

Research Article

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Corresponding Author:

***Mr. Urmilesh Jha,**
Dept. of Pharmacy, NIMS Institute
of Pharmacy, NIMS University,
Sobha Nagar Jaipur, Rajasthan

Mobile no. +919156549540



e-mail: Jha_urm@rediffmail.com

In-Vitro Antilithiatic Effect of Ethanolic Extract of Codiaeum Variegatum (L.) Blume

Urmilesh Jha*, Anoop Singh, Rajesh J Oswal

ABSTRACT

Objective: The aim of the study is to investigate the antilithiatic activity of ethanolic extract of Codiaeum Variegatum (L.) Blume by in-vitro method.

Methods: The ethanolic extract was evaluated for its antilithiatic activity by preparing synthetic urine. The crystal size development was monitored in sample drops every five minutes by polarized microscope. The experiments were carried in absence of plant extract and in presence of 25, 50, 75, and 100% of ethanolic extracts.

Result: In the presence of plants extracts, the length and the width of the crystals were reduced. The average length of the crystals grown in the presence of the inhibitors was less than that of the control sample.

Conclusion: In-vitro study shows that the ethanolic extract possesses antilithiatic activity and further in vivo study is required to confirm its mechanism of action.

Key-words: Codiaeum Variegatum, Antilithiatic Activity, Calcium Oxalate Monohydrate Crystallization, Synthetic Urine.

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INTRODUCTION:

Renal lithiasis is one of the oldest disease known to human beings and has been documented in ancient Greek. Urinary stones have been found in the remains of Egyptian mummies dating back as far as 7000 years¹. Renal lithiasis is defined as the consequence of an alteration of the normal crystallization conditions of urine in the urinary tract². Stone disease is common with the lifetime risk of stone formation exceeding 6-9% in men and 3-4% in women³. Kidney stone formation is the result of a physicochemical process that involves nucleation of crystals from a supersaturated solution. The common constituents of kidney stones are calcium, phosphate and oxalate. The factors that influence crystal generation are urine volume, concentration of stone constituents (a function of urine volume), the presence of a nidus and the balance among various physicochemical factors that inhibit or promote stone formation.

Garden crotons (*Codiaeum variegatum*) are a group of beautifully variegated leafy perennial ornamental shrubs found almost everywhere in India. It belongs to the family Euphorbiaceae which is an important plant family for diverse biological activities⁴.

Apart from its ornamental values, *Codiaeum variegatum* is also used for several medicinal purposes. Root decoction is taken for the treatment of gastric ulcers. Its leaves contain antibacterial and anti-amoebic properties and cures diarrhea⁵. A recent report also suggests that *Codiaeum variegatum* extracts are active against influenza virus and a bioactive cyanoglucoside was isolated as the active component⁶. *Codiaeum variegatum* also possesses alkaloids, anthraquinones, flavanoids, terpenes, steroid, phenol, saponins, tannins, phlobatannin and cardenolide and showed potent cytotoxicities in Brian shrimp lethality bioassays⁷. Phytochemical components, especially polyphenols (such as flavonoids, tannins, phenylpropanoids, phenolic acids etc) are known to be responsible for the free radical scavenging and antioxidant activities. Phenolic compounds are very well known plant constituents because of their scavenging ability⁸. It is demonstrated that polyphenolic compounds possess inhibitory effects on mutagenesis and carcinogenesis. In vitro studies also suggested that polyphenols may exert their inhibitory effects by acting as pro-oxidants on cancer cells or may inhibit the formation and growth of tumors by induction of cell cycle arrest and apoptosis⁹. The methanol and aqueous extract is effective against picrotoxin-induced convulsions in mice¹⁰.

MATERIAL AND METHODS:

Preparation of plant extract:

The shade dried leaves of *Codiaeum variegatum* was reduced to fine powder (# 40 size mesh) and around 500 gm of powder was subjected to successive continuous hot extraction (soxhlet) with Petroleum ether (40- 60°C), Ethanol and alcohol. Each time before extracting with the next solvent the powder material dried in a hot air oven at 50°C for one hour. After the effective extraction, the solvents were distilled off, the extracts then concentrated on water bath and extract obtained with each solvent weighed. Its percentage is calculated in term of air dried weight of plant material.

Qualitative chemical investigation:

Qualitative chemical tests were conducted in order to identify the various phytoconstituents.

