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**ANTIUROLITHIATIC PHARMACOLOGICAL STUDIES ON *PUNICA  
GRANATUM* LINN**

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**ABSTRACT**

Urolithiasis, often known as kidney stone disease, is a frequent clinical ailment. According to statistics, 12% of the world's population suffers from urinary stone illness. Disease frequency is increasing as a result of global warming, lifestyle, and eating habits. Traditional Botanical Knowledge (TBK) has assisted man in isolating medications for a wide range of ailments and will continue to be a valuable source for future drug development research. *Punica granatum* Linn. is one of the herbal remedies commonly used to treat urolithiasis. The latest study suggests that kidney stones begin as an aggregation of calcium phosphate crystals. In this context, rind extract of *Punica granatum* Linn. were subjected to screening of their antiurrolithiatic property by studying its potential for growth inhibition of Calcium Hydrogen Phosphate Dihydrate crystals (CHPD) crystals employing crystallographic techniques. For this, single diffusion gel growth technique was used. Reduction in growth of treated crystals was noticed with increasing concentrations of plant extract. The morphology of CHPD (brushite) crystals was studied by microscopy. Characterization of gel grown CHPD (control) and rind extract treated crystals by FT-IR, XRD and TGA/DTA analysis revealed the structural and chemical changes of the treated crystals.

**Keywords: Urolithiasis, *Punica granatum*, Calcium hydrogen phosphate dihydrate, FT-IR, TGA/DTA and XRD**

**INTRODUCTION**

Urinary stone is a serious problem of the world population [1]. The disease all over the world as it affects nearly 12% frequency is on the rise due to global

warming, life style and dietary habits [2, 3]. The present-day medical management of urinary stone includes lithotripsy and surgical procedures [4]. Regardless of these advances, recurrence rate seems to be high and without proper treatment and preventive measures, approximately 75% of these patients will have at least one recurrence [5, 6]. Even after extensive research in Urology, no satisfactory drugs have so far been developed in modern medicine to disintegrate/ dissolve the urinary stone and patients mostly rely on alternative systems of medicine for better relief [7]. An investigation among the traditional healers and herbal collectors of Kerala confirmed the potential of *Punica granatum* for the treatment of urolithiasis. However, there are no records of systematic pharmacological studies that support this claim. *In vitro* Gel growth of a few urinary stone constituents and the inhibitory role played by some extracts or juices of natural products in crystal growth were studied by earlier researchers [8-10]. Studies on *in vitro* crystallization of calcium phosphate deserve utmost significance since calcium phosphate being one of the major constituent and initiator of kidney stone. In this context, the present study was undertaken to assess the efficacy of the aqueous extract of the rind of *Punica granatum* on growth inhibition of calcium hydrogen phosphate dihydrate

[CaHPO<sub>4</sub>.2H<sub>2</sub>O, CHPD] using single diffusion gel growth technique.

## MATERIALS AND METHODS

**Preparation of extract:** *Punica granatum* Linn. Fruits were collected, authenticated, separated the rind and aqueous extract was prepared (10g/100ml). 1 to 4% solutions were used in treatments 1 to 4 (T1, T2, T3 and T4) respectively.

### Single diffusion gel growth technique:

For growing CHPD crystals, hydrogel method developed by Henisch *et al.*, (1998) [11] was used with appropriate modifications. The hydrogel was prepared from sodium metasilicate (Na<sub>2</sub>O<sub>3</sub>S<sub>1</sub>. 9H<sub>2</sub>O, M.wt.212) and specific gravity of the gel was adjusted to 1.06 using deionized water. Freshly prepared CaCl<sub>2</sub> (0.5 molar) and orthophosphoric acid (1 molar) were used for the study. The pH of the gel was adjusted to 6.5 by adding one molar orthophosphoric acid. The gel thus prepared was used as a medium for growing crystals employing sterile Petri- plate and Test tubes (20ml).

To study the initial stages of crystal growth the gel was placed on a sterile glass slide which is kept in the Petri dish. The gel is covered with cover slip and allowed to set slowly. Thereafter, suitable concentration of the calcium chloride solution (5ml) was poured up to the level of the cover slip. The poured solution diffused through the gel reacted with the

impregnated weak acid in the gel medium, which resulted in the formation and growth of micro-crystals within minutes. Different concentrations of the plant extract were then added to study the growth inhibition and dissolution of CHPD. The slides were observed after 5 minutes using a compound microscope (Olympus); the photographs were taken and were subjected to morphometric analysis using image analyzer software (Motic Images).

