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GROWTH CHARACTERIZATION OF RENAL STONE STRUVITE CRYSTALS AND INHIBITORY EFFECTS OF *Alpinia zerumbet* Pers., UNDER INVITRO CONDITIONS

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ABSTRACT

Urolithiasis is the presence of calculi in the kidney and is also found in the urinary tract, the ureters and bladder. The urinary stone formation known as urolithiasis is a serious attenuate problem throughout the world. Struvite is a triple phosphate stone found more frequently in women. Urinary stones are characterized by high repetition rate therefore requiring a preventive treatment by using the medicinal plants. *Alpinia zerumbet* Pers. commonly known as Shell Ginger belongs to the family Zingiberaceae. Medicinal uses of *Alpinia zerumbet* contains anti-pyretic, anti-depressant, analgesic, anti-inflammatory, anti-cancer and anti-ulcer activities. The inhibitory effect of ethanol extract of*Alpinia zerumbet* rhizome on the growth of struvite crystals were investigated. Struvite crystals have been grown by the single diffusion gel growth technique. The weight of the formed struvite crystals were gradually reduced from 2.36 g to 0.30 g with increase in the concentration of ethanol extract of *Alpinia zerumbet* rhizome. Presence of bioactive compounds like Tannin, Phlobatannins, Alkaloids, Flavonoids, Saponins, Phenols, Steroids, Carbohydrates, Xanthoprotein, Leucoanthocyanin, Anthoquinines, Emodine, Terepenoids and Anthocyanins was confirmed by Preliminary phytochemical analysis of the rhizome extract. The major bioactive compounds of Alkaloid, Flavonoid and Terpenoid are present. The struvite crystals have been harvested and characterized by Fourier Transform Infrared Spectroscopy (FTIR) to confirm the functional groups. The FTIR spectrum of harvested struvite crystals from various treatments showed shifts in bands, disappearance of existing and appearance of new peaks which indicated the activity of bioactive

components in the extract. This study confirms that using ethanol extract of rhizomes of *Alpinia zerumbet* rhizome can promote the formation of ammonium magnesium phosphate hexahydrate crystals and reduce the nucleation rate of struvite crystal is a main component of triple phosphate urinary stone.

Keywords: Urolithiasis, Struvite crystals, *Alpinia zerumbet,* Fourier Transform Infrared Spectroscopy

INTRODUCTION

Urolithiasis is a worldwide problem affecting people for several decades and also referred to as Nephrolithiasis or kidney stones. It is the formation of stones in the urinary tract. It majorly causes degrees of pain, bleeding, and further may lead to secondary infection. Hence, alternative treatments by using medicinal plants are highly welcome nowadays. The life time risk of developing urolithiasis is about 10-15% in the western world, but can be as high as 20-25% in the Middle East **[6]**. Kidney stones form by supersaturation of the urine with calcium and other salts chiefly oxalate. The stone size can grow and block in the urinary system. Though extracorporeal shock wave lithotripsy is the most effective treatment of kidney stone, the side effects of this method are deadly and can again lead to formation of kidney stones. Herbal medicines play significant amount benefits for the treatment of urinary calculi because of its efficiency,safety and better compatibility in the human body. Nature has been source of medicinal agents since times immemorial.The leaves and rhizomes of *Alpinia zerumbet* is a rhizomatous evergreen tropical perennial commonly known as shell ginger that grows in upright clumps8 to 10 ft in tropical climates have efficient hypotensive and antioxidant activity. It has potential benefits for the treatment of diabetes, patients with atherosclerosis, and a wide range of skin diseases. Its root stalk is historically applied as a viscous, carminative, astringent, tonic and sedative. In the present study an approach was made to study the growth characteristics of the most common types of urinary stones struvite crystals and the efficiency of ethanolic extract of *A. zerumbet* rhizome on struvite crystals to find its antiurolithiatic property.