1. Tests for phytosterols

The extract was refluxed separately with alcoholic solution of potassium hydroxide till complete saponification. The saponified extract was diluted with water and unsaponifiable matter was extracted with diethyl ether. The ethereal extract was evaporated and the residue (unsaponifiable matter) was subjected to the following test by dissolving the residue in the Ethanol.

- a) Salkowaski test: To the test extract solution added few drops of conc. sulphuric acid shaken and allowed to stand, lower layer turns red indicating the presence of sterols.
- b) Liebermann-Burchard test: The test solution treated with few drops of acetic anhydride and mixed, conc. sulphuric acid was added from the sides of the test tube, a brown ring at the junction of two layers is formed and the upper layer turns green.
- c) Sulphur test: Sulphur when added in to the test solution, it sinks in it.

2. Tests for steroidal glycosides

The solution of extract was prepared with water and 1 volume of 10 % v/v solution of sulphuric acid solution was added, heated on the water-bath for half an hour and extracted the hydrolyzed extract with the Ethanol as the solvent, Ethanol fraction was separated and concentrated, concentrated fraction was tested for steroids/ phytosterols.

3. Tests for glycosides

The test solution was prepared by dissolving extract in alcohol 90% or aqueous alcoholic solution.

- a) Baljet's test: The test solution treated with sodium picrate gives yellow to orange colour.
- b) Keller-Killiani test for digitoxose: The test solution is treated with few drops of ferric chloride solution and mixed, and then sulphuric acid containing ferric chloride solution is added, it forms two layers. Lower layer shows reddish brown while upper layer turns bluish green.

4. Tests for saponins

The test solution was prepared by dissolving extract in the water.

- a) Foam test: Test solution on shaking shows formation of foam, which is stable at least for 15 min.
- b) Haemolysis test: 2 ml of 18% sodium chloride in two test tubes is taken. To one test tube added distilled water and to other 2 ml test solution. Few drops of blood is added to both the test tubes, mixed and observed for haemolysis under microscope.

5. Tests for carbohydrates

The test solution was prepared by dissolving test extract with water, hydrolyzed with 2N hydrochloric and subjected to following tests.

- a) Molisch's test: Test solution with few drops of reagent and 2 ml of conc. sulphuric acid added slowly from the sides of the test tube shows a purple ring at the junction of two liquids.
- b) Barfoed's test: Test solution treated with reagent, boiled on a water-bath, shows brick red colour precipitate.
- c) Benedict's test: The test solution treated with reagent and boiling on water-bath shows reddish brown precipitate.
- d) Tollen's phloroglucinol test: 2.5 ml of concentrated HCl and 4 ml of 0.5% phloroglucinol are treated with 1-2 ml test solution. Heat, yellow to red colour appears.
- e) Cobalt-Chloride test: 3 ml of test solution is treated with 2 ml cobalt chloride. Boil and cool. Add few drops of sodium hydroxide solution. Solution appears greenish blue (glucose) or purplish (fructose) or upper layer greenish blue and lower layer purplish (mixture of glucose and fructose).

6. Tests for alkaloids

The test solution was prepared by dissolving extracts in dilute hydrochloric acid.

- a) Mayer's test: Test solution with reagent (potassium mercuric iodide) gives cream coloured precipitate.
- b) Hager's test: The acidic solution with reagent (saturated picric acid solution) gives yellow precipitate.

c) Dragendorff's test: The acidic solution with reagent (potassium bismuth iodide) shows reddish brown precipitate.

7. Tests for flavonoids

The flavonoids are all structurally derived from the parent substance called flavones. The flavonoids, which occur in the form of free, as well as, bound to sugars called glycosides. For this reason, when analyzing flavonoids, it is usually better to examine the flavonoids in hydrolyzed plant extracts.

Preparation of test solution

To small amount of extract equal volume of 2M hydrochloric acid is added and heated the test tube for 30-40 min at 100°C, allowed to cool, filtered and extracted with ethyl acetate. The ethyl acetate extract is concentrated to dryness, followed the test for flavonoids to ethyl acetate fraction by dissolving the residue with ethyl acetate.

a) Shinoda test: Test solution with few fragments of magnesium ribbon and conc. hydrochloric acid shows pink to magenta red colour.

b) Zn/Hcl reducing test: Test solution with zinc dust and few drops of hydrochloric acid shows magenta red colour.

