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Antiurolithiatic activity of *Ensete superbum* (Roxb.) Cheesman (wild banana) pseudostem on ethylene glycol induced urolithiasis in rats

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In this study, chloroform extract derived from pseudostem of ***Ensete superbum*** (Roxb.) Cheesman **(**Family: Musaceae) powder was investigated for treatment of calcium oxalate urolithiasis. An ***in vitro*** antiurolithiatic study was carried out by conductometric titrations of $CaCl_2$ with $Na_2C_2O_4$ in the absence and presence of chloroform extract of ***Ensete superbum*** (ES). Whereas, in an ***in vivo*** studies, urolithiasis was developed in animals by adding 0.75 % of ethylene glycol in drinking water for 28 days. The extract (100, 200 and 400 mg/kg) was administered orally along with ethylene glycol for 28 days. On 28 day, 24 hrs urine was collected from individual animals and various biochemical parameters were measured in urine (calcium, phosphate and oxalate), serum (creatinine, urea and uric acid) and kidney homogenate (renal oxalate). The paraffin kidney sections were prepared and subjected to histopathological analysis to observe the calcium oxalate deposits. The result of conductometric titration show shift in end point towards lower side due to reduction in free Ca^{2+} content as evidence of complexation with the extract. Treatment of ethylene glycol (Group II – negative control) cause significant ($P < 0.001$ vs. normal) increase in levels of urine calcium, creatinine, uric acid, and serum calcium, creatinine, magnesium and uric acid, as compared to normal. The treatment with extract, significantly ($P < 0.001$ vs. control) depleted the levels of urine calcium, creatinine, uric acid, and serum calcium, creatinine, magnesium and uric acid, in ethylene glycol induced urolithiasis after 28 days in dose dependent manner. The antiurolithiatic activity of the chloroform extract of ***Ensete superbum*** pseudostem is mediated possibly through the inhibition of calcium oxalate crystal formation and its effect on the urinary concentration of stone-forming constituents. The activity may be attributed due to the presence of β-carboline alkaloids.

Keywords: ***Ensete superbum*** (Roxb.) Cheesman**,** Chloroform extract, β-Carboline, Cystone, Ethylene glycol, Urolithiasis.

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The urinary tract stone formation is a common disorder of mankind and the third most prevalent disorder of the urinary system globally[1]. Metabolism of different minerals are important factors associated in the formation and growth of urinary stones. There are various modern the rapies like extracorporeal shock wave lithotripsy (ESWL) and various drug treatments in urological practices utilized for eliminating kidney stones[2]. The major limitation associated with shock waves is its traumatic effects and recurrence of infection due to persisted residual fragments in the kidney[3]. Therefore, it is worthwhile to look for alternative means from natural sources such as medicinal plants or phytotherapy[4]. Since ancient times, a large number of plants, herbal extracts and certain juices have been recommended for urinary stone treatments in India[5]. One of them is *Ensete superbum* (Roxb.) Cheesman **(**Family: Musaceae) commonly known as Wild Banana **(**syn-***Banakadli*****)**, found as monocarpic, non-stoloniferous perennial shrub in Western Ghats and North-eastern hills of India[6]. In *Ayurvedic* system of medicine the pseudostem and seeds of ***Ensete superbum*** were used for the treatment of various human ailments like, debility, diabetes, kidney stone, leucorrhoea, measles, stomachache and easy delivery[7-8]. Earlier report on phytochemical analysis on plants and its parts revealed the presence of alkaloids, steroids, phenolics, glycoside, colour pigments like chroman derivatives (contain non-steroidal phytosterol) fatty oil, triterpenoid esters, proanthocyanidin, pro-pelargonidin glucosides, pelargonidin and sugars[9-11].

E. superbum is a non-toxic (LD_{50} = 3235.9 mg/kg) and has been reported to possesses antiviral, antivariola, antivaccinia, antifertility, cholinergic and hypoglycemic activities[12-13]. However, no studies

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have so far been reported as antiurolithiatic effect of *E. superbum* pseudostem. In the absence of any scientific evidence, an attempt was made to investigate the antiurolithiatic effectiveness of chloroform extract of *Ensete superbum* pseudostem in rats in the present communication.