MATERIAL AND METHODS

Collection of plant material

***Alpinia zerumbet* Plant**

Rhizome of *Alpinia zerumbret*

Alpinia zerumbet Pers. plants were collected in the month of March from Kolli hills. The plant was identified and confirmed by Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirappalli, Tamil Nadu. The voucher specimen number (SS001 and SS002) dated 26.03.2018

Preparation of ethanol extracts

The rhizome of *Alpinia zerumbet* were washed in running water, cut into small pieces and then shade dried for a week at 35-40ºC, after that it was grinded to a uniform powder of 40 mesh size. The ethanol extract were prepared by soaking 100 g of the dried powder plant materials in 1 L of ethanol by using a soxhlet extractor for 10 hr continuously. The extract were filtered through whatmann filter paper No. 42 (125mm). The filtered extract was concentrated and dried by using a rotary evaporator under reduced pressure. The obtained extract of concentration 25mg/ml (rhizome) was used to prepare the series of (0.15%, 0.25%, 0.50%, 0.75%, 1.00%, F_3 and F_9) ethanol supernatant concentrations for in vitro studies (Table-1).

Phytochemical screening

Phytochemical screening for Tannin, Flavonoid, Terpenoid, Saponin, Phlobatanin, Steroid, Carbohydrate, Glycoside, Coumarin, Alkaloid, Protein, Emodin, Anthoquinone, Anthocyanine, Cardiac glycosides, Leuco-anthocyanine, Phenol, Xanthoprotein were performed using standard procedures [**7**].

Growth and characterization of Struvite crystals

Glass test tubes were used as a crystallization apparatus and the single diffusion reaction technique was employed. One of the reactants, 0.5 M ammonium dihydrogen-phosphate (ADP), was mixed with sodium metasilicate solution the density of 1.04g/cm3 at pH9.4, so that the pH of the mixture was maintained at 6 and left

undisturbed for 2-3 days. After gelation took place, the supernatant solution of 1 M Magnesium acetate was gently poured onto the set gel in various test tubes. After pouring on each supernatant solution, the test tubes were capped with airtight stopples. The experiments were conducted at room temperature (37˚C). The grown Struvite crystals were characterized using FTIR to verify the compound and structure of the grown crystal. FTIR was performed by Hitachi 570 FT-IR spectrophotometer technique to verify the proper formation of crystal and their purity.

Table 1: Supernatant solutions added to the set gels for struvite crystals.

Supernatant Solutions (SS) (Groups and Treatments)	Compositions
I (Control)	10 ml of 1 M magnesium acetate
II (Distilled water)	5 ml of 1 M magnesium acetate +5 ml of distilled water
III (0.15% ethanol extract)	5 ml of 1 M magnesium acetate +5 ml of 0.15% of ethanol extract of rhizome of *A. zerumbet* separately
IV (0.25% ethanol extract)	5 ml of 1 M magnesium acetate +5 ml of0.25% of ethanol extract of rhizome of *A.zerumbet* separately
V(0.50% ethanol extract)	5 ml of 1 M magnesium acetate +5 ml of 0.50% of ethanol extract of rhizome of *A. zerumbet* separately
VI(0.75% ethanol extract)	5 ml of 1 M magnesium acetate +5 ml of 0.75% of ethanol extract of rhizome of *A.zerumbet* separately
VII(1.00% ethanol extract)	5 ml of 1 M magnesium acetate 5 ml of 1.00% of ethanol extract of rhizome of *A. zerumbet* separately
VIII(Fraction 3ethanol extract)	5 ml of 1 M magnesium acetate +5 ml fraction 3 with ethanol extract of rhizome of *A. zerumbet* separately
IX (Fraction 9 ethanol extract)	5 ml of 1 M magnesium acetate +5 ml fraction 9 with ethanol extract of rhizome of *A. zerumbet* separately

Calculation of the percentage of inhibition (1%) was based on the formula:

1%= [(TSI-TAI)] /TSI) x 100

TSI represents the number of crystals without inhibitors and TAI the number of crystals after addition of inhibitors **[4]**.

RESULTS

The inhibitory effect of ethanol extract of *Alpinia zerumbet* rhizome on the growth of struvite crystals were investigated. Struvite crystals were grown by the single diffusion gel growth technique. The weight of the formed struvite crystals were gradually reduced from 2.36 g to 0.30 g with increase in the concentration of ethanol extract of *Alpinia zerumbet* rhizome.

