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Original Article

Evaluation of Calcium Oxalate Crystal Nucleation, Aggregation and Phytochemical Compositions of *Cissus adnata* Roxb. and *Cissus discolor* Blume

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ABSTRACT

Background: From time immemorial medicinal plants are used in treating urolithiasis in ayurvedic system and other forms of traditional health practitioners. In Manipur, local traditional health practitioners used *Cissus adnata* Roxb. and *Cissus discolor* Blume for treating kidney stone problems. However, proper scientific validation is not well studied and evaluated. **Methods:** The present study investigates the inhibitory effect of the chloroform extract of *Cissus adnata* Roxb. (CAc) and *Cissus discolor* Blume (CDc) on calcium oxalate (CaOx) crystal nucleation and aggregation using spectrophotometer. Furthermore, phytochemical studies of both plants were performed using Gas Chromatography-Mass Spectrometry (GC-MS), Graphite Furnace Atomic Absorption Spectrometry (GF-AAS), Inductively coupled plasma optical emission spectroscopy (ICP-OES) and Inductively coupled plasma atomic emission spectroscopy (ICP-AES). **Results:** CDc exhibited significantly higher inhibitory effect on nucleation and aggregation of CaOx than CAc and Cystone ($p<0.05$). GC-MS analysis of the CDc and CAc revealed presence of nine compounds, the highest area percentage occupied by stigmasterol in CAc and gamma-sitosterol in CDc. Elemental analyses of both plants detected twenty elements, strontium being the dominant element. The reduction in CaOx nucleation and crystal aggregation by CAc and CDc observed in this study could be attributed to calcium replacement by strontium. **Conclusion:** This study reported the inhibitory effect on CaOx crystal aggregation and nucleation by CAc and CDc in the *in vitro* assay. Further *in vivo* studies are necessary to validate the inhibitory effect of the studied plant extracts.

Key words: *Cissus adnata* Roxb., *Cissus discolor* Blume, Calcium oxalate, Crystal nucleation, Crystal aggregation.

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INTRODUCTION

A journey towards natural resources is an endless one. Likewise, digging for medicinal properties from the plants is also a never-ending path, and the outcomes are always exciting with lots of benefits. Remedies for various illnesses obtained from medicinal plants are being increasingly adopted by the masses over the past decade, as people believe that natural medicines are much safer than synthetic drugs.[1] Knowledge of plants having therapeutic benefits is drawn out mostly from traditionally used medicinal plants before the advent of modern medicines. These medicinal plants are also known as herbal plants. Depending on the illness, suitable herbal medicines are effectively used for treatment of human diseases. Most herbal products are either solid raw or crude extract, primarily mixtures of organic compounds.[2,3] Notably, different herbal product means different composition, and therefore the study of the medicinal plants' composition would help draw out the skeleton of phytochemical composition and their specific properties.

Kidney stone is a common human health problem with a high incidence of occurrence globally. Kidney stones, or renal calculi, are the crystal aggregates of dissolved minerals in the urine. Its formation depends on several factors such as retention time of aggregates, nucleation and aggregation of minerals in urine, crystal super saturation of urine, and urinary tract infection by urea-splitting bacteria.[4-6] The most commonly known varieties of renal calculi afflicting the human population are calcium phosphate, calcium oxalate (CaOx), struvite, and uric acid. Among these, CaOx stone occupies the highest with 93.5% occurrence.[7] The kidney stone problem affects 10-20% of the population globally, with a lifetime risk of 10% to 50% recurrence.[8,9] The major problem of kidney stones is recurrence after surgical removal or lithotripsy. Recurrence of kidney stones could lead to kidney failure or injury that could not be healed.[10] The percentage of kidney stone recurrence varies from 10% at one year to 50% at five years.[8]

The focus on herbal products is increasing. Many herbal medicines are also available at markets. Cystone is one of the successfully used herbal products for kidney stone (urolithiasis) treatment. However, potent herbal products suitable for CaOx crystal nucleation and crystal aggregation inhibition, which could stop the recurrence of kidney stones or avoid kidney failure, are still being explored.