8. Test for phenolics / tannins

The extract is dissolved in 90% alcohol.

a) Ferric chloride test: Test solution treated with few drops of ferric chloride solution gives dark colour.

b) Gelatin test: Test solution treated with gelatin gives white precipitate.

Preparation of synthetic urine:

The synthetic urine was prepared by mixing and stirring two equal volume of solution A and solution B (Solution A: $\text{Na}_2\text{C}_2\text{O}_4$ (2 m mol/1) and Solution B: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (10 m mol/1). The temperature was maintained at 37°C. The mixture is continuously agitated to prevent sedimentation.

Simulation of the sedimentary crystal formation:

The crystal size development was monitored in sample drops every five minutes by polarized microscope. Crystals were identified with 40X magnifying lens.

The experiments were carried at 25, 50, 75, and 100% of plants extracts. The crystal size development were observed at 5, 10, 20, 25 30 minutes of formation of crystals. Calculation of the percentage of Inhibition (1 %) was based on the formula¹¹.

$$I\% = [(TI - TAI) / TSI] \cdot 100$$

TI- represents the number of calcium oxalate monohydrate (COM) crystals without inhibitor.

TAI - represents the number of calcium oxalate monohydrate crystals after addition of inhibitor.

RESULT:

IN-vitro study of calcium oxalate monohydrate crystallization:

Kidney oxalate stone is the result of super saturation of urine with certain urinary salts such as calcium oxalate. Since crystallisable oxalate species are pH independent, the crystallization of oxalate in the absence of inhibitor, led to the formation of COM. In the crystal growth experiments sown, (nucleation, growth and aggregation), the rate of crystallization is usually controlled by the number of crystals of calcium oxalate as a function of time, Following the introduction of seed crystals.

Effect of Codiaeum variegatum extract on different stages of crystallization:

In order to assess the inhibiting potential of substances for oxalate crystallization and understand the mechanisms of action of Codiaeum variegatum, on oxalate crystallization steps (nucleation, growth, and aggregation), the effectiveness of extract was tested. In the presence of plants extracts, the length and the width of the crystals were reduced. The average length of the crystals grown in the presence of the inhibitors was less than that of the control sample. It was found that Codiaeum variegatum extract inhibits the process of nucleation and growth of the COM crystal while no effect on aggregation of crystals.

Effect of Codiaeum variegatum extract on crystallization after 30 minute:

30 minutes after the addition of Codiaeum variegatum extracts the inhibition of crystallization was noted. It was observed that Ethanol extract of Codiaeum variegatum shows 93.97, 94.87, 96.8, 96.86 percent of inhibition at corresponding conc. of 25% 50% 75% & 100% of concentration.

DISCUSSION:

Kidney stones are hard, solid particles that form in the urinary tract. In many cases, the stones are very small and can pass out of the body without any problems. However, if a stone (even a small one) blocks the flow of urine, excruciating pain may result, and prompt medical treatment may be needed.

Kidney stone formation is a complex process that results from a succession of several physiochemical events including super saturation, nucleation, growth, aggregation and retention within renal tubules.¹² Nucleation is the formation of solid crystal phase in a solution it is a essential step in renal stone formation. Crystal aggregation is very important step in the kidney stone formation as it occurs very rapidly and affects the crystal size and aggregated stone are present in the urine.¹³

Recurrent stone formers excrete clusters of crystals in the urine, caused by aggregation, also known as agglomeration, whereas urine from normal people contains mainly single crystal in the urine.

For the treatment of urolithiasis, there exist a large number of plant products as a prophylactic or curative agent in ethanomedicine, but there were very few plants which have been studied extensively.

In vitro crystal systems are widely used to study processes of crystal nucleation, growth and aggregation¹⁴.

COM is the primary constituent of the majority of stones formed in urolithiasis. The super saturation of urine with CaOx is the most common component of kidney stones ¹⁵ is an important factor in crystallization.

In the present study, the inhibitory potential of Codiaeum variegatum was tested for the COM crystal formation, which is predominantly present in most of the kidney stones, under in vitro conditions. The study was conducted at 25%, 50%, 75% and 100% of extract solution at the time interval of 5 minutes. The ethanol extract inhibits the growth of CaOx, at various concentrations. Ethanol extract (100%) shows high inhibitory percent.

