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ISSN: 2581-771X

In vitro evaluation of antiurolithiatic activity of *Euphorbia thymifolia* L. plant extracts

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ABSTRACT

Objectives: The plant *Euphorbia thymifolia* Linn., Family: Euphorbiaceae, is used in traditional medical practices of India to treat various diseases. It was suspected and found to evaluate the effect of *E. thymifolia* plant extracts for *in vitro* antiurolithic activity on generated calcium oxalate crystals.

Method: The aqueous, hydroalcoholic, and alcoholic (ethanolic) extracts of plant were tested for antiurolithiatic potential on generated calcium oxalate crystals by homogeneous precipitation method, and simultaneously, supporting two-step vice versa reactions were assessed (new method). The activity was assessed by studying the crystal dissolution by microscopy and quantitative alimental ions analysis for calcium and oxalates.

Results: They exhibited significant activity when compared to standard drug cystone-polyherbal formulation. The aqueous and alcoholic extracts significantly decreased ($P < 0.001$) crystal size and increased calcium and oxalate concentration in reaction setup of all tested groups as compared to normal control. Simultaneously, a supporting two-step vice versa reaction was assessed which have shown a significant inhibition of crystal formation.

Conclusion: All the interpretations of various result outcomes direct the use of this drug for urolithiasis prophylactic as well as curative treatments.

Key words: Antiurolithic, calcium, cystone, *Euphorbia thymifolia*, oxalate

INTRODUCTION

Urolithiasis is somewhat synonym to the renal calculi that are solid concretions, inside the lumen of renal calyces or toward the exit of renal pelvis. These are usually a combination of inorganic salts (such as calcium, oxalate, phosphorus, and ammonia) or organic salts (such as uric acid). The exact pathogenesis of it is still not known, but in some cases, it is known to be due to incorrect purines catabolism and gout. It leads to uricosuria and stagnation of some other salts in the kidney and their precipitation. The presence of such big crystals/stones in the kidney causes excruciating pain (renal colic) and determines the presence of pus or blood in urine, compromising the renal activity. If it is quite large and blocks the urine flow through renal pelvis and ureter, then it is often necessary to surgically intervene. Today, we rely on endoscopic lithotripsy, extracorporeal shock wave lithotripsy, or laser lithotripsy and ultrasound technologies which reduce the surgical intervention to a minimum. Since quite long time, medical approaches are being made to eliminate calculi by the use of drugs only excluding any painful surgical operations. If the calculus is made up of only salts of the uric acids and calcium oxalate, then a specific drug therapy can dissolve them. Some polyherbal drug formulations are available in the market.[1]

MATERIALS AND METHODS

Plant Material

The fresh complete plants of *Euphorbia thymifolia* were collected from the gardens of DBGI campus, Dehradun, Uttarakhand. It was identified and authenticated by taxonomist Dr. S. K. Srivastava, Botanical Survey of India, Dehradun, Uttarakhand. The Acc. No. was 115372.

Preparation of the Plant Material

The fresh complete plants of *E. thymifolia* were dried under shade at room temperature for 7 days. The dried plants were powdered and passed through the sieve (coarse 10/44). This powder was used for the preparation of aqueous, hydroalcoholic, and alcoholic extracts.

Preparation of Extracts

The dried plant powder was extracted by simple maceration followed by sonication method. Alcohol and distilled water were used as solvent. Alcoholic, hydroalcoholic (ratio 1:1), and aqueous extracts were prepared.

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Received: 30-03-2018; **Revised:** 10-05-2018; **Accepted:** 20-06-2018

Experimental Design

Method A

Test tubes of 10 ml capacity were used and marked the tubes as control and test. 5 groups (6 tubes), in each tubes, 1 ml of calcium chloride (Merck Specialities Pvt. Ltd., Mumbai) anhydrous and 1 ml sodium oxalate (RFCL Ltd., New Delhi) were added along with 2 ml of tris buffer (disodium hydrogen phosphate and potassium dihydrogen phosphate), adjusted at 7.4 pH similar to the kidney pH, and incubated at 36.7°C overnight. The next day, the test tubes were centrifuged for 10 min, decanted to remove a top liquid layer.[2,3]

