly to the clearance of uroliths in the lithiatic rats. Saponins (Kenner and Requena, 1996) boosts the immune system, tannins (El Marie and Jonah, 2001); alkaloids and flavonoids (Aliero *et al.*, 2008) possess antibacterial property and are phytotherapeutic agents capable of clearing uroliths. From the study, both the ethanol extract and its partitioned fractions could prevent the calculi formation at all the nucleation and aggregation stages of stone formation. As the concentration increases, the inhibitory effect also increased. The number of stones in the solution microscopy reduced progressively with the dose, confirming the anti-nephrolithiatic effect of *C. procera* leaf. Nafiu *et al.* (2008) similarly asserted that saponin extract of *Dianthus bausticus* exhibited the highest nucleation inhibitory activity based on its antioxidant-rich potentials. The antioxidant test demonstrated the plant's capacity to function as a free radical scavenger. *Calotropis procera* leaf extract and its fractions can prevent the development of stones because DPPH has a proton free radical and pairs with it in the presence of a free radical scavenger (Chang *et al.*, 2002).

Divakar *et al.* (2010) reported that experimental mice given ethylene glycol and ammonium chloride experienced elevated renal oxalate retention and excretion as well as the development of calculi primarily made of calcium oxalate. Urinal microscopy validates the potency of the n-hexane fraction at reducing the sizes and quantity of calculi in the urine. The stones clearance at 25 mg/kg body weight with a further increase at higher dose concentrations confirms the anti-nephrolithiatic activity of HFCPL. Previous studies indicate that plants lower kidney stone formation by causing a reduction in stone number and size (Rushton and Spector, 1982). Kidney stones block the urinary tract, and hence the obstruction in the passage of urine.

The production of stones in the urine is frequently accompanied by a low amount of magnesium. By joining with calcium oxalate to produce a soluble complex and lowering the concentration at which calcium oxalate can form, magnesium has been found to effectively inhibit the crystallization of calcium oxalate in vitro (Lemann *et al.*, 1991). The increase in urinary magnesium concentration in all doses indicates the efficacy of C. *procera* in the management of kidney stones by clearing the oxalate concentration available for stone formation. This supports studies that magnesium levels recover to normal following medication therapy, with decreases in CaOx growth and nucleation rate in nephrolithiasis-affected rats (Grases *et al*., 1989; Patel *et al.*, 2016).

The danger of precipitation, stone formation, and stone growth are reduced because calcium and uric acid are eliminated through the urine. Hypercalciuria at all the doses validates the potency of *C. procera* in the inhibition and regulation of stone development characteristic of nephrolithiasis. This is further substantiated by the urinary microscopy (Fig. 5) result, which showed the quantity and sizes of stones across all groups. In this study, uric acid level showed no significant difference, contrary to reports by Patel *et al.* (2016) that uric acid level in lithiatic rats decrease after drug treatment. This suggests that kidney stone disease poses no threat to the production and elimination of uric acid.

According to Wright and Plummer (1974), alkaline phosphatase is a plasma membrane marker enzyme that is necessary for healthy organ function in particular levels and is essential to evaluating the integrity of the plasma membrane. The metabolism of xenobiotics and the preservation of biological homeostasis in an organism both depend heavily on the liver. Due to these important roles,

liver enzymes are used as makers in the assessment of drugs (Satyapal *et al.*, 2008). The reported increase in AST and ALT activity across all dose groups indicate impairment of the liver function (Huang *et al.*, 2002). At 50 mg/kg body weight dose, the observation of no significant difference in the serum suggests absence of hepatic injury which could cause elevation of ALP levels in the blood.

It was reported that antioxidant enzyme activity decreases in kidney stone patients, hence an increase in lipid peroxidation. An increase in MDA characterizes calculi formation with a progressive reduction in levels of SOD, catalase and GSH in CaOx induced rats. The observed decrease in MDA (Huang *et al.*, 2002) and the corresponding increase in antioxidant enzymes level (Patel *et al.*, 2016) are in line with previous nephrolithiatic studies that used phytotherapy as a drug alternative in the disease management. The HFCPL potency is also supported by its DPPH scavenging potentials, hence protecting against oxidative stress.