Cissus adnata Roxb. and *Cissus discolor* Blume are two medicinal plants used traditionally for urolithiasis in Manipur, India (23.80 N-25.68 N and 93.03 E-94.78 E). Both these plants are perennial climbing shrubs that belong to the Vitaceae family. The traditional medicine practitioners used either water or hydro-alcohol extract, which is a polar solvent. The phytochemical constituents from non-polar solvent extracts, such as chloroform (polarity index 4.1, boiling point 61.2°C), of both *C. adnata* Roxb. and *C. discolor* Blume have not been reported.

Therefore, in this study, we aimed to analyse the various compounds present in the crude chloroform extract of *C. adnata* Roxb. and *C. discolor* Blume leaf. Inhibitory effect of the crude chloroform extract on CaOx crystal nucleation and crystal aggregation was also studied.

MATERIALS AND METHODS

Collection, *Identification, and Preparation of Plant Sample*

The plant samples were collected and identified at the Botanical Survey of India, Shillong. *C. adnata* Roxb. and *C. discolor* Blume were collected in September 2018 (No. BSI/ERC/Tech/Identification/2018/67). The leaves of *C. adnata* Roxb. and *C. discolor* Blume were washed properly and were shade dried for one week. 25 gm of the dried plant leaves were used for Soxhlet extraction. Chloroform (500 mL) extractions were carried out at 30 - 40°C for 8 hr. After extraction, the solvent was evaporated using Rotatory Vacuum Evaporator. The extract yield was calculated as (gm/100gm) = $(W_1X\ 100)/W_2$, where W_1 is the weight of extract residue after solvent removal, and W_2 is the weight of the sample. The dried extract residues were collected in sterilized Eppendorf tubes and stored at -20°C.

Nucleation Assay

The CaOx crystal nucleation inhibition was studied using nucleation assay. Briefly, calcium chloride (5 mmol/L) and sodium oxalate (7.5 mmol/L) were dissolved in aqueous solution containing Tris (0.05 mol/L) and sodium chloride (0.15 mol/L), and the solution was adjusted to pH 6.5. The artificial urine for the nucleation assay study was prepared using the method of Burns and Finlayson (1980). The dried chloroform extracts of *C. adnata* Roxb. (CAc), *C. discolor* Blume (CDc) and Cystone (The Himalaya Drug Company, Batch No.:19900321, used as positive control) were dissolved in ethanol (70% v/v). The concentrations used for CAc, CDc, and Cystone in nucleation assay were 100, 200, 300, 400, and 500 µg/mL. The final solution mixtures were incubated at 37°C for 10 min, and absorbance was measured at 620 nm using Spectrophotometer (Spectrophotometer 104, Systronics). Microscopic observation (Leica DM 2500) of final solution of Cystone, CDc, and CAc was performed for 500 µg/mL. The percentage inhibition of nucleation assay was calculated as $\{(OD_{control} - OD_{extract}/OD_{control}) \times 100\}$.[11]

Aggregation Assay

CaOx crystals were made by dissolving 1 mg/mL of calcium oxalate monohydrate in a Tris (0.05 mol/L) and NaCl (0.15 mol/L) solution, and pH was adjusted to 6.5. The plant extract concentrations were similarly taken as that of nucleation assay. The absorbance of the final mixture was measured at 620 nm after 90 min (Spectrophotometer 104, Systronics). The inhibitory rate for aggregation was calculated similarly to the above nucleation assay.

GC-MS Analysis

Analysis of chloroform extracted active compounds was undertaken using GC-MS equipped with Column J&W 122-5532, DM-5MS of 30 X 250µm X 0.25µm (GC-M 5975 C Agilent). 600 mg sample leaf extract was analysed for this at a total GC-MS running time of 35 minutes. The oven was maintained at 325°C and programmed for 3 min at 70°C, then 10°C/min to 300°C for 9 min. Helium was used as carrier gas. National Institute of Standards and Technology Library source was used for identification of the compounds.

