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EVALUATION OF ANTI-UROLITHIASIS ACTIVITY OF *WOODFORDIA FRUTICOSA*

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ABSTRACT

Euphorbia hirta L. is a common weed plant that belongs to the family Euphorbiaceae characterized by the presence of milky latex in leaves and stems. Phytochemical screening of leaves of Euphorbia hirta L. confirmed the presence of alkaloids, cardiac glycosides, flavonoids, phenolic compounds, tannin and terpenoids. FTIR confirms the formation of calcium oxalate crystals with major peaks at 3331.07, 1608.63, 1317.38, and 775.38, which corresponds to asymmetric O-H bending, C=O stretching, C-O stretching and C-H bending. Urinary stone formation takes place due to changes in urinary chemistry, such as hyperoxaluria and hypercalciuria, leading to urinary super saturation, which later crystallizes, aggregates and ends up in stone formation. Evidences in previous studies indicated that, in response to 14 days period of ethylene glycol (0.75%v/v) administration, young albino rats form renal calculi composed mainly of CaOx. The principal precursor of oxalic acid in mammals is glyoxalic acid. The enzymatic oxidative conversion of glycolate to oxalate via glyoxylate is the major metabolic pathway involved in endogenous oxalate synthesis. The enzymatic disturbances are the causative factors for the idiopathic hyperoxaluria; while, the defective intestinal absorption of oxalate plays a vital role in enteric hyperoxaluria and lead to an increase in the urinary oxalate concentration. In the present study, chronic administration of 0.75% ethylene glycol aqueous solution to male Wistar rats resulted in hyperoxaluria. Oxalate and calcium excretion in urine were grossly increased in calculi- induced animals.

Keywords: *Euphorbia hirta L.*, Phytochemical, Nucleation, Aggregation, Calcium oxalate

INTRODUCTION

Urolithiasis is the third most prevailing and painful disorder of global concern. It is the process of kidney stone formation in the urinary tract. Supersaturation of the urine with crystal-forming substances and imbalance between promoters and inhibitors are two major causes of kidney stone formation. Nucleation is the first step in kidney stone formation in which the smallest unit of crystal *i.e.* "nuclei" or "nidus" of calcium oxalate stones formed [1, 2]. When the nuclei of calcium oxalate started binding to each other and formed larger particles, a process called aggregation. Strong intermolecular forces of crystals not allowed

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nuclei to get separated easily, and now these crystals are large enough to behave like a stone [1, 2]. Kidney stones vary in composition and hence can be of different types like calcium oxalate, calcium phosphate, uric acid, and mixed (magnesium,ammonium, calcium, and phosphate), but calcium oxalate stones are most abundant [3]. Many plants have been used in the treatment of kidney stones, and various plants are reported to have antiurolithiatic activity [4, 5]. Plant *Euphorbia hirta* L.commonly known as dhudhi, asthma weed and dugdhika, belongs to family Euphorbiaceae withmany activities reported in the literature. Present study has been designed to bring light on the urolithiatic potential of leaves of *Euphorbia hirta* L.

MATERIALS AND METHODS

Collection of Plant and Preparation of Extract:

Plant *E. hirta* has been collected from the garden the Department of Botany, the University of Rajasthan in the month of July-August.

Phytochemical Screening:

Leaves of the plant were shade dried and powdered using a mixer grinder and extracted in ethanol by the soxhlet method.

Table 1: Methodology for Phytochemical Screening

S. no.	Secondary metabolite	Name of test	Methodology	Observations
1	Alkaloids	Wagner test	2ml extract + 1% HCl + steam + 1ml Wagner's reagent drop by drop	Brownish red Precipitate[6]
2	Cardiac glycosides	Kellar-Killiani test	2ml extract + 2 ml of chloroform + H_2SO_4 to form a layer	Brown ring at interphase[7]
3	Flavanoids	NaOH test	Extract + dilute NaOH, + dilute HCl	Yellow solution on NaOH turns colorless on HCl[7]
4	Phenolic compounds	Lead acetate test	Extract + few drops of 10% lead acetate solution	Formation of white precipitate[8]
5	Saponin	Frothing test	0.5ml extract + 5ml distilled water and shake well	Persistence of frothing[6]
6	Tannin	Braemer's test	10% alcoholic $FeCl_3$ + 2-3ml of methanolic extract (1:1)	Dark blue or greenish grey coloration[6]
7	Terpenoids	Salkowski test	5ml extract + 2ml Chloroform + 3ml conc. H_2SO_4	Reddish Brown color of interface[6]
8	Anthroquinone	Ammonia test	1 ml dilute (10 %) ammonia + 2ml extract	A pink-red color in the ammoniacal (lower) layer[7]
9	Phlobatannin	HCl test	Extract boiled with 2 ml of 1% hydrochloric acid	Formation of red precipitate[8]
10	Starch	Iodine test	2ml extract + 2ml iodine solution	Formation of Blue color[9]

RESULTS AND DISCUSSION:

Phytochemical Screening:

Phytochemical screening of leaves of *Euphorbia hirta L.* confirms the presence of different phytochemical groups Table 2.