The calcium oxalate crystals formed in the test tube were checked using the compound microscope under ×45 magnification. The crystal formed resembled prism shape. 5 ml (5 mg/ml) (equivalent to 25 mg) of the extracts of plant *E. thymifolia* were introduced to the tubes, and the same amount of polyherbal formulation cystone was added to the test tube. All the above-treating agents were administered as an aqueous suspension using Tween-80 as suspending agent and incubated at 36.7°C for 3 days. On the 4th day, all the test tubes were taken and checked for dissolution of the crystals under the microscope at the same superimposition; to this test, a drop of concentrated HCl was added to separate the oxalate ion and calcium; and both the ions were spectroscopically analyzed.

Group 1 - Generated calcium oxalate crystals and referred as control.
Group 2 - Generated calcium oxalate crystals + 5 ml cystone.
Group 3 - Generated calcium oxalate crystals + 5 ml aqueous extract of *E. thymifolia.*
Group 4 - Generated calcium oxalate crystals + 5 ml alcoholic extract of *E. thymifolia.*
Group 5 -Generated calcium oxalate crystals + 5 ml hydroalcoholic extract of *E. thymifolia.*

Crystal dissolution was observed under ×45 microscope, and prism shape calcium oxalate crystals were sized/measured by eyepiece and stage micrometer. Mean size of >50 crystals was observed.[4]

Method: B

A reaction setup was established to evaluate the influence of drugs on calcium oxalate crystal generation. This is a vice versa protocol to check the drug effects in both the ways.[5]

Step A

Calcium chloride solution 1 ml (saturated)

↓

Drug solution 1 ml (10 mg/ml)

↓

Incubated for 24 h

↓

Sodium oxalate solution 1 ml (saturated)

↓

Further incubated for 24 h

↓

Observed microscopically the calcium oxalate crystal formation/ generation and their size

Step B

Sodium oxalate solution 1 ml (saturated)

↓

Drug solution 1 ml (10 mg/ml)

↓

Incubated for 24 h

↓

Calcium chloride solution 1 ml (saturated)

↓

Further incubated for 24 h

↓

Observe microscopically the calcium oxalate crystal formation/ generation and their size

Microscopical Examination

Crystal dissolution was observed under ×45 microscope, and prism shape CaOx crystals were sized/measured by eyepiece and stage micrometer. Mean size of > 50 crystals were observed.

Elementalion Analysis

Calcium

Calcium, in an alkaline medium, reacts with o-cresolphthalein to form an intense chromophore which absorbs light at 575 nm (570–580 nm). Set the autoanalyzer instrument with the parameters given along with the kit (AGAPPE Diagnostics Ltd., Kerala). Prepare the working, standard, and test solutions as per the protocol. Incubate for 5 min at room temperature. Mix and read at 575 nm.[6]

Oxalate

Oxalate is coprecipitated with calcium sulfate, reduced to glycolic acid by boiling dilute sulfuric acid and a zinc pellet, and estimated colorimetrically with chromotropic acid. Set the autoanalyzer instrument with the parameters given along with the kit (AGAPPE Diagnostics Ltd., Kerala). Prepare the working, standard, and test solutions as per the protocol. Incubate for 5 min at room temperarture. Mix and read at 570 nm.[7]

Statistical Analysis

The results of the study were subjected to one-way ANOVA followed by Dennett's *t*-test.

RESULTS

Calcium Oxalate Crystal Size Analysis

The effect of drug extracts (5 mg/ml-5 ml) and cystone (5 mg/ml-5 ml) on size and dissolution of calcium oxalate crystals was determined by microscopy and chemical analysis.

The prism shape calcium oxalate crystals were sized/measured by eyepiece and stage micrometer using ×45 magnification. Mean size of >50 crystals was assessed as shown in Tables 1-9 and Figures 1-7.