GF-AAS, ICP-OES and ICP-AES

0.5 g each of *C. adnata* Roxb. and *C. discolor* Blume leaf powder was separately acid-digested using a Teflon digestion vessel by taking 10 mL concentrated HNO_3. The digested samples were kept for 30 min and made to cool. Further, the digested sample solutions were diluted to 12 mL with concentrated HNO_3. Finally, the volume was made 50 mL by adding double-distilled water, and the solutions were filtered. The filtered solutions were readied for elemental analysis using GF-AAS (Analytik Jena Vario 6), ICP-OES (Thermo Scientific™ iCAP™ 7600), and ICP-AES (Thermo Fisher iCAP RQ ICP-MS).

Statistical Analysis

The experimental results were analysed by ANOVA followed by the Tukey's multiple comparison test at the significance level of $p<0.05$. All values were represented as Mean ± SEM/ Mean ± SD.

RESULTS

Plant Extraction

On Soxhlet extraction, using 500 mL chloroform, 25 gm each of *C. adnata* Roxb. and *C. discolor* Blume leaves yielded 70.98±0.37 mg and 89.2±0.33 mg of crude extracts respectively. The yield of the *C. discolor* Blume is significantly higher than the *C. adnata* Roxb. leaf ($p<0.01$).

Nucleation Assay

The overall result of nucleation assay revealed that chloroform extract of *C. discolor* Blume (CD_C) leaf showed significant inhibitory potential on CaOx nucleation than Cystone ($p<0.01$) and CAc ($p<0.05$) (Figure 1 and 2).

Aggregation Assay

The aggregation assay also showed a similar result as that of the nucleation assay, i.e., *C. discolor* Blume (CD_C) leaf showed significant inhibitory potential of CaOx aggregation than Cystone ($p<0.01$) and CAc ($p<0.05$) (Figure 3).

GC-MS Analysis

Studies of phytochemical compounds using GC-MS revealed nine compounds on the chloroform extract of *C. adnata* Roxb. and *C. discolor* Blume leaves. The compounds identified from chloroform extract of *C. adnata* Roxb. were stigmasterol, campesterol, squalene, 9-octadecenamide (Z), Vitamin E, phytol, 1, 19-eicosadiene, octadecane and n-hexadecanoic acid. Stigmasterol occupied the highest area percentage (10.65%), while n-hexadecanoic acid (1.24%) showed the lowest area percentage. The compounds detected from chloroform extract of *C. discolor* Blume leaves were gamma-sitosterol, campesterol, stigmasterol, cyclo-tetracosane, 9-octadecenamide (Z), phytol, squalene,

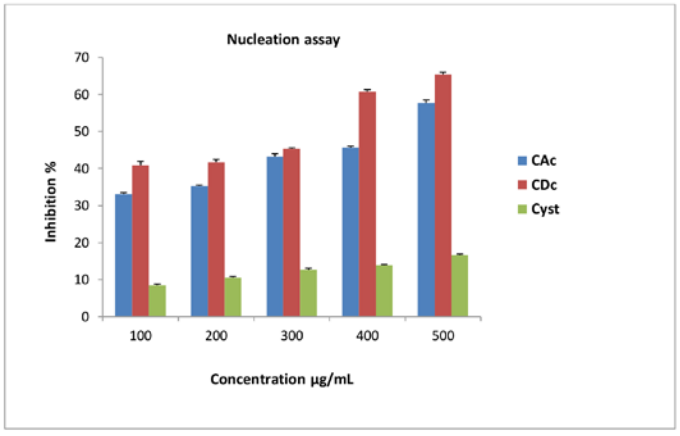

Figure 1: Inhibition of Calcium oxalate crystal nucleation under different experimental conditions.
The nucleation inhibition percentages are given in Mean ± SEM (n=5). CAc and CDc denote the chloroform extracts of C. adnata Roxb., C. discolor Blume respectively. Cyst (Cystone) is used as standard.