However, crystalluria alone is not a risk factor for lithiasis because it is common in healthy subjects and stone-formers^{16, 17}. The limiting factors in stone formation could be those processes that affect the size of the particles formed, because particles may become large enough to occlude the urinary tract, leading to stone formation. The extracts of medicinal plant induced more crystals in whole urine, thereby reduced super saturation and the size of the particles. This property of the extracts is therefore advantageous, preventing urinary stone formation by inducing the excretion of small particles from the kidney and reducing the chance of retention in the urinary tract. The herb extract may contain substances that

inhibit the growth of COM crystals. This property of plants extract may be important in preventing kidney stone formation; crystals induced by urinary macromolecules were less tightly bound to epithelial cell surfaces than were COM particles¹⁸. The plants extracts may also contain substances that inhibit CaOx crystal aggregation; the agglomeration of particles is a critical step in urinary stone formation, as larger crystals are less likely to pass spontaneously in urinary tract^{19, 20}. If the extract keeps CaOx particles dispersed in solution they are more easily eliminated.

The plant extract when tested for chemical constituents shows the presence of tannins, glycosides, steroid, flavanoids, triterpenoids, saponins, alkaloids, carbohydrate and phenols.

Different activities observed in the plant crude extract might be due to the presence of these phytochemicals. For example, flavonoids are known to possess antispasmodic and Ca²⁺ channel blocking, antioxidant and antidiuretic activities. Saponins are known to have anti crystalline properties by disaggregating the suspension of mucoproteins, the promoters of crystallization^{21,22}. Antiurrolithiatic activities have been attributed to triterpenes, lupeols and phenolic and polyphenolic compounds like quercetin²³.

The plants extracts may also contain substances that inhibit CaOx crystal aggregation; the agglomeration of particles is a critical step in urinary stone formation, as larger crystals are less likely to pass spontaneously in urinary tract. If the extract keeps CaOx particles dispersed in solution they are more easily eliminated²³.

CONCLUSION:

It was observed that the ethanol extract of *Codiaeum variegatum* caused dissolution of CaOx crystal nucleation these results could be considered positives because the herb extract inhibits crystallization and prevents stones formation, revealing that the *Codiaeum variegatum* extract could reduce the size of the crystals formed indicative of antilithiatic activity.

Table 1: Physico-chemical study of *Codiaeum variegatum*.

Sr. no	Parameters studied	% Value
1.	Extractive values	
	Ethanol	4.76 % w/w
2.	Ash value	
	Total Ash	5.12 % w/w
	Water soluble Ash	4.06 % w/w
	Acid insoluble Ash	1.48% w/w
3.	Loss on drying	28%

Table 2: Results of qualitative chemical investigation of *Codiaeum variegatum*.

Sr. No.	Phytoconstituents	Procedure	Observation	Ethanol Extract
1	Test for Tannins	1. Drug + lead acetate + water	Formation of white ppt	+
2	Test for Glycosides	1. Anthrone + H ₂ SO ₄ + Heat	Purple or green	+
3	Test for Steroid	1. Liebermann Test 2. Salkowski Test	Bluish green Red & Fluorescent	+

4	Test for Flavanoids	1.Shinodaw's Test 2.Zn-HCl acid reduction Test	Red colour Magenta colour	+ +
5	Test for Triterpenoids	1.Noller's test	Pink colour	+
6	Test for Saponins	Drug + water + shaking	Formation of honey comb like froth	+
7	Test for Alkaloids	1.Drug + Dragondroffs reagent 2.Mayer's reagent	Orange colour White ppt.	+ +
8	Test for Carbohydrate	1.Drug + Molishs reagent+ conc.H2SO4 2.Fehling's solution A&B	Purple colour Brick red colour	+
9	Phenol	Fecl3	Intense colour	+

Table 3: Effect of Ethanolic extract of Codiaeum variegatum on different phases of calcium oxalate monohydrate crystallization.

Extract	Nucleation	Growth	Aggregation
Ethanolic extract of Croton coedum	+	+	-

(+) Action on the stage, (-) No action on the phase, (+-) More or less action

Table 4: Study of calcium oxalate crystallization without inhibitors.

Time (min)	5	10	15	20	25	30
Number of (COM)/mm ³	667	715	843	705	692	772
(COM) aggregation /mm ³	103	107	117	115	88	79
Total	770	822	960	820	780	851

Table 5: Effect of Codiaeum variegatum extract on crystallization at different concentrations of the extract.

Concentration Codiaeum variegatum extract	25%	50%	75%	100%
% Inhibition of crystallization	93.97	94.87	96.8	96.86

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