Materials and methods

Chemicals and reagents

All chemicals and reagents were of analytical grade and purchased from SD Fine Chem, Hi-media, and Sigma Aldrich (Mumbai, India).

Plant materials and preparation of extracts

The fresh pseudostem of *Ensete superbum* were collected in July 2013, from Vadodara, Gujarat, India and was identified and authenticated by Dr Nagar, Botany Department, The M S University of Baroda, Vadodara, Gujarat (India). A voucher specimen (PG/KB/HDT-1-2013) was retained in Pharmacy Department, The M S University of Baroda, Vadodara, Gujarat (India) for future reference. Coarsely dried powder (1000 gm) of ES pseudostem was subjected to hot continuous sequential solvent extraction using soxhlet apparatus with petroleum ether (60-80 °C) to defat, followed by chloroform (40- 50 °C).The solvent was completely removed under reduced pressure by rotary evaporator. The percentage yield of petroleum ether and chloroform extracts was found to be 1.26 % (w/w) and 3.79 % (w/w), respectively, calculated in terms of dried weight.

Characterisation of extract for alkaloids

Thin layer chromatographic (TLC) studies were performed using various solvent systems, and finally chloroform: methanol: toluene (7:2:1, v/v) was found to be suitable solvent system for the proper separation of alkaloid (R_f = 0.46) from chloroform extract. Presence of alkaloid was confirmed by Dragendorff's reagent.

Determination of the total alkaloid content

The total alkaloid content was determined in triplicate by using gravimetric method of analysis[14].

Isolation of compounds

The chloroform extract (1.5 gm) obtained was mixed with silica gel (10 gm) to form an admixture and chromatographed using silica gel as an adsorbent in glass column (500 mm length × 20 mm diameter, 100–200 mesh size). Isocratic elution was done using chloroform: methanol: toluene (7:2:1, v/v), to obtain 76 fractions (25 ml each). Fractions 17–48 (shown the presence of alkaloid spot in TLC), were combined, and the solvent evaporated to give 550 mg of crude alkaloid fraction of ES (AFES). The fraction so obtained was further purified by preparative chromatography to get compound 1 (yield-100 mg, R_F 0.46, blue fluorescent band under 254 nm and become orange brown after treatment with Dragendorff's reagent).The purity of compound was further measured in terms of area under curve using high performance liquid chromatographic (HPLC) fingerprinting method. The solvent system optimized was acetonitrile: water (50:50, v/v) with a flow rate of 1 ml/min (alkaloid peak R_T - 6.74) and detected at 284 nm. Characterization of compound 1 was done by melting point determination, UV–Visible spectrophotometry, elemental analysis, FT-IR, NMR and mass spectral analysis.

HPTLC standardization and quantification of compound 1

HPTLC standardization was performed using aluminium backed pre-coated silica gel $60F_{254}$ HPTLC plates as stationary phase and chloroform: methanol: toluene (7:2:1, v/v) as mobile phase. Developed plates were scanned at 254 nm using CAMAG SCANNER III[15-17]. Presence of alkaloid was confirmed by dipping the developed plate in dipping chamber containing Dragendorff's reagent. For quantitative analysis stock solution (1 mg/mL) of compound 1 was prepared and utilised further to get standard solutions containing 100, 200, 300, 400, 500, and 600 ng/spot. A measured quantity of the CEES and AFES was dissolved in methanol and filtered to get a final concentration of 1 mg/mL. This solution was used for the quantitative TLC analysis.

In vitro inhibition of calcium oxalate crystallization

Conductometric titration

Conductometric titration of $CaCl_2$ was carried out with $Na_2C_2O_4$ in absence and presence of CEES. 10 ml of 0.05M $CaCl_2$ and 10 ml of 0.05M $Na_2C_2O_4$ was added and conductance was measured in absence and presence of 5 mL of CEES (100 µg/ml). This was done to study the interaction of extracts with $CaCl_2$ during precipitation of calcium oxalate[18].