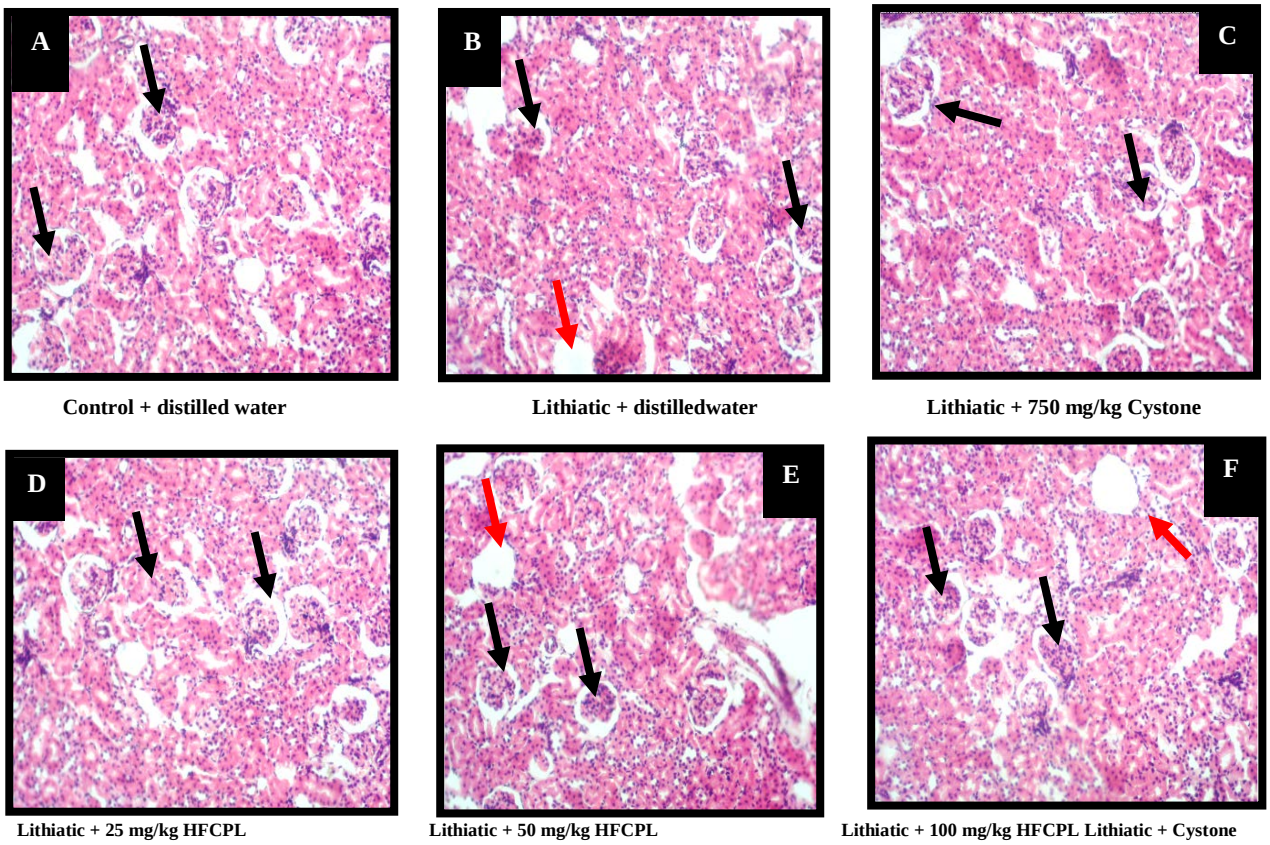

Figure 5: Photomicrographs of the kidney of experimental rats showing the renal cortex and distribution of renal corpuscles (black arrow) and possible corpuscular degeneration (red arrow). Figures A, C and D represent normal renal cortical morphology and histo-architecture with intact renal corpuscles and staining characteristics. B, E and F present corpuscular degeneration suggesting pathological alteration. H and E stain (Mg x 400).