Qualitative phytochemical analysis

The present study carried out on the rhizome of *Alpinia zerumbet* revealed the presence of medicinal active constituents. The phytochemical active compounds were qualitatively analyzed for rhizome of *Alpinia*

zerumbet. Phytochemical compounds such as terpenoids, flavonoids, saponins, tannins, coumarins, leucoanthocyanin, xanthoprotein, steroids, alkaloids, emodine, phlobatanin, anthocyanine, anthoquinone, carbohydrate are present in ethanol extracts of rhizome of *Alpinia zerumbet*, proteins, glycosides, and cardiac glycosides are absent **(Table 1)**.

Morphology of the harvested crystals after addition of rhizome of *Alpinia zerumbet* as shown in Fig.2.a.The largest single struvite crystals having dimensions of 1.6cm was observed in Fig.2.b. The sizes of the struvite crystals is no additive in distilled water 2cm.to 1.3 cm at 1% concentration of extracts, 1.0 cm in 2%, 1.1 cm in 3%, 0.7cm in 4%, 0.6cm in 5% and 1.0cm in fraction 3and 0.8 cm fraction 9 were observed. With an increase in the concentration of the rhizome of *Alpinia zerumbet* from 1% to 5% (v/v), the weight of the formed crystal were gradually reduced from 1.21gm to 0.51gm.

The percentage of inhibition of Struvite crystals by the rhizome of *Alpinia zerumbet* are shown in **(Table 2)**. In control there is 0% inhibition, In control+H_2O is16.5%, in 0.15% is 48.5%, in 0.25% is 55.7%, in 0.50% is 68.9%, in 0.75% is 76.1%, in 1% is 95.3%, in fraction 3 is 80.8% and fraction 9 is 87.2%. In the present work, Struvite crystals growth was reduced due to the inhibitory effect of the rhizome of *Alpinia zerumbet* under *in vitro* conditions.

Characterization of Struvite crystals

The FTIR spectra of Struvite crystals obtained in the presence and absence of the *Alpinia zerumbet* are show in **Figure 4(a)** without an additive, a storng band at 3256 cm-^{1} is due to O-H stretching, intermolecular. The peak at 2927 cm^{-1} is due to weak bond O-H stretching intramolecular and the peak at 2368 cm-^{1} due to the antisymmetric and symmetric vibrations of NH_4 units. The band at 1613 cm^{-1} is due to HOH deformation of water and the peak 1438 cm^{-1} is due to the HNH deformation modes of NH_4 units. The peak at 1004 cm^{-1} is due to V_3antisymmetric stretching vibration and the peak at 881 cm^{-1} is due to wagging modes of coordinated water molecules. The peak at 759 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 569 cm^{-1} is due to the V_4bending mode of the PO_4 units. The peak at 459 cm^{-1} is due to the component of V_2 mode of the PO_4 units. **Figure 4(g)** a peak at 3215 cm^{-1} is due to strong broad O-H stretching, intermolecular. The peak at 2922 $cm^{-1}$is due to weak bond O-H stretching, Intramolecular. The band is shift to 2325 $cm^{-1}$is due to the antisymmetric and symmetric vibrations of NH_4 units. The band at1739 cm^{-1} is due to the

HOH deformation of water and the peak 1596^{cm-1} it have been attributed to N-H bending vibration. The peak is 1431cm $^{-1}$ and 1370 cm^{-1} is due to the HNH deformation modes of NH_4 units. The peak at 978 cm^{-1} is due to V_3antisymmetric stretching vibration. The band is shift to 748 cm^{-1}is due to the water liberational and NH_4rocking modes. The peak at 600 cm^{-1}is due to the V_4 bending modes of the PO_4 units. The peak at 554 cm^{-1} is may be due to metal oxygen bond. The band at 469 cm^{-1} is due to could be attributed to the component of V_2 mode (PO_4) in the presence of 1% rhizome extract of *Alpinia zerumbet.*

Table 2: Qualitative phytochemical analysis of ethanol extract of rhizome of *A. zerumbet*