Table 1: Generated calcium oxalate crystals referred as control (Group 1)

	1	2	3	4	5	6	7	Total
1	20	14	15	19	19	13	23	107
2	30	24	14	21	30	9	24	126
3	12	6	13	15	25	11	13	109
4	8	10	12	24	7	12	14	114
5	14	26	18	14	16	13	12	116
6	17	18	11	17	23	18	24	128
7	22	16	20	28	11	17	25 & 9	135

Mean - 846/50=16.92 µm

Table 2: Crystal size in cystone-treated group (Group 2)

	1	2	3	4	5	6	7	Total
1	7	6	4	11	6	9	12	49
2	8	8	3	13	5	7	9	63
3	6	7	7	3	11	6	8	75
4	6	12	6	4	10	5	11	74
5	7	11	7	4	8	11	5	54
6	9	10	8	7	13	13	15	65
7	12	5	11	8	12	4	7 & 10	56

Mean - 407/50=8.14 µm

Table 3: Crystal size in the aqueous extract of *Euphorbia thymifolia* L.-treated group (Group 3)

	1	2	3	4	5	6	7	Total
1	11	14	9	5	7	6	5	50
2	7	9	10	8	9	8	6	50
3	10	14	12	9	5	4	6	44
4	13	8	6	9	6	14	12	56
5	6	12	6	9	7	10	11	66
6	13	10	5	7	6	8	5	52
7	9	14	11	6	13	11	7 & 9	57

Mean - 437/50=8.74 µm

Table 4: Crystal size in hydroalcoholic extract of *Euphorbia thymifolia* L.-treated group (Group 4)

	1	2	3	4	5	6	7	Total
1	5	6	4	5	6	5	4	58
2	5	7	10	9	8	11	5	75
3	12	9	8	7	10	8	12	47
4	10	10	12	8	5	7	5	45
5	10	8	5	7	8	6	6	89
6	3	5	11	13	10	9	13	66
7	11	5	6	7	5	12	5	41

Mean - 382/50=7.6 µm

Elemental Calcium Ions Analysis[8]

Calcium ions were calorimetrically and spectroscopically analyzed using calcium detection kit (AUTOPAK Siemens Ltd., Vadodara, Gujarat).

Method - 2

In this method, microscopically, we observed that due to the influence of drug treatment on both the reactants, it was not proper calcium oxalate crystal growth compared to normal reaction setup.

Thus, there was no crystal formation observed in alcoholic and hydroalcoholic extract-treated groups. The formed crystals size in aqueous extract treated was very minute measuring <5 µm at ×45 magnification, and the number of crystal size was very

Table 5: Crystal size in the alcoholic extract of *Euphorbia thymifolia* L.-treated group (Group 5)

	1	2	3	4	5	6	7	Total
1	8	11	7	7	12	10	5	51
2	11	9	7	12	11	9	10	60
3	4	9	6	10	5	7	6	47
4	5	4	8	5	7	4	10	49
5	9	5	8	7	6	12	4	67
6	9	6	5	7	6	3	13	33
7	6	10	4	11	7	6	10 & 4	66

Mean - 377/50=7.54 µm

Table 6: Effect of *Euphorbia thymifolia* L. bark extracts on generated calcium oxalate mean crystals size

Group	Mean crystal size (µm)±SEM
Normal control	16.92±0.120
Standard control (cystone)	8.14±0.417
Test-1 (aqueous extract)	8.74±0.400***
Test-2 (hydro alcoholic)	7.65±0.383***
Test-3 (alcoholic)	7.54±0.367***

Values are in mean±SEM (n=50) ***P<0.001, *P<0.05 versus, normal control.
SEM: Scanning electron microscope

Table 7: Effect of *Tectona grandis* L.f. bark extract treatment on free calcium concentration

Group	Calcium (mg/dl) ±SEM	Oxalate (mg/dl) ±SEM
Normal control	10.55±2.560	23.73±1.020
Standard control (Cystone)	31.030±1.811	69.81±1.032
Test-1 (Aqueous extract)	29.170±1.323***	65.63±2.002***
Test-2 (Hydroalcoholic extract)	36.055±3.482***	81.11±1.100***
Test-3 (Alcoholic extract)	30.678±3.471***	69.00±2.011***