CHPD crystal growth inhibition studies were also conducted in sterile test tubes. For that 10ml of the gel was poured in and was allowed to set slowly at room temperature. Calcium chloride solution (0.5molar) was added into each tube. Different concentrations of the additives were added to the test tubes and the inhibitory effects were analyzed based on the changes noted with respect to number, size and morphology of crystals. After 3 weeks, the gel grown crystals were harvested by washing in double distilled water and filtered through Wattman no.1

filter paper; air dried and was characterized using FTIR, TG/DTA and XRD techniques.

## RESULT AND DISCUSSION

The result obtained from the present study indicates the synergetic action of bioactive compounds of *Punica granatum* on *in vitro* growth inhibition of calcium phosphate urinary crystals (Fig.CHPD and T4). The result given in graph 1 reveals that the size of the crystal got reduced and the number of crystals increased as concentration of the additive increased. Moreover, the frequency of the sharp-edged crystals is much reduced in treatments (**Table 1**). The crystals in the treatments were with smooth edges and were less transparent. According to Bharat *et al* (2007) [12] change in morphology of crystal is an important phenomenon because, if the painful star type or spiky, needle, irregular stones were converted into smooth spherical or oval grain like ones, then, their passage through the urethra is less painful.



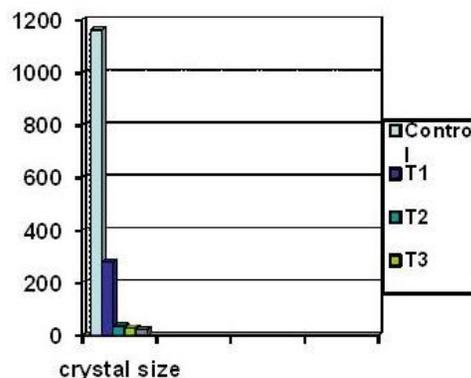
Control (CHPD)

Treatment (T4)

**Table 1: Results of morphometric studies**

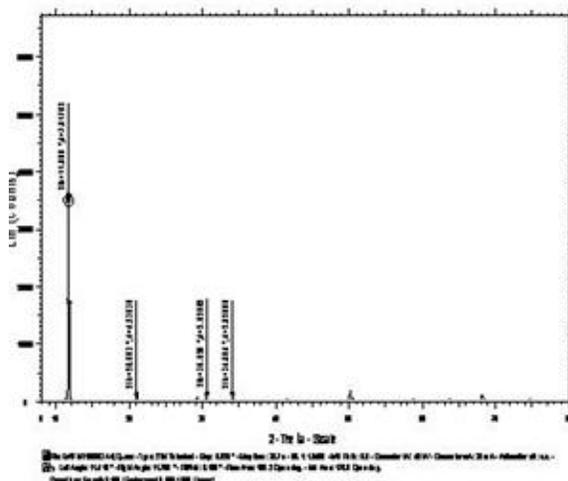
Treatments	Crystal characters		Average number of crystals
	Shape	Area range ( $\mu\text{m}^2$ )	
CHPD (C)	Irregular Star Sword Leaflike	18-1162	21
T1	Spindle Round	2-280	20
T2	Spindle oval	2-36	28
T3	Spindle oval	1-30	32
T4	Round Spindle	1-24	54

**Graph:1 Variation of crystal size among different treatments.**

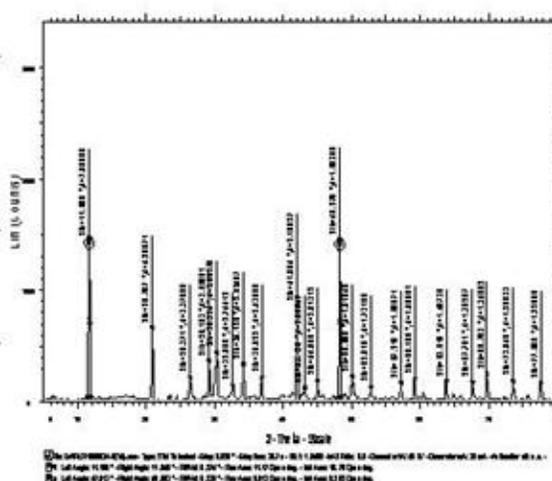


The graph II&III shows the X-ray diffractograms of the grown CHPD and T4 crystals respectively. The peak in the figure represents the positions where the x-ray beam has been diffracted by the crystals. The 'd spacing' value given in the table II is the distance between the adjacent planes of

atoms, which represents the unique “finger prints” of the crystals. As evident from the table II&III and graph II&III the XRD pattern of T4 shows more peaks compared to that of CHPD crystals which is an indication of structural change in the treatments.