Table 2: Phytochemical Screening

S. no.	Secondary metabolite	Name of test	Result
1	Alkaloids	Wagner test	+
2	Cardiac glycosides	Kellar- Killiani test	+
3	Flavanoids	NaOH test	+
4	Phenolic compounds	Lead acetate test	+
5	Saponin	Frothing test	-
6	Tannin	Braemer's test	+
7	Terpenoids	Salkowski test	+
8	Anthroquinone	Ammonia test	-
9	Phlobatannin	HCl test	-
10	Starch	Iodine test	-

In Vitro Method :

The experiment consisted of the following test tubes of 10 ml capacity and marked the tubes as control and tests into 12 groups, each group has 6 test tubes, in each tube 1ml of calcium chloride anhydrous and 1ml sodium oxalate were added to the tubes and 2 ml of tris buffer (disodium hydrogen phosphate and potassium dihydrogen phosphate) adjusted at 7.4 pH which to the kidney pH and incubated at 36.7^0C over night. The next day the test tubes were centrifuged for 10min to decant to remove top liquid layer. The calcium oxalate crystal formed in the test tube will be checking using the compound microscope under 45x magnification, the crystal formed will resembling the prisms shape, to this 5ml (5mg/ml) equivalent to 25mg to each test tube of the different sequential extracts of plant *Kigelia africana* will introduce to the tubes and at the same quantity the synthetic drugs Spironolactone, Furosemide and the Poly herbal formulation Cystone will administered to the test tube, all the above treating agents will administered as aqueous suspension using tween 60 as suspending agent and again it will incubated 36.7^0C for 3 days on the fourth day all the test tubes will taken and checked for dissolution of the crystals under the microscope at the same superimposition, to this test a drop of con. HCl will add to separate the oxalate ion, calcium and both the ions will spectroscopically analyzed.

Table 3: Groupings

Group- I	Generated calcium oxalate crystals and referred as control.
Group- II.	Generated calcium oxalate crystals + 5ml **Cystone**
Group- III	Generated calcium oxalate crystals + 5ml **Aqueous Extract of** *Woodfordia Fruticosa*
Group-IV	Generated crystals + 5ml **Alcoholic Extract** of , *Woodfordia Fruticosa*

Elemental Ions Analysis:

Calcium, magnecium and oxalate ions will spectroscopically analyzed.

In Vivo Method:

Animals:

Adult male albino wistar rats will be used for this study with the weight range of 150-200g. they will be maintained under 12h dark/light cycle in well ventilated polypropylene metabolic cages(3-4/cage)

Preparation of the extract:

The dug extract will be suspended in water solvent

Experimental design:

Lithiasis will be inducing in rats by using the method of selvam et al, as ethylene-glycol, being reported to be renotoxic. The selected animals divided into 4 groups of 6 each.

Table 4: Animals groups

S. No.	Group	No. of Animals (Rats)	Treatment	Route of Administration
1.	Group-I (Normal- Control)	06	Commercial pellet feed&ordinary drinking water	Orally
2.	Group-II (lithiatic- Control)	06	0.75% ethylene glycolated water ad libitum for 28 days	Orally
3.	Group-III (Positive- Control)	06	0.75% ethylene glycolated water ad libitum + test drug extract	Orally
4.	Group-IV	06	Extract with ordinary drinking water	Orally

Urine will be collected on days 7,14,21 and 28 for 24h by keeping the animals in metabolic cages. the collected urine will analyzed for calcium (using calcium liquid kit; raichem method) , magnesium (Erba magnesium Aresnazo method,), oxalates (Hodkinson and Williams procedure), inorganic phosphates(using phosphorous reagent kit; raichem method) and protein (at 650 nm) by U.V. spectroscopy using standard

methods. The volume of urine collected from all groups also recorded. The prevalence of lithiasis will confirmed by histopathological studies of the kidneys isolated from the sacrificed animals.