Ethylene glycol-induced urolithiasis (*in vivo* studies)

Animals

The male albino rats of Wistar strain, weighing 200–250 gm, were obtained from Zydus Cadila

Laboratory, Ahmedabad, India. The rats were housed at temperature (25 ± 1 °C) with 50 ± 55 % of relative humidity and light (12 hrs light–dark cycles) were used. Rats were fed on standard chow diet and water *ad libitum*. The experimental protocols were approved (Reg. No. MSU/PHARM/IAEC/2013/40; dated, 24th August 2013) by the Institutional Animal Ethics Committee (Reg. No. 404/01/a/CPCSEA), The M S University of Baroda, Vadodara, Gujarat (India), in accordance with the guidelines for the care and use of laboratory animals set by CPCSEA.

Acute toxicity studies

The acute toxicity studies was performed as per the guideline set by the Organization for Economic Cooperation and Development (OECD) number 425 for determination of maximum tolerable dose (MTD)[2].

Grouping

The animals were divided into 6 groups, each group containing 6 animals.

Group I; normal rats; received vehicle only.

Group II; (negative control) received ethylene glycol (EG) 0.75 % in drinking water alone.

Group III; (positive control) received standard drugs Cystone 750 mg/kg.

Groups IV-VI; fed orally CEES (100, 200 and 400 mg/kg), respectively, for 28 days.

To induce urolithiasis and generate calcium oxalate deposition into kidneys, groups II–VI, received 0.75 % ethylene glycol (EG) in drinking water *ad libitium* for 28 days, respectively[19].

Biochemical parameters

Analysis of urine samples

Urine sample of 24 hrs was collected on 28th day by placing animals individually in separate glass metabolic cages. The collected urine samples were acidified (3 N HCl), then centrifuged (1500 rpm for 10 min) to remove debris and resultant supernatant was stored in deep freezing (-20 °C), until analyzed. Urine was analyzed for the presence of calcium, creatinine and uric acid contents[2].

Analysis of blood samples

The blood samples (2 ml) were collected from each animal in centrifuge tubes without adding any anticoagulant by puncturing the retro-orbital venous plexus and allowed to clot at room temperature. The serum was separated by centrifugation (1500 rpm for 15 min) and used for estimation of the serum calcium, creatinine, magnesium and uric acid[2].

Hispathological analysis

Both kidneys were removed by dissection after sacrificing animals and washed (under cold) with 0.15 M KCl. Left and right kidney were fixed in a 10 % solution of buffered formalin (*p*H.7.4) solutions separately. The tissues were processed for paraffin embedding sectioned at 5 µm thickness using microtome and subsequently stained with hematoxylin-eosin for examination under binocular microscope. Sections were examined for renal tubular necrosis and presence of calcium oxalate crystals[19-20].

Statistical analysis

All results were reported as mean ± SEM. The variation in a set of data has been estimated by performing Dunnett's Multiple Comparison test post-test to measures one-way ANOVA using non-parametric methods in Graph pad prism 5.01software (GraphPad software, San diego, CA, USA). The $P < 0.001$ regarded as significant.

Results and discussion

The total alkaloid content of CEES was measured at 110.34 ± 15.52 mg in 100 gm of the air dried sample of ES pseudostem (RSD = 14.06 %). The chromatographic analysis of the CEES identified one alkaloid band (R_f 0.46), which was targeted and processed to get AFES using column chromatography with silica gel as the adsorbent. The AFES was further purified by preparative chromatography on silica gel glass plate (mobile phase: chloroform: methanol: toluene, 7:2:1) to obtain compound 1(R_f 0.46, quenching under 254 nm, become orange brown after treatment with Dragendorff's reagent). HPLC (R_T = 6.74, 284 nm and using flow rate of 1ml/min) chromatogram in acetonitrile: water (50:50 v/v) showing difference in peak area of alkaloid, viz. CEES (581580), AFES (598270) and compound 1 (661882), respectively, suggest the increase in content of alkaloid in each purification steps (Fig. 1). The % purity of compound 1 was found to be 94.12 % and the physical and spectroscopic data for probable identity were in agreement with those of tetrahydro-β-carboline with reference to previous literature (Figs. 2 & 3) [21-23].