Values are in mean±SEM (n=6) ***P<0.001, *P<0.05 versus normal control.
SEM: Scanning electron microscope

Step A

Table 8: Observed crystal size under ×45 magnification

Group	Observation	Crystal size
Control solution	Large size crystals were observed	=30 µm
Cystone solution	Very small and few crystals were observed	<1 µm
Aqueous extract	Small and few crystals were observed	<5 µm
Hydroalcoholic extract	No crystals were observed	0 µm
Alcoholic/ethanolic extract	No crystals were observed	0 µm

Step B

Table 9: Observed crystal size under ×45 magnification

Group	Observation	Crystal size
Control solution	Large size crystals were observed	<30 µm
Cystone solution	Very small and few crystals were observed	<1 µm
Aqueous extract solution	Small and few crystals were observed	<5 nm
Hydroalcoholic extract	Very small and few crystals were observed	<1 µm
Alcoholic/ethanolic extract	Very small and few crystals were observed	<1 µm

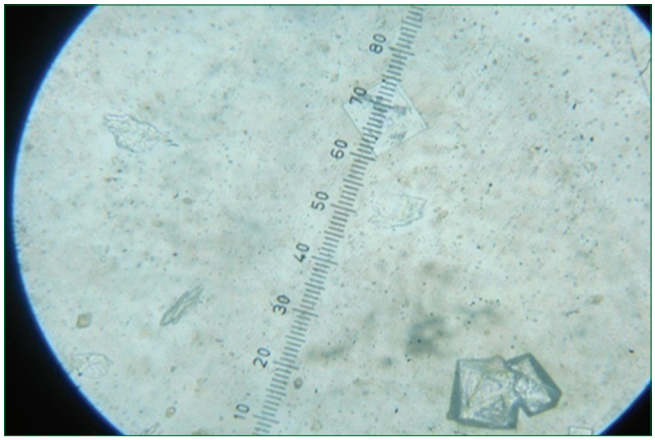

Figure 1: Observed crystal sizes in control at ×45

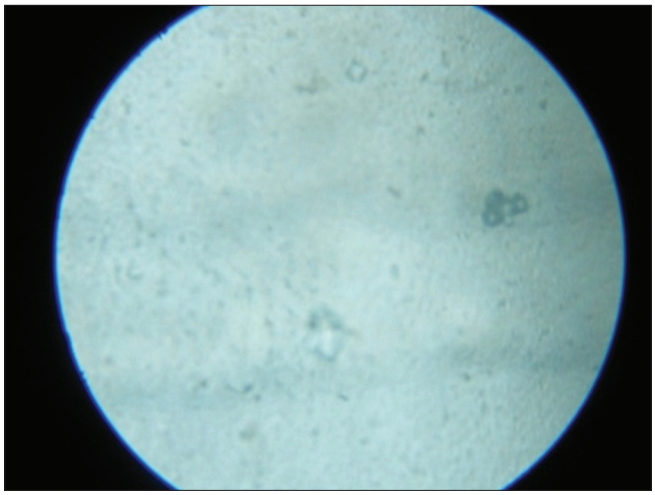

Figure 2: Observed crystals in cystone-treated group under ×45

less. That is, this drug may help in prophylactic regimen of urolithiasis.

DISCUSSION

The alcoholic and hydroalcoholic extracts of *E. thymifolia* L. plant inhibited the precipitation of calcium and oxalate. The results of our study clearly showed the utility of *E. thymifolia* L. plant for the treatment of renal calculi.

In the microscopical examination, the reduction of crystal size in the alcoholic and hydroalcoholic extract was more significant