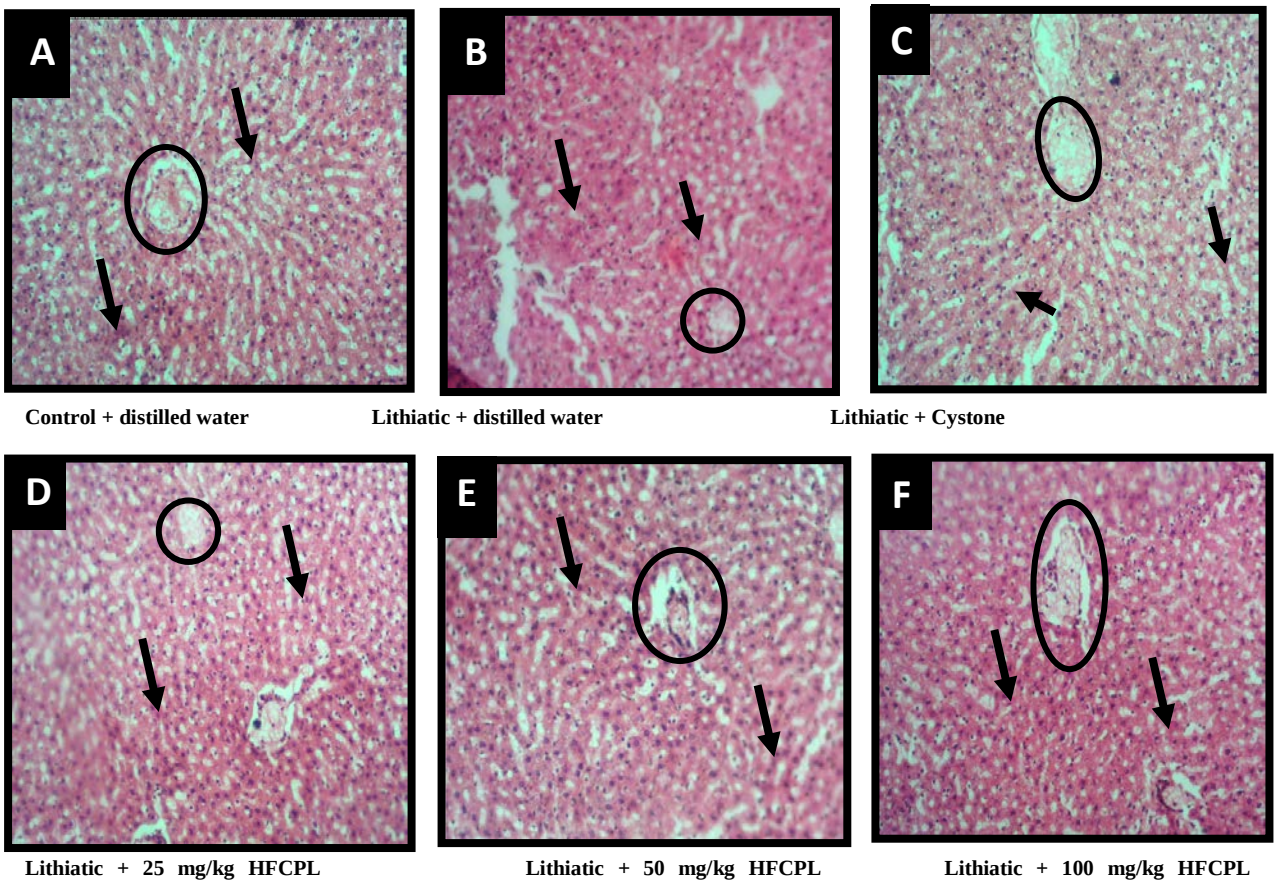

Figure 6: Photomicrograph of the liver of lithiatic rat treated with HFCPL ethanol extract showing the central veins (black circles) and densely packed hepatocytes (black arrow). The photomicrographs appear characteristically normal with no pathological alteration though with sings of cellular delineation, fatty liver and cholestasis.

