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Antilithiac activity of *Chrysopogon zizanioides* Brushite crystallization *in vitro*

Research Article

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Abstract

Urolithiasis or the formation of calculi at any level of urinary tract is a common disorder found throughout world. Brushite minerals, probably the initiator of stone formation under favorable physiological environment, are one of the most commonly occurring stones along with calcium oxalate mono and di-hydrates. An attempt was made to know the effectiveness of methanolic extract of *Chrysopogon zizanioides* L. (Vetiver) roots on the growth of brushite crystals. Brushite crystals grown by single gel diffusion technique were treated with different additives, and with increasing concentration of the herbal extract, the crystals showed reduced size, weight, transparency and complexity in morphology. Preliminary phytochemical analysis confirmed the presence of active components like flavonoids, saponins, phenols and anthocyanins. The Fourier Transform Infrared Spectroscopy (FTIR) spectrum of harvested brushite crystals from various treatments revealed band shifts, disappearance of existing and appearance of new peaks which indicated the action of extract. The work established the inhibitory activity of vetiver root extract on the formation of brushite and supports the conventional knowledge of using vetiver root for control and treatment of kidney stones.

Key Words: Urolithiasis, Calcium Phosphate, Chrysopogon zizanioides, Herbal medicine, Single Gel Diffusion, Fourier Transform Infrared Spectroscopy (FTIR).

Introduction

Urolithiasis or the formation of calculi at any level of urinary tract is a common disorder worldwide. Around 12% of world population experience renal stone disease (1). Calcium-containing stones, especially calcium oxalate mono & dihydrates and calcium phosphate are the most commonly occurring ones (75-90%) followed by struvite (10-15%), uric acid (3-10%) and cysteine (0.5-1%) (2). Calcium hydrogen phosphate dihydrate ($CaHPO_4.2H_2O$), known as Brushite, is a stable form of calcium phosphate and is found beneath many pathological conditions including kidney stones and arthritis. Brushite is hypothesized as a transient precursor of minerals like octacalcium phosphate and hydroxylapatite which are thought to be the initiator of stone formation, under favorable conditions (3). Also calcium oxalate nucleation usually happens over initial calcium phosphate layer which precipitates with increase in urinary pH. So the treatment for the condition should lower the super-saturation of both calcium oxalate and phosphate (4).

Herbal medicines have gained attention in urolithiasis treatment because they are reported effective in decreasing the recurrence rate of renal calculi and also they are cheap and easily available (5). Plants produce various secondary metabolites naturally which give people a ray of hope on facing limitations in modern medicine (6). Herbals drugs exert their beneficial effect on urolithiasis being diuretic, analgesic, antispasmodic, antibacterial, and having antioxidant activity which can inhibit the crystallization, nucleation and aggregation of stones (2, 7, 8). *Chrysopogon zizanioides* (L.), commonly known as Vetiver is widely recommended for its medicinal values. An ancient ayurvedic work Charaka Samhita mentions it as an herb that enhances urinary excretion and relieves genital disorders including urolithiasis and dysuria. This plant helps in reducing oxidative stress and also exhibits antimicrobial, analgesic and anti-inflammatory properties (9, 10). But works reporting the antilithiatic potential of Vetiver are scarce. In the present work an *In vitro* approach was framed to examine the anti-urolithiatic property of methanolic extract of Vetiver roots on brushite crystallization by single gel diffusion technique.

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Materials and Methods

Preparation of Herbal extract

Root of *C. zizanioides* collected from Valparai, Coimbatore, was air-dried at room temperature for 2 weeks and was ground to a uniform powder of 40 mesh size. 100 g of dry powder was extracted with 1 L of methanol in a Soxhlet extractor continuously till the solvent falling out of the siphon tube became colorless. Extract was filtered by Whatman filter paper and was

concentrated using rotary evaporator to get viscous semi-solid masses.

Phytochemical Analysis

Preliminary Phytochemical screening on the plant extract was done by simple qualitative tests following the methodology of Sofowara (1993), Trease and Evans (1989) and Harborne (1973). The methanolic extract was tested for Tannins, Phlobatannins, Saponins, Flavonoids, Steroids, Terpenoids, Alkaloids, Anthroquinone, Phenols, Carbohydrates, Proteins, Cardic Glycosides, Coumarins, Anthocyanin and Leucoanthocyanin. The condensed methanolic extract was subjected to column chromatography over silica gel. Elution with hexane followed by ethyl acetate and finally with methanol yielded many fractions among which F4 and F5 were selected for treatment.