**Graph II: XRD of Control (CHPD)**



**Graph III: XRD of Treatment (T4)**

**Table 5 XRD analysis of T4**

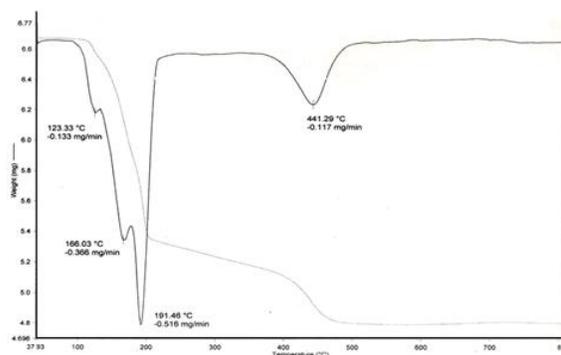
<u>2θ value</u>	<u>d-spacing</u>	<u>2θ value</u>	<u>d-spacing</u>
11.550	7.65508	48.270	1.88390
20.787	4.26971	50.095	1.81945
26.371	3.37699	52.818	1.73189
29.103	3.06591	57.218	1.60871
30.234	2.95370	59.195	1.55961
32.605	2.74412	63.815	1.45739
34.129	2.62497	67.701	1.38287
36.822	2.43896	69.783	1.34662
41.984	2.15027	73.640	1.28533
43.136	2.09547	77.658	1.22855
44.995	2.01312		

**Table4 :XRD analysis of CHPD crystal**

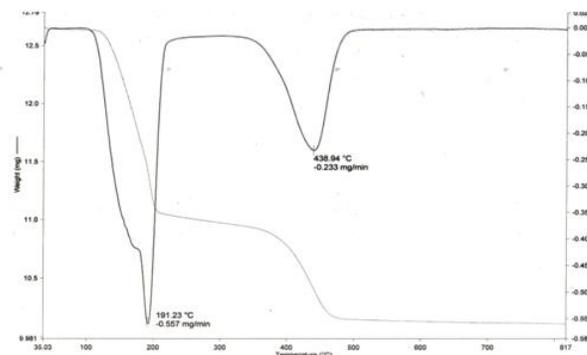
<u>2θ value</u>	<u>d-spacing</u>
11.608	7.61702
20.953	4.23638
30.529	2.92582
34.064	2.62986

Thermogravimetric analysis was performed on powdered samples. The thermogram is shown in graph IV&V. In CHPD 25% of the hydration of water is lost between 85 and 140 °C, 50% between 115 and 170 °C and total dehydration is achieved between 130 and 240 °C. Therefore, in CHPD the peak at 123.33 °C,

166.03 °C and 191.46 °C were related to the dehydration process. As clear from the graph IV&V, the T4 shows 21% total degradation whereas it is only 15% degradation in C; which indicates complexation of bioactive compounds in T4.