HPTLC standardization and quantification of compound 1

The results obtained by standardization and quantification of compound 1 are shown in Table 1

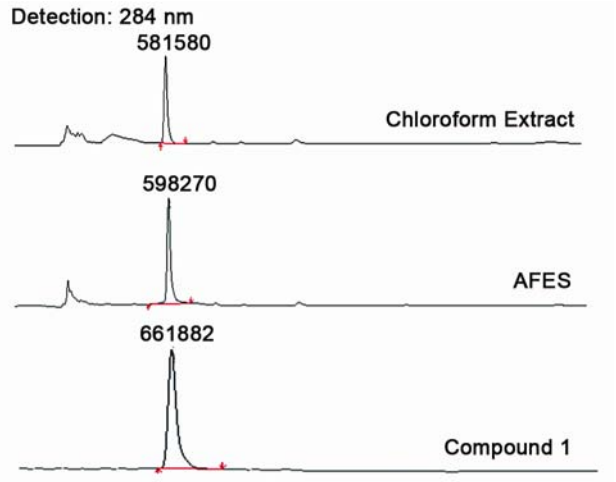

Fig. 1—HPLC chromatogram showing difference in peak area of alkaloid (R_T = 6.74) in acetonitrile: water (50:50 v/v) at 284 nm with flow rate 1ml/min.

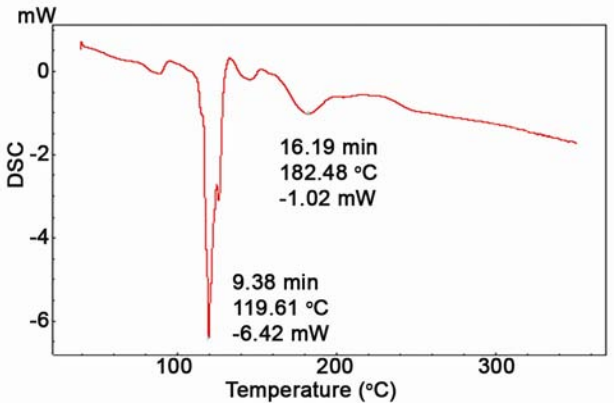

Fig. 2—DSC melting curve of compound 1 recorded in a dynamic nitrogen atmosphere (50 mL/min), and at a heating rate of 10°C min^{-1}.

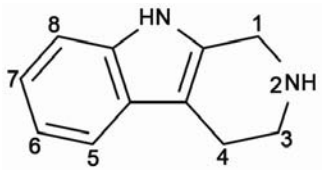

Fig. 3—Probable chemical structure of compound 1

and Fig. 4. The calibration plots were linear in the range 100-600 ng/spot and the correlation coefficient (r) of 0.9945 was indicative of good linear dependence of peak area on concentration.

Inhibition of calcium oxalate crystallization by conductometric titration (in vitro studies)

In vitro antiurolithiatic study by conductometric titrations of $CaCl_2$ with $Na_2C_2O_4$ in the absence and presence of CEES shows a decrease in conductivity (Table 2). The data presented for conductometric titration demonstrate shift in end point, cause reduction in free Ca^{2+} content due to complexation with the constituents of the extract. It was reported earlier that aqueous seed extract of ES show inhibitory effects on *in vitro* crystallization and growth patterns of calcium hydrogen phosphate dihydrate ($CaHPO_4.2H_2O$, CHPD) crystals using single diffusion gel growth technique. Reduction in growth of CHPD crystals was noticed with increasing concentrations of seed extract[8].

Table 1—Quantitative determination of compound 1 by HPTLC

Substance alkaloid @ 254 nm Regression mode: Linear

Regression via Area Y = 17.293 + 2.296 * X; r = 0.9945; sdv = 11.85%

Track	R_f	Amount (ng)	Area	X(calc)	Samples ID/Remarks
1	0.46	100	470.33		
2	0.46	200	470.33		
3	0.46	300	737.58		
4	0.46	400	954.90		
5	0.46	500	1127.92		
6	0.46	600	1339.37		
7	0.46		1504.45	647.58 ng	CEES
8	0.46		3948.47	1.712 µg	AFES

Acute toxicity studies

From the acute toxicity study, the cut-off dose (no signs of any abnormal behaviour or any mortality) was found to be 2000 mg/kg of body weight for the CEES. Three doses 100, 200 and 400 mg/kg of CEES were selected for future study by dose fixation method.