Growth of Brushite crystals

Glass test tubes of 2.5 cm diameter and 15 cm length were used for growing crystals. Sodium meta-silicate solution of specific gravity 1.06 was added with 1M orthophosphoric acid to set a pH of 6.0. After gelation, freshly prepared 1M Calcium chloride was added as supernatant and was allowed to diffuse slowly through the gel to initiate nucleation, aggregation and growth of micro-crystals.

Antilithiatic activity

Equal volume of Plant extract in different concentrations was added through the sides of test tubes and were capped airtight (Figure 1). After 21 days, the crystals were harvested from the gel, washed, filtered and then air dried. The inhibitory effects were monitored based on the changes with respect to number & nature of the Liesegang rings and size, weight & morphology of crystals. The mass of the crystals measured in gram and the statistical significance among different groups was tested by One-way Analysis of Variance (ANOVA) using JASP Version 0.14.1. The dried crystals were characterized by Fourier Transform Infrared Spectroscopy (FTIR) to know about the presence of any structural change in functional groups on treatment. FTIR spectrum was recorded by KBr pellet technique using Perkin Elmer FTIR spectrometer in the range 4000 - 400cm^{-1}.

Figure 1: Effect of *C. zizanioides* on brushite crystallization

A- Control; B- Control+H_2O; C- CZ1; D- CZ2; E- CZ3; F- CZ4; G- CZ5; H- CZ-F4; I- CZ-F5

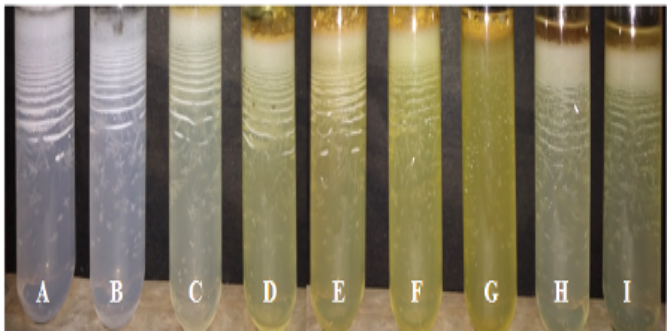


Table 1: Composition of Supernatant in Different Treatments

Sl. No.	Group	Treatment	Supernatant Additives		
			Volume of $CaCl_2$ (ml)	Volume of Extract (ml)	Volume of Deionized water (ml)
1	A	Control	5	0	0
2	B	Control + H_2O	5	0	5
3	C	CZ1	5	1	4
4	D	CZ2	5	2	3
5	E	CZ3	5	3	2
6	F	CZ4	5	4	1
7	G	CZ5	5	5	0
8	H	CZ-F4	5	5	0
9	I	CZ-F5	5	5	0

Results

The preliminary phytochemical analysis revealed the presence of tannins, alkaloids, cardiac glycosides and coumarins in it; Saponins, flavonoids, terpenoids, phenols, anthocyanin and leucoanthocyanin were also found comparatively in a higher amount (Table 2).

Observation of precipitation stages, Liesegang rings formation and crystal growth in different treatment for a period of 21 days helped to understand the activity of *C. zizanioides* extract on Brushite crystals. $CaCl_2$ diffusion resulted in formation of 8 to 10 Liesegang rings of thickness 0.5 mm. Liesegang rings were more granular and the inter ring width got reduced with increasing concentration of extract. In treatment CZ5 there was no ring formation (Table 3). The formed Liesegang rings disappeared with the formation of crystal aggregates.

With increase in concentration of extract the crystals showed reduced size and yield weight. Also, a finite variation in the crystal morphology was observed. The inhibitory effect was around 95.83% in CZ5. The weight (Mean ± SD, n = 3), length & width, and the morphological characters of harvested crystals and the percentage inhibition in each treatment are presented in Table 4.