RESULT & DISCUSSION

Effects on in-vitro CaOx crystallization The effect of administration of ethanolic and methanolic extract (5 mg/ml-5 ml) / Cystone (5 mg/ml-5 ml) on size and dissolution of Calcium-Oxalate (CaOx) crystals was determined by microscopy and chemical analysis. The prism shape Calcium-Oxalate (CaOx) crystals were sized/measured by eye piece and stage micrometer. In microscopic examination the crystal size reduction in aqueous extract (2.98 µm) is more significant ($p < 0.001$) than both alcoholic extract (4.06 µm; $p < 0.001$) and standard cystone (3.46 µm; $p < 0.001$) compared to normal untreated crystal size (9.84 µm). Values are in mean ±SEM (n=50) ***p <0.001, *p <0.05 vs. Normal control. The ethanolic extract shows more activity then methanolic extract in examination of both the analytical parameters. In alimental ion analysis also the ethanolic extract shows maximum hike in concentration of calcium and oxalate ions (44.2; 99.47 mg/dl) than methanolic extract (42.104; 94.73 mg/dl) and slight bit near to the standard cystone control (44.033; 98.28 mg/dl) compared to the normal control (13.185 28.78 mg/dl). T he increase in calcium and oxalate concentration in both test drug extracts and standard drug cystone treated groups were found to be very significant ($p < 0.001$) compared to normal untreated group.

Table 5: Effects on *in-vitro* CaOx crystallization

Group	Calcium (mg/dl) ±SEM	Oxalate (mg/dl) ±SEM
Normal control	13.185 ±3.240	28.78 ±1.0
Standard control (cystone)	44.033 ±0.3059***	98.28 ±1.0***
Test-1(Methanolic extract)	42.104 ±0.526***	94.73 ±1.0***
Test-2 (Ethanolic extract)	44.20 ±0.526***	99.47 ±1.0***

Effect Observed In Animal Model (In Vivo)

Body weight, water intake, urine volume and pH before the start of experiment were not significantly different among the study groups. The parameters recorded during the experimental period are shown in Table 12. The weight of the untreated group decreased significantly ($p < 0.01$) compared to the normal and treated groups. However the WF treated groups have shown significant increase in body weight. There was no significant increase or decrease in water intake in all groups. EG treatment reduced the urine pH in the untreated group compared to that of the control group, although not significantly. Co-treatment with KAFE increased urine volume ($p < 0.05$) in a dose dependant manner, although these parameters remained higher than those of the untreated animals. Our results are in agreement with these studies, as shown by the significant increase in urinary calcium and oxalate levels were found to be highly significant. WF treatment at a dose of 100 and 250 mg/kg b. wt. revealed a dose related response. WF treatment at dose levels of 250 mg/kg b. wt showed a better protective effect. However, there was no significant difference observed between 100 and 250 mg/kg b. wt of WF treatment. This finding revealed that 100 mg/kg b. wt of WF is the minimum dose required for eliciting an optimal activity. Cystone (std. drug) treatment significantly lowered the oxalate values ($p < 0.001$) compared to lithiatic control group animals, probably by its inhibitory action on glycolate oxidase. WF (alcoholic and aqueous) treatment also significantly lowered the oxalate values ($p < 0.001$) compared to lithiatic control. The increase in calcium excretion may be due to defective tubular reabsorption in the kidneys. Cystone and WF treatment markedly reduced the levels of calcium and phosphorus ($p < 0.001$) in urine (Table 13). The excretion of magnesium (0.30 ±0.64 mg/24h) decreased gradually in group-2 animals after 4^{th} week following EG treatment which is quite significant ($p < 0.001$) compared to normal control group. But in other animal treatment groups (3,4 & 5), it showed an enhanced excretion of magnesium and values are significant ($p < 0.001$) compared to lithiatic control animals.

Table 6: Various parameters recorded for assessment of antiurolithiasis activity during 28 days of study

Parameter		Normal control	Lithiatic control	Cystone (std.)	Alcoholic Ext.	Aqueous Ext.
General	Change in body weight (gm)	3.76 ±1.92	-7.23 ±44.43b	2.13 ±2.13	4.57 ±1.82b	6.14 ±2.47c
	Water intake (ml/24hr)	11.2 ±0.23	13.73 ±0.25b	17.33 ±0.55bd	15.42 ±1.08b	16.92 ±0.62bd
	Urine volume (ml/24hr)	6.67 ±0.38	11.15 ±0.84b	16.05 ±0.36	13.74 ±0.48b	14.06 ±1.32bd
	Urine pH	6.8 ±0.03	6.4 ±0.06	6.8 ±0.04	6.8 ±0.03	6.8 ±0.07
Kidney homogenate analysis	Weight (gm)	0.63 ±0.03	1.44 ±0.22b	0.74 ±0.12	0.79 ±0.02	0.67 ±0.15
	Calcium (mg)	0.20 ±0.35	1.27 ±0.34b	0.40 ±0.56d	0.47 ±0.64d	0.34 ±0.42d
	Oxalate (mg)	0.44 ±0.06	3.04 ±0.15b	1.12 ±0.05d	1.27 ±0.21d	0.94 ±0.08d
	Phosphorus (mg)	2.39 ±0.01	2.53 ±0.17	2.35 ±0.02	2.17 ±0.03	2.89 ±0.78

Table7: Effect of drugs on various urinary parameters recorded for assessment of antiurolithiasis activity during 28 days of study