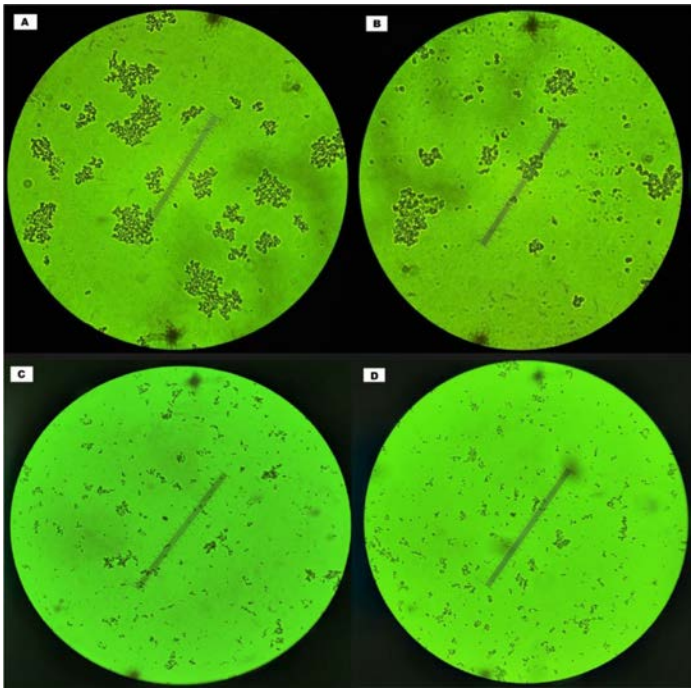

Figure 2: CaOx crystals as seen under microscope at different treatment conditions.
(A) Calcium oxalate solution, (B) Cystone treated solution, (C) Cissus adnata Roxb. treated solution, and (D) Cissus discolor Blume treated solution. Microscopic observation of final solution of Cystone, CDc, and CAc was performed for 500 µg/mL.

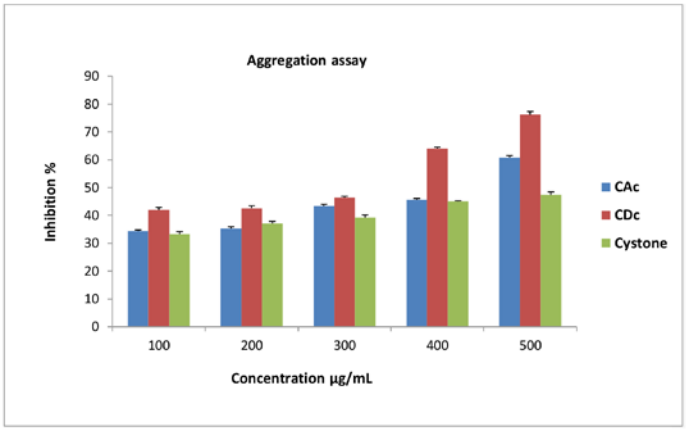

Figure 3: Inhibition of Calcium oxalate crystal aggregation under different experimental conditions.
The CaOx aggregation inhibition percentages of the chloroform extract of C. adnata Roxb. (CAc) and C. discolor Blume (CDc) as well as Cystone (Cyst) are given as Mean ± SEM (n=5). Cystone is used as standard.

9, 12, 15-octadecadienoic acid (Z, Z, Z)- and octadecanoic acid. In the case of CDc, gamma-sitosterol showed the highest area percentage (13.39%) and octadecanoic acid had the lowest area percentage (1.26%). The compounds present in both CAc and CDc were stigmasterol, campesterol, squalene, and 9-octadecenamide (Z). The area percentage of stigmasterol, campesterol, squalene in chloroform extract of *C. adnata* Roxb. were higher than that of the chloroform extract of *C. discolor* Blume leaf. Only the area percentage of 9-octadecenamide (Z) in the chloroform extract of *C. discolor* Blume was higher than the chloroform extract of *C. adnata* Roxb. (Table 1 and 2).