Graph IV: Thermogram of CHPD



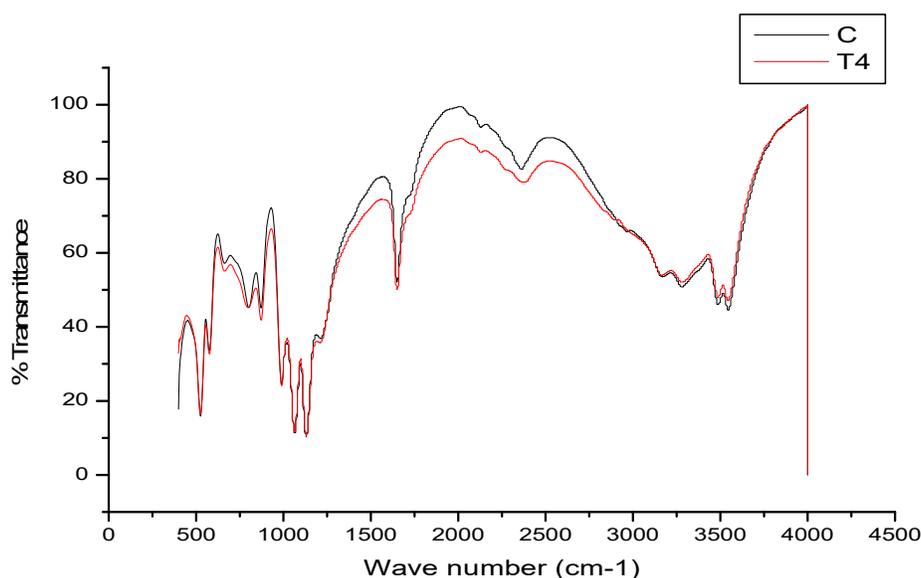
Graph V: Thermogram of T4

Several workers have reported IR spectroscopy analysis of calcium hydrogen

phosphate dihydrate (CHPD) crystals [13-15]. According to Rajendran and Dale,

2010 the FT-IR of the CHPD crystals grown by single diffusion gel method (sodium metasilicate solution –SMS) resembles with that of commercially available CHPD [16]. FT-IR spectra of KBR pellets of crystals grown in the present study (CHPD and T4) are shown in **Graph VI**. In C (CHPD) the presence of water of crystallization was referenced from absorptions at 3543.16, 3487.57 and 3282.79  $\text{cm}^{-1}$ , which are due to

intermolecular and weakly H bonded OH. The absorption at 1650.29  $\text{cm}^{-1}$  is due to H-O-H symmetric bending vibrations and P=O associated stretching vibrations were observed at wave numbers 1211.2, 1129.96 and 1064.34  $\text{cm}^{-1}$ . Likewise, the P-O-P asymmetric stretching vibrations were observed at 873.10 and 790  $\text{cm}^{-1}$ . The absorption at 665  $\text{cm}^{-1}$  is due to (H-O-) P=O and the strong absorption at 578 and 525  $\text{cm}^{-1}$  are due to acid phosphates.



Graph VI: FTIR pattern of CHPD and T4

Wave numbers (cm-1)		Bonds / Vibrations
CHPD	T4	
3543.16	3546.37	Weakly H bonded OH vibrations
3487.57	3486.62	Weakly H bonded OH vibrations
3282.79	3279.30	Weakly H bonded OH vibrations
3169.12	-----	Weakly H bonded OH vibrations
2363.34	-----	Combination
1650.29	1650.30	H-O-H Symmetric bending vibrations
1211.20	1214.47	PO4 P=O associated stretching vibrations
1129.96	1134.00	PO4 bond, P=O stretching vibrations
1064.34	1061.66	PO4 bond, P=O stretching vibrations
990.20	986.20	PO4 bond, P=O stretching vibrations
873.10	874.66	P-O-P asymmetric stretching bond
790.09	795.13	P-O-P asymmetric stretching bond
664.00	661.85	(H-O-) P=O
578.82	576.55	(H-O-) P=O bond (strong absorption) acid phosphates
525.33	525.05	(H-O-) P=O bond (strong absorption) acid phosphates

A preliminary phytochemical analysis of the rind of *Punica granatum* L. revealed presence of alkaloids, tannins, saponins, and triterpenes [17]. Pomogranate peel is a rich source of antioxidants, especially polyphenols, such as ellagic acid queerecetin and punicalagin [18-20]. According to the present investigation *Punica granatum* rind extract is effective in controlling the formation, growth and aggregation of calcium phosphate crystals and also modifies the shape and thermal behaviour of CHPD crystals as evident from the TG/DTA and XRD analysis results. Therefore, it can be a possible therapeutic agent for prevention of recurrent stone diseases.

## CONCLUSION

The rind extract of *Punica granatum* was evaluated for its antilithic activity employing single diffusion gel growth technique. The gel was prepared from hydrated Sodium metasilicate solution. The gel framework acts like a three-dimensional crucible in which the crystal nuclei are held in the position of their formation and nutrients are supplied for growth. Hence this technique can be utilized as a simplified screening static model to study the growth, inhibition and dissolution of urinary stones under *in vitro* conditions. The current study's findings reveal a synergistic impact of *Punica granatum* bioactive components on the growth

inhibition of calcium phosphate urinary crystals under *in vitro* conditions. The frequency of sharp-edged and large crystals was much reduced in *Punica granatum* rind extract treatments. This crystal shape change is an essential phenomenon because if the painful star type or spiky, needle, irregular stones were changed into smooth spherical or oval grain like ones, their transit through the urethra would be easier. Furthermore, the results of XRD, FTIR and TGA/DTA analysis of the gel grown crystals revealed the change in thermal behaviour and altered crystal structure. Therefore, *Punica granatum* rind extract is found to be effective in controlling the formation, growth and aggregation of CHPD under *in vitro* conditions.

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