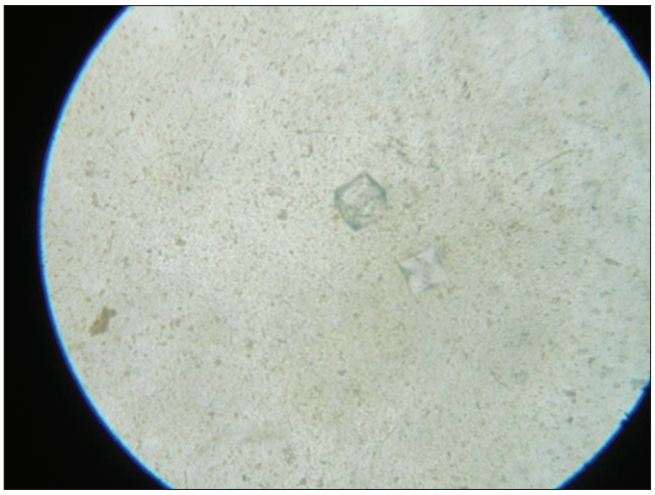

Figure 3: Observed crystal size in an aqueous extract of *Euphorbia thymifolia* L.-treated group at ×45

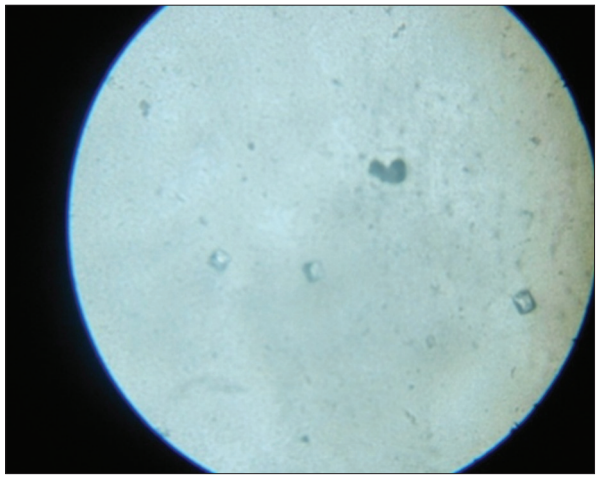

Figure 4: Observed crystal size in hydroalcoholic extract of *Euphorbia thymifolia* L.-treated group at ×45

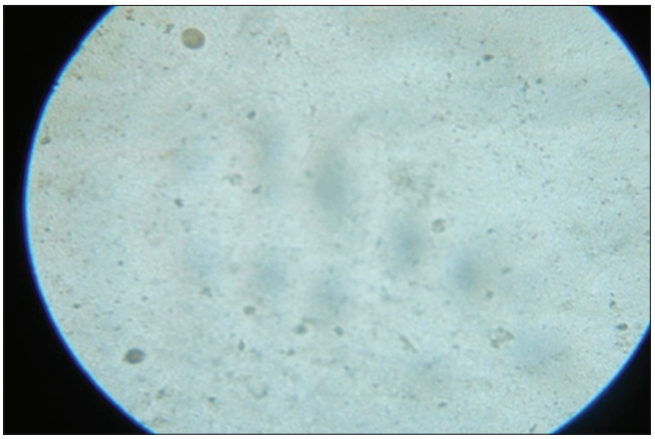

Figure 5: Observed crystal size in alcoholic extract of *Euphorbia thymifolia* L.-treated group at ×45

than both aqueous extract compared to normal untreated crystals. All three test extracts decreased the crystal size with the potency order of alcoholic, hydroalcoholic,and aqueous extracts,

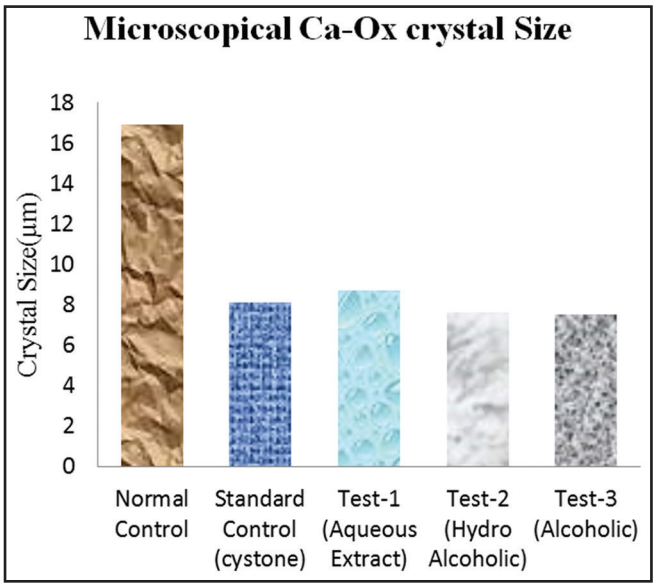

Figure 6: Effect of *Euphorbia thymifolia* L. bark extracts on generated calcium oxalate mean crystal size