Element Analysis

Elemental analysis of *C. adnata* Roxb. and *C. discolor* Blume leaves showed 20 similar elements, but the content amount was different (Table 3). Out of 22 elements analysed (i.e., Ca, Co, Cu, Cr, Fe, Zn, Mn, Mg, K, Na, Se, Ni, Al, As, B, Li, Mo, Pb, Sn, Sr, Ti, and V), Mo and Ti were absent in both the medicinal plants. The dominant element in *C. adnata* Roxb. and *C. discolor* Blume leaves was Sr (strontium) with values of 104 and 92 ppm respectively.

DISCUSSION

Nucleation is the first step for CaOx stone formation, followed by aggregation of CaOx crystals. Nucleation and aggregation of the CaOx crystal eventually happen and increases at the supersaturation state of the solution.[12,13] Thus, inhibition of nucleation and crystal aggregation, i.e., reducing CaOx crystals, is the primary focus for preventing urolithiasis. The result from the nucleation and aggregation assay indicated that dose-dependent inhibition potential was observed in all the extracts, and CDc showed significant inhibitory potential than CAc ($p<0.05$) and standard Cystone ($p<0.01$) (Figure 1 and 2). The difference in the inhibitory potential of CDc and CAc might be the difference in the composition of the extract.

The phytochemical composition of CAc and CDc might be an essential parameter in reducing CaOx nucleation, aggregation, and increased antimicrobial properties. The differences in the content of compounds in CAc and CDc could be one of the main reasons for their variation in percentage reduction of CaOx nucleation and aggregation and increased antimicrobial property. Thus, the phytochemical composition of CDc could be a significant factor for showing higher potentiality on – (1) reducing the nucleation of CaOx crystals, (2) reducing the aggregation of CaOx crystals than that of CAc. The compounds detected from both the plant extracts have well-known bioactive compounds. Stigmasterol has been implicated as an anti-osteoarthritic and inhibitor of pro-inflammatory cytokines.[11,14] Squalene has antioxidant and antimicrobial properties.[15] Phytol has diuretic and antioxidant activity.[16] In addition to the common bioactive compounds mentioned above, the CAc had Vitamin E, a potent anti-inflammatory and antioxidant agent,[17] and n-hexadecanoic acid which also possesses antioxidant properties.[15,16] CDc contained 9, 12, 15-octadecadienoic acid (Z, Z, Z), which has exhibited anti-inflammatory and anti-arthritic potentials.[15]

Chemical elements present in medicinal plant extracts play significant roles in biological activities, either directly or indirectly. Sujatha, D. *et al.* (2015)[18] suggested that a possible mechanism for anti-urolithiasis activity is that plant extracts could form complexes with free calcium/oxalate and prevent CaOx crystals. Elemental studies support the initiation of kidney stones by some primary trace and heavy elements after the phenomenon of insertion and replacement of foreign elements on the crystal lattice of salt.[19] Likewise, insertion and replacement of elements can also take place on the inhibition process of CaOx crystals nucleation and aggregation. In this study, 20 elements were detected from a total of 22 analyses in both CAc and CDc. Four elements are of particular interest concerning our study on anti-urolithiasis properties of CAc and CDc. These are K, Na, Li, and Sr, which are more reactive than Ca. These four elements can replace Ca from CaOx.

On the other hand, oxalate ion in its free state carries a negative charge to form a complex with any of the 20 metals present in CAc and CDc. The dominant element content in CAc and CDc is Sr which is more reactive than Ca. Thus, Sr might replace Ca from CaOx. Certain studies confirmed the presence of Sr in calcium oxalate stone as the sign

Table 1: Phytochemical compositions of the chloroform extract of *C. adnata* Roxb. as determined by GC-MS. The retention time is in minutes.