FT-IR spectra and the vibration assignments of the brushite crystals were identified (Table 5). There were some shifts and new peaks found after the treatment compared to the control (Figure 3). In CZ5 treatment the crystals exposed absorption bands similar to the control with some deviations including a new

peak at 1333.01 cm^{-1} (Figure 3-A). In treatment CZ-F4 (Figure 3-B), there was disappearance of peak at 3322.01 cm^{-1} and appearance of two new peaks representing the O-H stretch shift. Another new band at 1828.11 cm^{-1} and disappearance of bands 1597.23 and 1412.63 cm^{-1} was observed. In treatment CZ-F5 (Figure 3-C), many peaks disappeared and new peaks appeared.

Table 2: Preliminary Phytochemical Screening

Sl. No.	Phytochemical Constituents	Results
1	Tannins	+
2	Phlobatanins	-
3	Saponins	++
4	Flavonoid	++
5	Steroid	+
6	Terpenoid	++
7	Alkaloids	+
8	Anthraquinone	-
9	Phenols	++
10	Carbohydrate	-
11	Protein	-
12	Cardiac Glycosides	+
13	Coumarins	+
14	Anthocyanin	++
15	Leucoanthocyanin	++

'++' – Highly Present; '+' – Present; '-' – Absent

Table 3: Characteristics of Liesegang rings

Sl. No.	Group	Treatment	No. of Liesegang rings	Inter ring Width at Upper end (mm)	Inter ring Width at Lower end (mm)
1	A	Control	11	1	4
2	B	Control + H_2O	10	1	4
3	C	CZ1	10	1	5
4	D	CZ2	10	1	5
5	E	CZ3	8	0.8	5
6	F	CZ4	8	0.8	3
7	G	CZ5	0	-	-
8	H	CZ-F4	7	2	5
9	I	CZ-F5	5	2	5

Table 4: Crystal Characteristics, Inhibitory effect and ANOVA statistical analysis

Sl. No	Group	Treatment	Weight of crystals (g) (Mean ± SD)	Size of the crystal		Shape of crystals	Percentage Inhibition
				Length (mm)	Width (mm)		
1	A	Control	2.21±0.098	10-18	2-4	Irregular, Platy and needle aggregates	0
2	B	Control + H_2O	1.93±0.072	7-18	2-4	Irregular, Platy aggregates	10.64
3	C	CZ1	1.32±0.067	6-18	1-2	Long, Rod, Platy	38.88
4	D	CZ2	0.64±0.060	6-17	0.5-2	Long, Blunt needle aggregates	70.37
5	E	CZ3	0.43±0.055	5-.8	0.5-2	Platy, Rod, Star	80.09
6	F	CZ4	0.23±0.059	5-16	1-2	Platy, Rod	89.35
7	G	CZ5	0.09±0.031	2-4	1-2	Granular, Star, Blunt	95.83
8	H	CZ-F4	0.29±0.035	3-6	1-2	Platy aggregates, Granular, Star	86.57
9	I	CZ-F5	0.31±0.045	3-15	0.5-1.5	Platy, Rod aggregates	85.64

The difference in mean mass of all the samples (n = 3), was statistically significant at $P < 0.05$. Differences between the groups A vs B-I, B vs C-I, C vs D-I, D vs E-I, E vs F-G and G vs H-I were statistically significant at $P_{tukey} < 0.05$, where E vs H-I, F vs G-I and H vs I were not significant.

Figure 2: Harvested Brushite Size and Structure; A- Control; B- Control+H_2O; C- CZ1; D- CZ2; E- CZ3; F- CZ4; G- CZ5; H- CZ-F4; I- CZ-F5