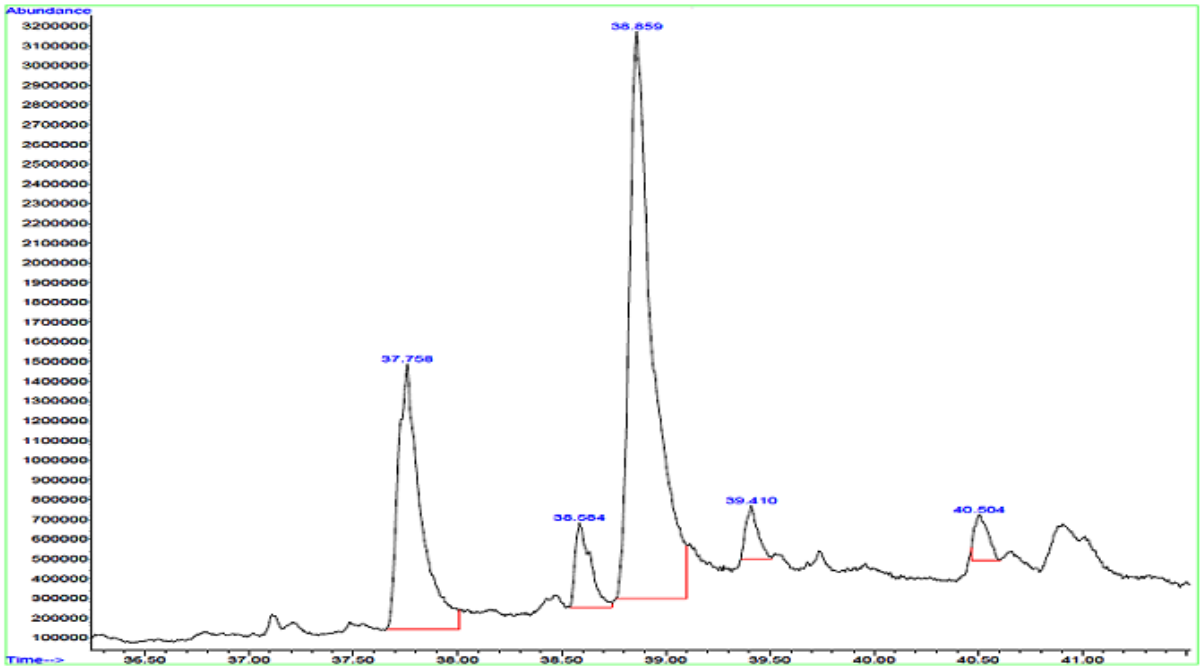

Figure 7: Gas Chromatography-Mass Spectrophotometry (GC-MS) chromatogram of hexane fraction of *Calotropis procera* leaf (HFCPL) ethanol extract.

Normal urea concentration at all dose groups in relation to the control could be indicative of adequate kidney functioning in excretory, regulatory and endocrine processes (Judykay, 2007). According to Adebayo *et al*. (2003), elevated urea levels predict a decline in the nephrons' capacity to filter waste, leading to waste accumulation, whereas a drop could be ascribed to insufficient protein catabolism that results in lesser urea production. Whelton *et al.* (2002) stated that an increase in creatinine concentration suggests impaired renal function. The increase observed in creatinine level is, therefore, suggestive of a distorted function of the kidney in the HFCPL treated rats.

To assess an animal's proper liver function, a combination of molecules called albumin, total bilirubin, and globulin can be employed (Rasekh *et al.*, 2008). With both biological and diagnostic relevance, bilirubin is a significant byproduct of blood catabolism. The increase in bilirubin generation at 25 mg/kg dose may be due to oxidative stress, hepatic injury, or impaired liver function (Moudgil *et al.*, 1989). The main serum protein components are albumin and globulin, and their concentrations can provide some insight into the health of the immune system and diet. Low albumin to globulin ratios can indicate either excessive globulin production, as in autoimmune illnesses, or albumin deficiency, as in cirrhosis, or even selective albumin removal from circulation. The lack of any discernible variation in serum albumin and globulin indicates adequate protein synthesis.

The kidney corpuscular degeneration at higher doses after the histological examination is suggestive of pathological alteration. The aggregation of stones in the kidney of the rats after induction causes severe damages in the kidney pending treatment because of impaired urine excretion. However, the normal liver morphology without pathological alteration shows that interactions between promoters and inhibitors had no effect on the tissue; hence, HFCPL displayed a good hepatoprotective agent.

The analysis of the active ingredients in the n-hexane fraction indicated the presence of chemical compounds that might enhance the plant's therapeutic qualities. Phytol acts as an antioxidant by the removal of hydroxyl radicals which may be capable of inhibiting cell damage caused by this radical (Camila *et al.*, 2013). The presence of this compound corroborates the antioxidant potential of the plant which was able to lower lipid peroxidation *in vivo*. It occurs naturally in green tea and is used in the synthesis of vitamins E and K. Hexadecanoic acid is a surfactant used in soap making, which might account for the high foaming capacity of the plant. Vasudevan *et al.* (2012) reported that hexadecanoic acid possesses anti-inflammatory property upon its kinetic assessment.

5. Conclusion

From this study, *Calotropis procera* leaf may be used in the management of kidney stone disease, a novel outcome in the phytotherapy potentials of the plant. This is probable because of its antioxidant, nucleation and aggregation properties that promote inhibition of stone formation. The n-hexane fraction is effective in stone clearance with no alteration in biochemical indices. When administered at higher dose concentrations, the fraction poses a threat to tissue morphology; hence, pathological alterations occur.

Conflicting Interests

The authors certify that they have no business or personal connections that would have improperly impacted their authorship of this work.

Data Availability Statement: The corresponding author, Nafiu MO., will provide the data supporting the study's conclusions upon reasonable request.

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