Sl. No.	Chemical compounds	Molecular formula	*C. adnata* Roxb.	
			Area %	Retention time
1.	Stigmasterol	$C_{29}H_{48}O$	10.65	26.700
2.	Campesterol	$C_{28}H_{48}O$	7.57	26.477
3.	Squalene	$C_{30}H_{50}$	2.81	23.817
4.	9-Octadecenamide, (Z)	$C_{18}H_{35}NO$	2.69	20.341
5.	Vitamin E	$C_{29}H_{50}O_2$	2.50	25.838
6.	Phytol	$C_{20}H_{40}O$	2.45	18.231
7.	1, 19-Eicosadiene	$C_{20}H_{38}$	2.06	23.855
8.	Octadecane	$C_{18}H_{38}$	1.49	22.881
9.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	1.24	16.805

Table 2: Phytochemical compositions of the chloroform extract of *C. discolor* Blume as determined by GC-MS. The retention time is in minutes.

Sl. No.	Chemical compounds	Molecular formula	*C. discolor* Blume	
			Area %	Retention time
1.	Gamma-Sitosterol	$C_{29}H_{50}O$	13.39	27.725
2.	Campesterol	$C_{28}H_{48}O$	7.05	26.477
3.	Stigmasterol	$C_{29}H_{48}O$	6.27	26.692
4.	Cyclotetracosane	$C_{24}H_{48}$	6.13	25.593
5.	9-Octadecenamide, (Z)	$C_{18}H_{35}NO$	4.70	20.341
6.	Phytol	$C_{20}H_{40}O$	4.60	18.231
7.	Squalene	$C_{30}H_{50}$	2.41	23.817
8.	9, 12,15-Octadecadienoic acid (Z,Z,Z)-	$C_{19}H_{32}O_2$	2.28	18.640
9.	Octadecanoic acid	$C_{18}H_{36}O_2$	1.26	18.640

of substitution reaction.[20] However, strontium oxalate's growth and homogeneous nucleation at supersaturation conditions are low.[21]

Therefore, the reduction in CaOx nucleation and CaOx crystal aggregation by CAc and CDc observed in this study could be attributed to calcium replacement by strontium. All the elements detected in this study across different concentrations of the plant extracts were within the average daily requirement as per the Dietary Reference Intakes (DRIs).

The CDc extract exhibited significantly higher inhibition of nucleation and aggregation of calcium oxalate crystal (CaOx) than the CAc and Cystone. The reason could be the differences in the composition of active compounds and elements that can form complexes with free calcium/oxalate and prevent the formation of CaOx crystals. Thus, the phytochemical constituent of the CDc might play an essential role in the inhibitory action on nucleation and aggregation of calcium oxalate (CaOx) crystals. Further *in vivo* studies are required to investigate the role of CDc in the prophylaxis of urolithiasis as a potent inhibitor of nucleation and aggregation of calcium oxalate (CaOx) crystals.

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Table 3: Elements present in the chloroform extract of *C. adnata* Roxb. and *C. discolor* Blume.

Element	*C. adnata* Roxb. (ppm)	*C. discolor* Blume (ppm)
Ca[a]	0.50±0.01	0.47±0.01
Co[a]	0.15±0.01	0.17±0.00
Cu[a]	0.04±0.01	0.04±0.00
Cr[a]	2.01±0.81	2.57±0.14
Fe[a]	0.44±0.01	0.15±0.01
Zn[a]	0.31±0.04	0.25±0.01
Mn[a]	0.24±0.00	0.20±0.01
Mg[a]	1.91±0.03	2.01±0.01
K[a]	0.91±0.01	1.06±0.06
Na[a]	5.90±0.04	0.19±0.00
Se[a]	0.60±0.07	0.49±0.01
Ni[a]	0.02±0.01	0.08±0.01
Al[b]	0.041±0.00	0.053±0.01
As[b]	0.057±0.00	0.013±0.01
B[b]	0.347±0.02	0.348±0.05
Li[b]	0.245±0.02	0.247±0.03
Mo[b]	ND	ND
Pb[b]	0.038±0.00	0.046±0.00
Sn[b]	0.094±0.00	0.091±0.00
Sr[c]	104±0.00	92±0.00
Ti[b]	ND	ND
V[b]	0.630±0.02	0.345±0.05

GFAAS[a], ICP-OES[b] and ICP-AES[c]. Values are expressed as Mean ± SD; *(n=5)*. ND-Not detected.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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