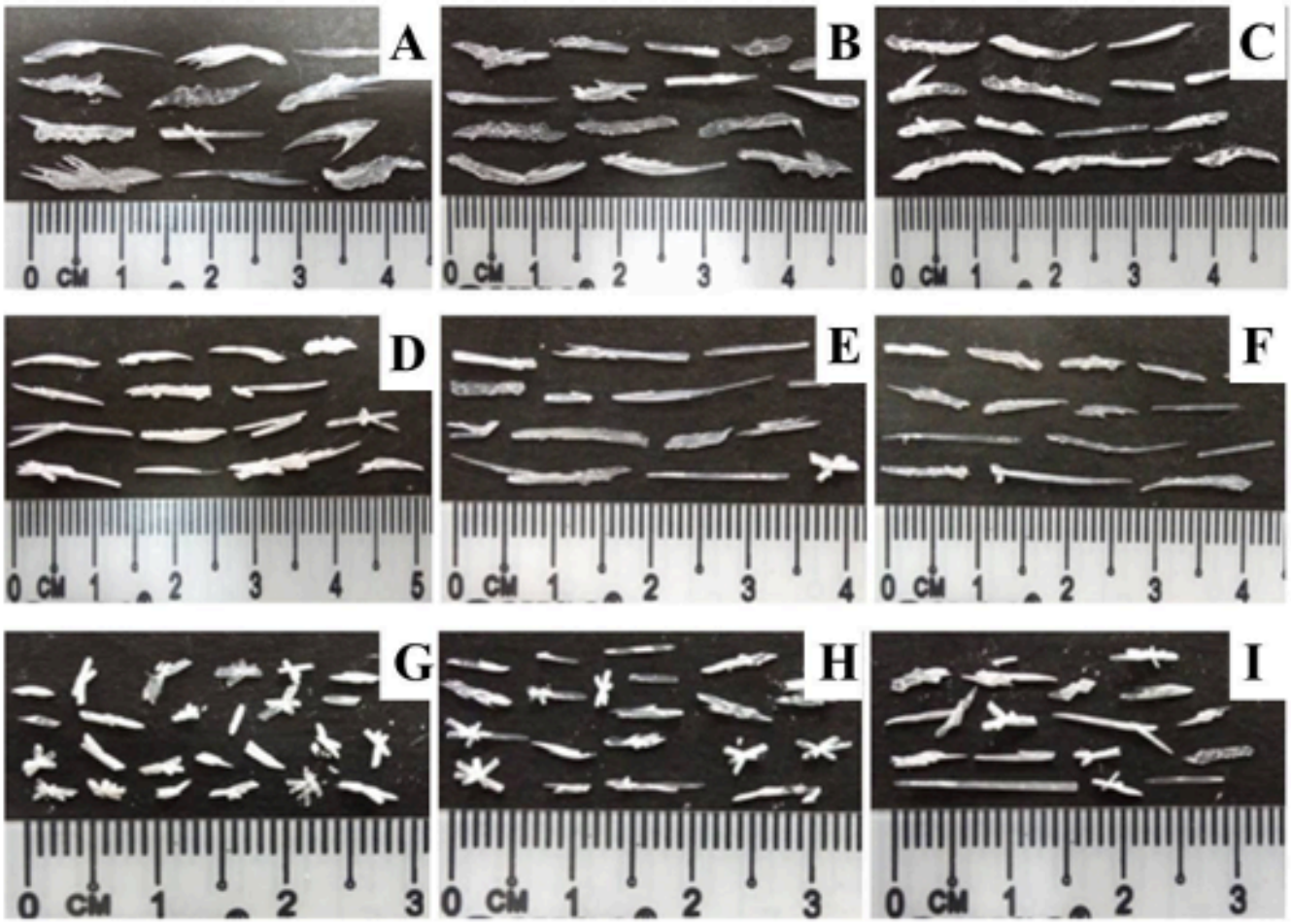
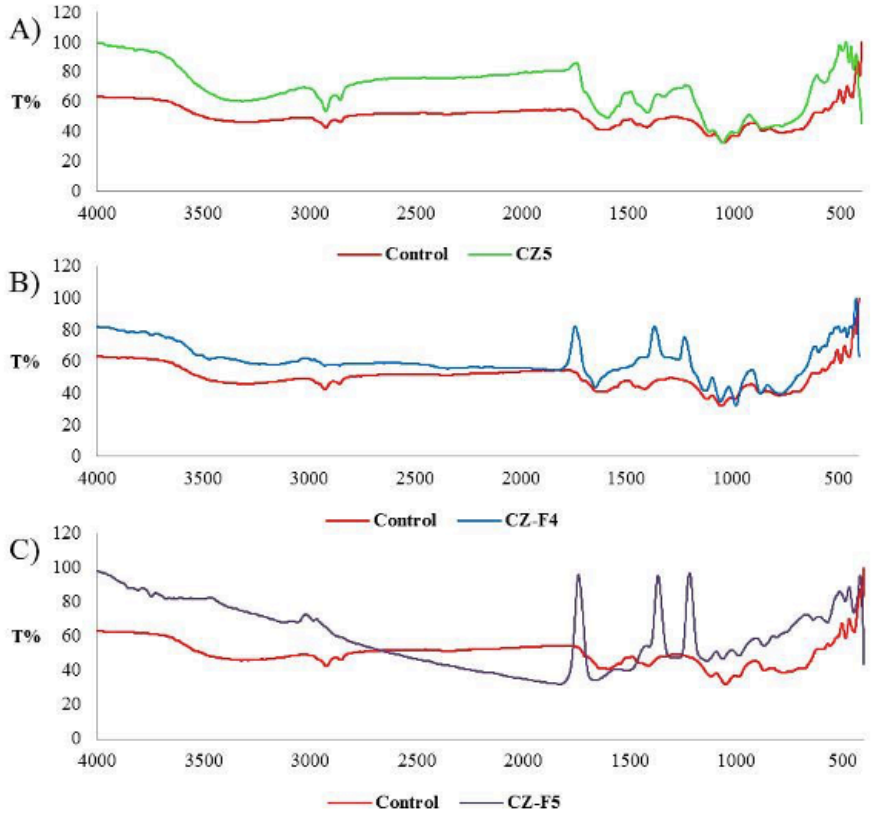