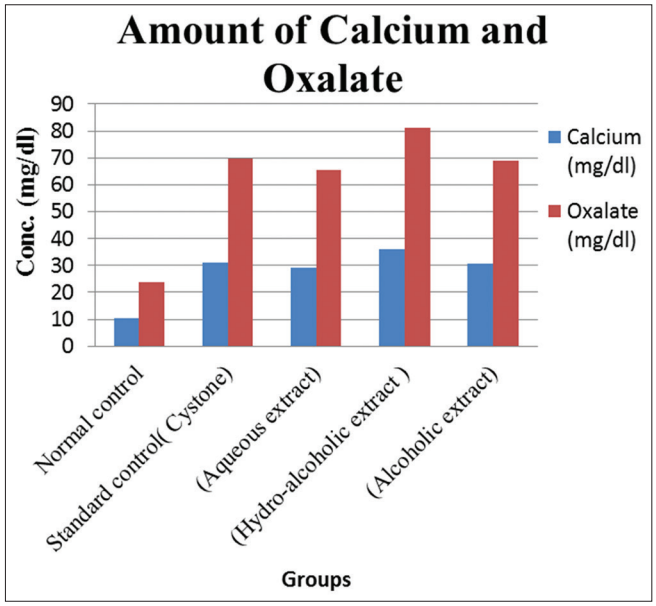

Figure 7: Effect of *Euphorbia thymifolia* L. bark extracts treatment on free calcium concentration

respectively, with comparable results of standard cystone drug treatment.

Since our test drug extract shown activity comparable to standard, but the results of alcoholic and hydroalcoholic extract were very near and significant to the standard drug. The aqueous extract also shown mild activity than compared to standard.

Simultaneously, elemental calcium ion analysis also supports the result outcomes in the same way as discussed above.

Simultaneously, supporting two-step vice versa reaction was assessed as per method two that described significantly the inhibition of calcium oxalate crystallogenesis as well as crystal growth in all the treatment groups.

Under physiological conditions of the reaction system inhibited calcium and oxalate ion precipitation. Our results conclude that these inhibitors of crystallization along with crystal dissolution would potentially contribute in the ailment of urolithiasis.

SUMMARY AND CONCLUSION

Morden synthetic medicines are not only very effective in curing a number of diseases but also cause serious side effects. Crude drugs are less efficient with respect to the cure of disease but are relatively free from side effects. A number of medicinal plants are evaluated mainly against calcium phosphate and calcium oxalate type kidney stones, employing various experimental models of urolithiasis.

In the present study, the plant *E. thymifolia* L. was selected for its considerable tannins content and diuretic activity, and thus, an attempt was made to evaluate anticalcinogenic potential. Different extracts of *E. thymifolia* L. showed anticalcinogenic action in the growth of calcium oxalate crystals. Alcoholic extract was more potent than hydroalcoholic extract and aqueous extract at tested crystal dissolution and quantitative elemental ion analysis in *in vitro* study.

Plants with tannins as their major active constituents have been shown to exhibit anticalcinogenic activities. Therefore, the anticalcinogenic activity of *E. thymifolia* L. plant can be attributed to their tannin content. Although, to establish *E. thymifolia* L. as an anticalcinogenic drug studies with other methods of the evaluation of urolithiatic action needs to be undertaken, but still the study projects *E. thymifolia* L. as a potential lead in search of active compounds for treatment of urolithiasis.

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How to cite this Article: Gupta AK, Dobriyal R, Victorian TD. *In vitro* evaluation of antiurolithiatic activity of *Euphorbia thymifolia* L. plant extracts. Int. Res. Med. Health Sci., 2018; 1(1):3-7.

Source of Support: Nil, **Conflict of Interest:** None declared.