S. No	Phytochemical constituents	Ethanol extracts of rhizome of *A. zerumbet*
1	Tannin	++
2	Flavonoid	+
3	Terpenoid	+
4	Saponin	+
5	Phlobatanin	++
6	Steroid	+
7	Carbohydrate	+
8	Glycoside	–
9	Coumarin	+
10	Alkaloid	++
11	Protein	–
12	Emodin	+
13	Anthoquinone	+
14	Anthocyanine	+
15	Cardiac glycosides	+
16	Leucoanthocyanin	+
17	Phenol	+
18	Xanthoprotein	++

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Table 3: Analysis for the percentage of inhibition of struvite crystals

Crystal	Group	Treatment	Percentage of inhibition	Mean (gm)±SD
Struvite	A	Control	0%	2.36 ± 0.0454
	B	Control+ dis H_2O	16.5%	1.96 ± 0.0329
	C	Control+ 1% extract	48.5%	1.21 ± 0.0205
	D	Control+ 2% extract	55.7%	1.03 ± 0.0449
	E	Control+ 3% extract	68.9%	0.74 ± 0.0339
	F	Control+ 4% extract	76.1%	0.56± 0.0163
	G	Control+ 5% extract	95.3%	0.21 ± 0.0124
	H	Control+ fraction3	80.8%	0.45± 0.0249
	I	Control+ fraction 9	87.2%	0.30 ± 0.0169

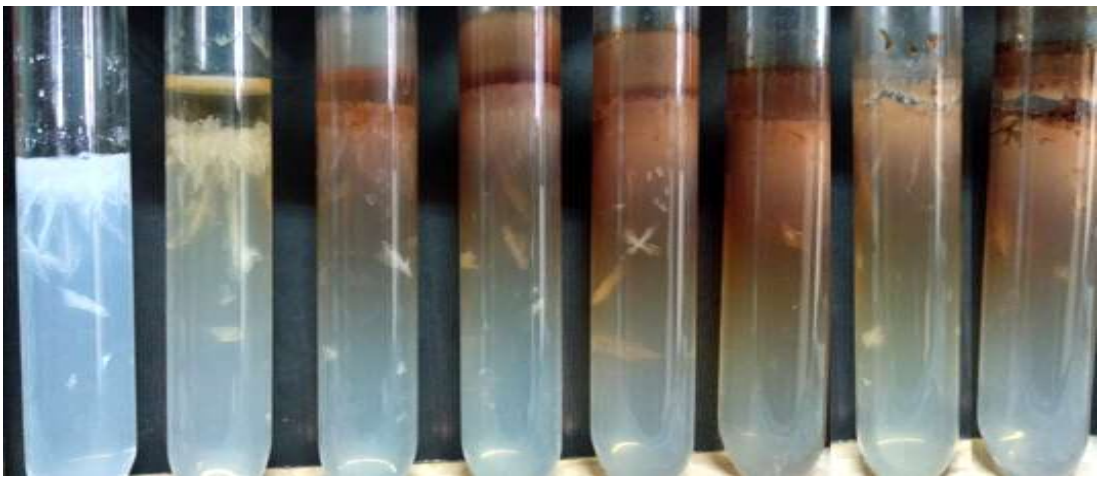

Figure 1: The effect of rhizome of *Alpinia zerumbet* on struvite crystals in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% ethanol extract (d) with the 0.25% ethanol extract (e) with the 0.50% ethanol extract (f) with the 0.75% ethanol extract (g) with the 1.00% ethanol extract (h) with the fraction 3 and(i) fraction 9 after 7days