Table 5: FTIR vibrations assignment of Brushite crystals

Sl. No.	CZ1	CZ5	Vibrations
1	3322.01	3316.36	intermolecular O-H stretch
2	2922.99	2922.52	intramolecular O-H stretch
3	2854.31	2863.92	intramolecular O-H stretch
4	2341.41	2328.08	Combination
5	1597.23	1596.66	H-O-H bending vibrations
6	1412.63	1408.28	O-H bend
7		1333.01	O-H bend of Phenol / alcohol
8	1117.26	1117.87	P=O associated stretching vibrations of PO4 bond
9	1051.21	1055.59	P=O associated stretching vibrations of PO4 bond
10	987.38	990.8	P-O-P asymmetric stretching vibrations
11	866.47	869.47	P-O-P asymmetric stretching vibrations
12	775.77	776.08	P-O-P asymmetric stretching bond
13	558.49	574.76	(H-O-)P=O
14	486.22	491.99	(H-O-)P=O bond (strong absorption) of acid phosphates
15	445.17	460.57	(H-O-)P=O bond (strong absorption) of acid phosphates
16	409.64	437.53	(H-O-)P=O bond (strong absorption) of acid phosphates

Figure 3: FTIR Characterization of Crystals



Discussion

Single gel diffusion technique is a promising method to grow brushite crystals *in vitro*, to study the crystal aggregation process and inhibitory effects of drugs easily. On growing crystals in test tubes, increasing concentration of plant extract reduced the number of Liesegang rings forming and created variations in the inter ring width. This cause decrease in nucleation and the number of crystals formed. Similar effect was also found by Diana K.J. and George K.V., in the study using seed extract of *Ensete superbum* (11). High presence of flavonoids, anthocyanins and other phenols in the extract is a valuable reason behind the antilithiatic effect of *C. zizanioides*. It is possibly due to the antioxidant, diuretic and anti-inflammatory activities of the phytochemicals which effects by modulating the synthesis and expression of activators or inhibitors of stone formation (2, 15).

In the FT-IR spectrum brushite is characterized by the splitting of phosphate bands in the region below 1600 cm^{-1} (16). Comparing the IR spectrum of the brushite crystals from control and treatments reveals peak shifts which again is an indication of the activity of plant extract. The phytochemical investigation of vetiver oil reported about the presence of various hydrocarbons including alcohols and ketones predominantly. The common constituents of vetiver oil detected by Gas Chromatography – Mass Spectrometry are vetiverols like khusinol, spathulenol, khusimol and khusol, vetivones like α-vetivone and β-vetivone, khusimone and nootkatone derivatives (10, 18).

Conclusion

The results revealed the inhibitory effectiveness of vetiver on brushite crystallization by reducing the number of Liesegang rings, nucleation sites, structural complexity and size & weight of crystals. High presence of flavonoids, anthocyanins and phenols was detected in the extract. The IR spectrum of treated crystals indicated band shifts, disappearance of existing and appearance of new peaks. The study exposed the efficiency of *Chrysopogon zizanioides* root as an effective antilithiatic agent for control and treatment of kidney stones and support the traditional knowledge on use of these roots for Urolithiasis. The further studies are required to delineate the mechanism on how the active phytochemicals promisingly inhibit the crystal growth.

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