	Group	Week-1	Week-2	Week-3	Week-4
	Dose→	100mg/kg	100mg/kg	100mg/kg	250mg/kg
Urine Oxalate	Normal control	14.48 ±0.54	14.081 ±0.27	14.28 ±0.43	13.3 ±0.1
	Lithiatic control	24.28 ±0.1***	22.24 ±0.08***	23.24 ±0.72***	24.12 ±0.08***
	Cystone (std.)	17.14 ±0.26***	16.53 ±0.01***	16.83 ±0.62***	15.79 ±0.11***
	Alcoholic Ext.	18.16 ±0.22**	17.55 ±0.06***	17.75 ±0.64***	16.02 ±0.68***
	Aqueous Ext.	14.28 ±0.43***	14.48 ±0.08***	14.08 ±0.91***	13.22 ±1.2***
Urine Calcium	Normal control	4.43 ±0.23	4.25 ±0.54	4.73 ±0.26	4.32 ±0.22
	Lithiatic control	13.2 ±0.14***	12.28 ±0.72***	12.84 ±0.33***	11.93 ±0.37***
	Cystone (std.)	8.68 ±0.54***	8.18 ±0.35***	8.33 ±0.38***	5.92 ±0.62***
	Alcoholic Ext.	7.832 ±0.9***	7.28 ±0.87***	7.55 ±0.15***	6.02 ±0.74***
	Aqueous Ext.	7.83 ±0.53***	6.53 ±0.86***	7.08 ±0.55***	5.87 ±0.87***
Urine Inorganic phosphorus	Normal control	0.579 ±0.59	0.723 ±0.47	0.661 ±0.37	0.87 ±0.38
	Lithiatic control	1.436 ±0.14***	1.336 ±0.28***	1.286 ±0.36***	1.496 ±0.54***
	Cystone (std.)	1.145 ±0.25**	1.142 ±0.56**	1.043 ±0.34***	1.121 ±0.12***
	Alcoholic Ext.	1.123 ±0.23**	1.228 ±0.32***	1.075 ±0.62***	1.136 ±0.36***
	Aqueous Ext.	1.18 ±0.87***	1.128 ±0.42**	1.004 ±1.0***	1.032 ±0.9***
Urine Magnesium	Normal control	1.17 ±0.73	1.2 ±0.43	1.35 ±0.84	1.22 ±0.25
	Lithiatic control	1.3 ±0.45*	0.9 ±0.57**	0.7 ±0.14***	0.3 ±0.64***
	Cystone (std.)	0.73 ±0.14**	0.81 ±0.28***	0.84 ±0.24***	0.96 ±0.34***
	Alcoholic Ext.	0.78 ±0.42**	0.88 ±0.32***	0.89 ±0.49***	0.92 ±0.39***
	Aqueous Ext.	0.63 ±0.24**	0.78 ±0.29**	0.84 ±0.28***	0.97 ±0.25***

Kidney stone formation is a physicochemical process including various events that starts with super saturation, nucleation, growth, aggregation, and retention within renal tubules. Different *in-vitro* models were used to study various physic- chemical events and simulate the urinary conditions by various authors. We have evaluated the effect of WF on CaOx crystallization kinetics by the time course measurement of crystal size and alimental ion analysis. The Methanolic and ethanolic extracts of fruit of ***Woodfordia Fruticosa*** strongly inhibited the precipitation of calcium and oxalate. The result of our study clearly showed the utility of ***Woodfordia Fruticosa*** in the treatment of renal and urinary calculi.In microscopical examination the crystal size reduction in aqueous extract was more significant ($p < 0.001$) than both alcoholic extract and standard cystone compared to normal untreated crystal size. Thus it can be inferred that the test drug extract contribute to heal renal calculi by crystal/stone size reduction . In alimental ion analysis, the ethanolic extract also shows maximum hike in concentration of calcium and oxalate ions than methanolic extract and standard cystone compared to the normal control. The increase in calcium and oxalate concentration in both test drug extracts and standard drug cystone treated groups were found to be very significant ($p < 0.001$) compared to normal untreated group. That is under physiological condition of the reaction system inhibited calcium and oxalate ions precipitation. Our results conclude that these inhibitors of crystallization along with crystal dissolution would potentially contribute in ailment of urolithiasis. Renal CaOx deposition induced by EG in rats is frequently used by various researchers to mimic the urinary stone formation in humans. Oxalate is produced during metabolism and excreted harmlessly in normal individuals.

CONCLUSION

Plant E. hirta shows significant antiurolithiatic potential against calcium oxalate kidney stones and hence can be considered as a better herbal alternative for the treatment of kidney stones. It can be further studied by in-vivo methods for more clarity as per its clinical significance and also its role as an herbal drug in human health and medicine.

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