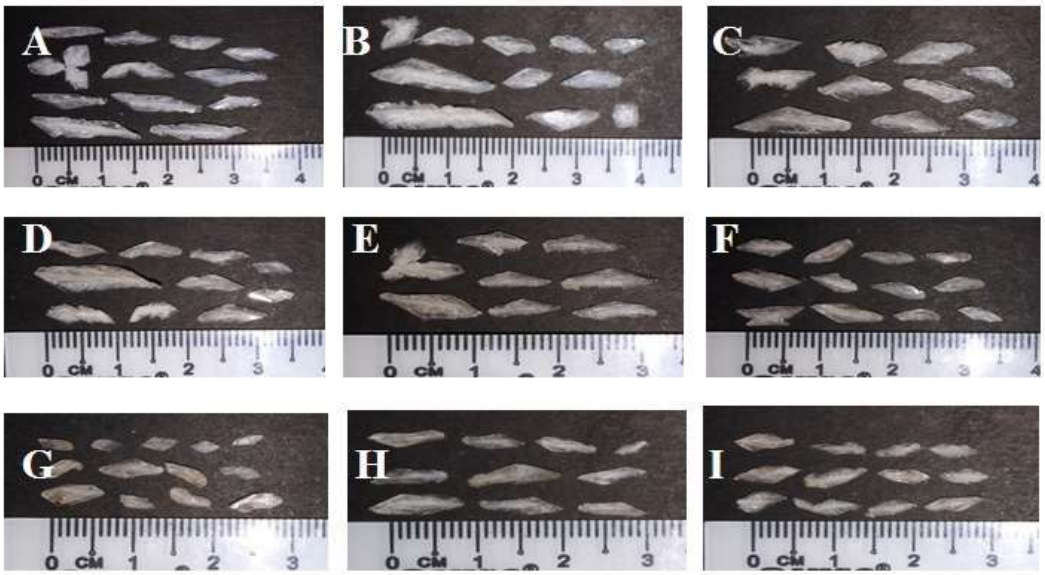

Figure 2: The measurement of struvite crystals obtained from *Alpinia zerumbet* in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% ethanol extract (d) with the 0.25% ethanol extract (e) with the 0.50% ethanol extract (f) with the 0.75% ethanol extract (g) with the 1.00% ethanol extract (h) with the fraction 3 and (i)fraction 9 after 28 days

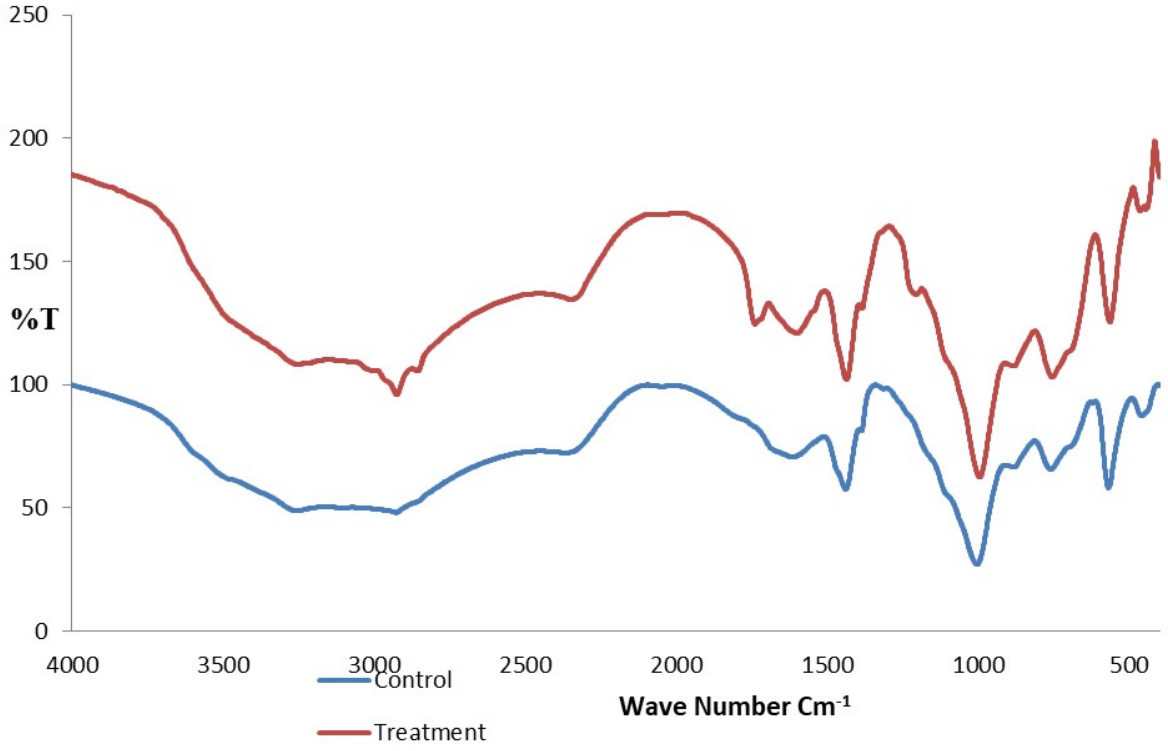

Figure 3: FTIR Characterization of Crystals comparing control and 1% of ethanolic extract of *A. zerumbet*