Ethylene glycol-induced urolithiasis (in vivo studies)

The concentration of urine calcium, creatinine and uric acid present in group I-VI, were shown in Table 3. In the present study, calcium oxalate crystals were absent in the 24 hrs urine of the vehicle-control animals whereas, in the lithogenic treatment, administration of EG of 0.75 % v/v in drinking water to male rats were caused increase in calcium, creatinine and uric acid concentration in the urine showing significant CaOx crystalluria, which shows abundant and larger crystals. It was reported earlier that administration of ethylene glycol causes urolithiasis due to an intracellular increase in the urinary concentration of calcium, creatinine and uric acid, which lead to nucleation and precipitation of calcium oxalate from urine. However, treatment with CEES (Group-IV-VI) at 100, 200 and 400 mg/kg reduced significantly ($P < 0.001$ vs. negative control)

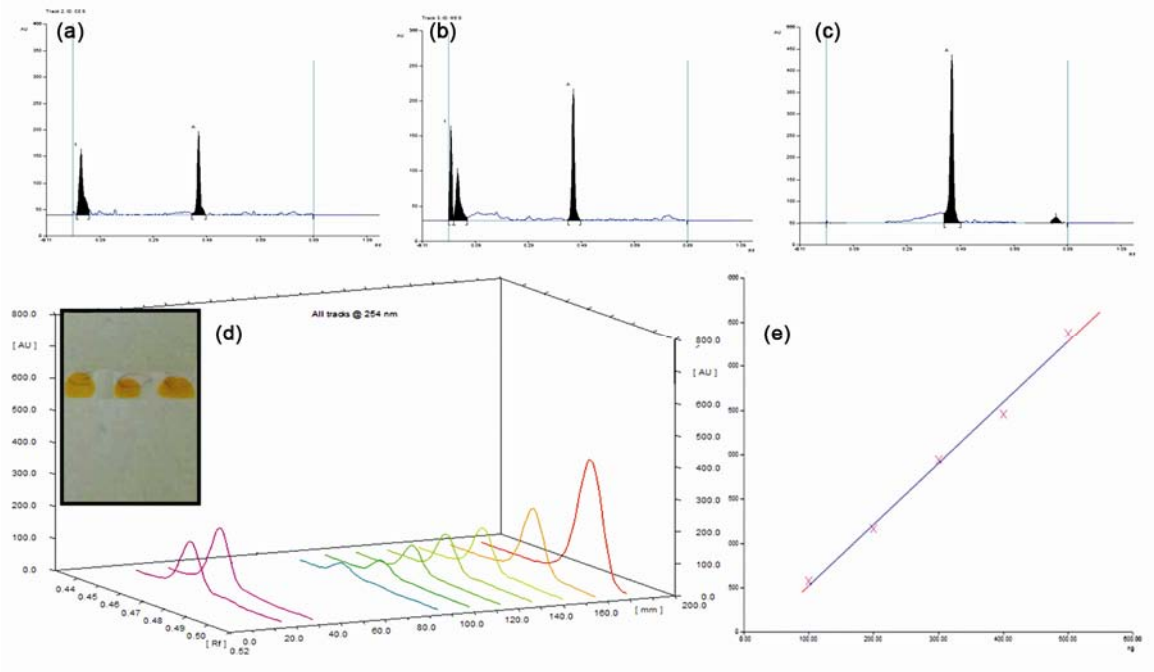

Fig. 4—HPTLC chromatogram at 254 nm; (A). CEES; (B). AFES; (C). Compound 1; (D). Densitogram of CEES, AFES and compound 1 (R_F = 0.46); (E). Calibration curve peak area versus concentration.

Table 2—Effect of CEES on conductometric titration

Volume (ml) of 0.05 M $Na_2C_2O_4$ added	Conductance (mmhos)	
	0.05 M $CaCl_2$	0.05 M $CaCl_2$ + CEES (100 µg/ml)
0	11.02	11.57
1	10.89	11.49
2	10.86	11.27
3	10.76	10.61
4	10.37	10.53
5	9.69	10.08
6	9.47	9.80
7	9.23	9.30
8	9.01	9.20
9	8.83	9.01
10	8.28	8.75
11	8.19	8.45
12	7.98	7.72
13	7.56	6.64
14	6.80	7.43
15	6.56	7.65
16	7.23	8.06
17	7.82	8.71
18	8.60	8.89
19	8.94	9.03
20	9.04	9.22

calcium, creatinine and uric acid excretion in urine. CEES (200 mg/kg) reduced calcium, creatinine and uric acid levels, were comparable to the Cystone treated rats (Group-III), whereas CEES (400 mg/kg) reduced calcium, creatinine and uric acid levels higher than that of Cystone treated rats (Group-III). Renal function was assessed by measuring serum calcium, creatinine, magnesium and uric acid in normal, control and treated rats and the results were shown in Table 4. The serum calcium, creatinine, magnesium and uric acid levels were significantly ($P < 0.001$ vs. Group-I) elevated in urolithiatic negative control (Group-II) when compared with (Group-I) indicating renal damage. Treatment with CEES, significantly ($P < 0.001$ vs. Group-II) reduced the levels of these substances. CEES (200 mg/kg) significantly reversed the serum calcium, creatinine, magnesium and uric acid closer to standard drug Cystone values, whereas CEES (400 mg/kg) reduced calcium, creatinine, magnesium and uric acid levels higher than that of Cystone treated rats (Group-III). These results indicate that the pseudostems of ES improve renal function in (Group-IV–VI) as compared to urolithiatic control (Group-II).

According to recent reports, plant extracts rich in polyphenolics and alkaloids can cause smooth muscle relaxation specifically to the urinary and biliary tract which could facilitate the expulsion of stones from both kidneys. ES revealed the presence of alkaloids, steroids, phenolics, glycoside, colour pigments like chroman derivatives and sugars[6].

In urolithiasis, non-protein nitrogenous (NPN) substances such as calcium, creatinine, magnesium

Table 3—Effect of CEES on urine calcium, creatinine, and uric acid in ethylene glycol induced urolithiasis in rats

S.No.	Groups	Calcium (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
1	Normal	$3.49 \pm 0.13^{a***}$	$0.820 \pm 0.02^{a***}$	$1.81 \pm 0.03^{a***}$
2	Negative control	13.07 ± 0.30^{b}	10.09 ± 0.20^{b}	3.95 ± 0.02^{b}
3	Positive control (Cystone)	$3.72 \pm 0.06^{c***}$	$1.39 \pm 0.04^{c***}$	$1.91 \pm 0.06^{c***}$
4	CEES 100 mg/kg	$4.49 \pm 0.12^{c***}$	$1.77 \pm 0.06^{c***}$	$2.33 \pm 0.04^{c***}$
5	CEES 200 mg/kg	$3.83 \pm 0.12^{c***}$	$1.41 \pm 0.21^{c***}$	$2.07 \pm 0.05^{c***}$
6	CEES 400 mg/kg	$3.54 \pm 0.11^{c***}$	$1.02 \pm 0.03^{c***}$	$1.87 \pm 0.05^{c***}$

All values are mean ± SEM (n = 6), One way analysis of variance test (ANOVA) followed by Dunnette's multiple comparison test. CEES, Chloroform extract of ***Ensete superbum***; 'a' is compared with 'b' and 'b' is compared with 'c'. *** $P < 0.001$ statistically significant, $P > 0.05$ are non-significant and ns is non-significant.

Table 4—Effect of CEES on serum calcium, creatinine, magnesium and uric acid in ethylene glycol induced urolithiasis in rats

S.No.	Groups	Calcium (mg/dl)	Creatinine (mg/dl)	Magnesium (mg/dl)	Uric acid (mg/dl)
1	Normal	$8.61 \pm 0.08^{a***}$	$0.97 \pm 0.01^{a***}$	$2.14 \pm 0.10^{a ns}$	$2.56 \pm 0.17^{a***}$
2	Negative control	13.83 ± 0.17^{b}	3.01 ± 0.06^{b}	2.38 ± 0.04^{b}	6.63 ± 0.10^{b}
3	Positive control (Cystone)	$8.85 \pm 0.03^{c***}$	$1.08 \pm 0.03^{c***}$	$2.24 \pm 0.05^{c ns}$	$3.71 \pm 0.19^{c***}$
4	CEES 100 mg/kg	$9.53 \pm 0.09^{c***}$	$1.26 \pm 0.10^{c***}$	$2.31 \pm 0.06^{c ns}$	$4.11 \pm 0.07^{c***}$
5	CEES 200 mg/kg	$9.42 \pm 0.03^{c***}$	$1.12 \pm 0.05^{c***}$	$2.30 \pm 0.05^{c ns}$	$3.82 \pm 0.07^{c***}$
6	CEES 400 mg/kg	$8.82 \pm 0.09^{c***}$	$1.03 \pm 0.02^{c***}$	$2.22 \pm 0.03^{c ns}$	$2.89 \pm 0.01^{c***}$