DISCUSSION

Kidney stone is a common urinary disorder in human. Quantitative analysis of major bioactive compounds of Saponins protect against hypercholesterolemia and antibiotic properties. Steroids and terpenoids show the pain reliving properties. The ethanolic and aqueous extracts showed the presence of flavonoids, carbohydrates, phenolic compounds and sterols were the major phytoconstituents. In the present study ethanol extracts of rhizome of *A.zerumbet*are screened for phytochemical analysis. As there is increase in the inhibitory effect of struvite crystals when treated with the rhizome of *Alpinia zerumbet* at 5% ethanolic extract a peak at 3215 cm-1 is due to strong broad O-H stretching, intermolecular. The peak at 2922 cm-1is due to weak bond O-H stretching, intramolecular. The shifting further supports that the can promote the formation of a major component of triple phosphate urinary stone and reduce the nucleation rate of struvite crystals as compared with collected fractions.

This result indicates that distilled water did not show any inhibitory activity with regard to crystal growth, whereas the methanol extract of rhizome of *A.zerumbet* possessed inhibitory activity due to the presence of

bioorganic molecules diterpenes such as calcaratarins, sesquiterpenes such as shyobunone and coumarins such as herniarin.This study confirmed that the rhizome of *A. zerumbet* ethanolic extracts can promote the formation of hydroxyapatite crystals and treat urinary stone by inhibiting the formation of struvite crystals, a major component of calcium urinary stone. Recently, growth inhibition studies of Struvite crystals in the presence of some of the herbal extracts **[1, 5]** were attempted in literature. In the present work, Struvite crystals growth was reduced due to the inhibitory effect of the leaves of *A. zerumbet* under in vitro conditions.

Alpinia zerumbet rhizome can exhibit antioxidant activity because of the presence of phytochemical such as alkaloids, terpenoids and flavonoids in the plant extract. *A. zerumbet* also proved to exhibit anti-microbial activity toward several microorganism strains. In this study the presence of these active phytocompounds also showed inhibitory effect or anti urolithiatic activity towards struvite crystals that is kidney stone formation. To gain an understanding of infrared spectral interpretation, it is instructive to start at the root of most organic compounds, namely the fundamental backbone or the parent hydrocarbon structure. The spectrum can provide information on the existence of most of these structures, either directly or by inference.

Among the functional groups observed in the extracts, OH group was found to be present uniformly only in the ethanol extracts of all plants. As OH group has got the ability of forming hydrogen bonding capacity, presence of OH group particularly in ethanol extract of rhizome of *A. zerumbet* probably indicates the higher potential of ethanol extract towards inhibitory activity against struvite crystals. Higher antimicrobial activity of ethanol extracts of leaf of all those four plants have been already demonstrated together with low IC50 value **[2, 3]**.

Crystallization characterization of struvite crystals treated with the rhizome of *A.zerumbet*was observed using FTIR techniques. In Control the band a at 3256 cm^{-1} is due to O-H stretching, intermolecular. As there is increase in the inhibitory effect of struvite crystals when treated with the rhizomeof *Alpinia zerumbet,* the band at The peak at 2927 cm^{-1} is due to weak bond O-H stretching intramolecular and the peak at 2368 cm^{-1} due to the antisymmetric and symmetric vibrations of NH_4 units . The shifting further supports that the can promote the formation of triple phosphatecrystals and

reduce the nucleation rate of struvite crystals as compared with collected fraction.

CONCLUSION

This research is focused to find new alternative medicine rhizome of *Alpinia zerumbet* for the treatment of urinary stone. The purpose of this work is to investigate the beneficial effects rhizome of *A. zerumbet* at a different dose and single compounds for the prevention of kidney stone formation. However further research on detailed isolation of another active phytoconstituents possessing the therapeutic activity and clinical study for the evaluation of safety and efficacy of the drug needs to be assessed.

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