All values are mean ± SEM (n = 6), One way analysis of variance test (ANOVA) followed by Dunnette's multiple comparison test. CEES, Chloroform extract of ***Ensete superbum***; 'a' is compared with 'b' and 'b' is compared with 'c'. *** $P < 0.001$ statistically significant, $P > 0.05$ are non-significant and ns is non-significant.

and uric acid accumulate in the blood[19]. In this study, we find that the concentration of NPN substances, viz. calcium, creatinine, magnesium and uric acid increases in the serum of ethylene glycol treated control rats. This suggests that the ethylene glycol causes renal tubular damage and decreases glomerular filtration rate (GFR). The groups (IV–VI) (Table 4) suggest that CEES treated rats brings significant decrease in the calcium, creatinine and uric acid up to the normal limit at the dose of 200 and 400 mg/kg. This may be due to the muscular damage caused by oxidative stress in experimental rats. These findings suggest that the kidney function were improved in CEES treated rats. Low levels of magnesium are also encountered in stone formers as well as in stone-forming rats[2]. The magnesium level sreturn to normal on drug treatment was observed in the present study. Histopathological examination of the paraffin kidney sections under light polarized microscope showed many crystalline deposits in renal tubules of all regions of kidneys of all the animals in the untreated negative control group compared to normal control group (Figs. 5A&B). In CEES treated groups, such deposits were found in rats receiving 100 mg/kg, while Cystone, CEES (200 mg/kg) and CEES (400 mg/kg) treated group showed few or none of crystal deposits compared to normal (Figs. 5C-F).

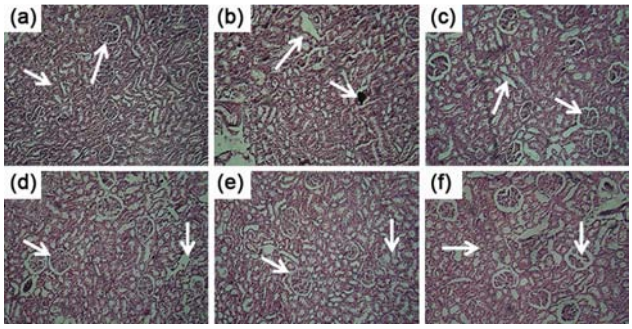

Fig. 5—Microscopic images (100 ×) of kidney sections from (A) Normal: Arrow marks indicate normal glomerular structure and renal tubules; (B) Negative control: Arrow marks indicate oxalate renal stone, tubular dilation and renal tubular damage; (C) Cystone: Arrow mark indicate little tubular dilation and normal glomeruli; (D) CEES 100 mg/kg: Arrow mark indicate Bowmen's capsule space with tubular dilation; (E) CEES 200 mg/kg: Arrow mark indicate partial atrophy of glomeruli little tubular dilation and normal glomeruli; (F) CEES 400 mg/kg: Arrow mark indicate normalisation of tubular dilation and glomerular structure.

Histopathological analysis revealed that administration of Cystone, CEES (200 mg/kg) and CEES (400 mg/kg) gradually decreased deposition as well as damage from lithogenic treatment and prevent the lithogenic induced renal tissue injuries.

Conclusion

In conclusion, chloroform extract of ***Ensete superbum*** pseudostem has the antiurolithiatic effects

in dose dependent manner, which may relate to several mechanisms, such as inhibition of calcium oxalate crystal formation, reduction in urinary concentration of stone-forming constituents, increasing the bioavailability of nitric oxide to sequester calcium via competitive absorption with ethylene glycol and nephrolithiasis inducing factors. It is worthwhile to evaluate further the effects as demonstrated here are due to the synergistic effects of the alkaloid with other substance or individual alkaloid might have better effects. However, further investigations are needed to pin-point the exact structure of alkaloid (β-carboline) and also for structural elucidation for substitutions present on β-carboline ring.

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Declarations of Interest

The authors report no declarations of interest.

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