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Inhibition of the growth of urinary calcium hydrogen phosphate dihydrate crystals with aqueous extracts of *Tribulus terrestris* and *Bergenia ligulata*

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Abstract Urinary type calcium hydrogen phosphate dihydrate (CHPD) or Brushite crystals were grown by the single diffusion gel technique in silica hydro-gels. The gel framework acts as a three dimensional crucible in which the crystal nuclei are delicately held in the position of their formation and nutrients are supplied for their growth. This technique can be utilized as a simplified screening model to study the growth and dissolution of urinary stones in vitro. The action of the putatively litholytic medicinal plants *Tribulus terrestris* and *Bergenia ligulata* on the growth of CHPD crystals was studied. The effects of artificial reference urine (ARU) and human urine (HU), along with the plant extracts, are also reported. Attempts were made to understand the role of these inhibitors on urinary crystal formation. HU, ARU, extracts of *B. ligulata* and *T. terrestris* exhibit appreciable amounts of inhibition, but *B. ligulata* and *T. terrestris* with ARU and HU do not show inhibition at all.

Keywords Urinary crystals · Gel growth · Urine · Calcium hydrogen phosphate dihydrate · Extracts of *Tribulus terrestris* · Extracts of *Bergenia ligulata*

Introduction

Urinary calculi are mainly composed of crystalline components. Multiple steps are involved in the pathogenesis of the crystals: nucleation, growth and aggrega-

tion. Nucleation implies the nidation of the crystals on the loci of proteinaceous material, cell debris, crystals, foreign bodies or other particulate matter. Nucleation is of two types, homogenous and heterogeneous. Heterogeneous nucleation occurs on diverse surfaces such as epithelial cells, urinary casts, other crystals or red blood cells in urine; this requires less energy and occurs in relatively less saturated urine. The process, by which nuclei form in a pure solution is known as homogeneous nucleation. Stone formation in vivo is dependent on, besides supersaturated urine, certain lithogenetic conditions, such as urinary pH, ionic strength, the solute concentration of certain glycoproteins, complexation and pathogenic factors, which are quite complex and well explained by Menon et al. [1]. Once nucleation has taken place, the next phase is growth of the crystal through the aggregation of more and more material.

There are several theories for the growth of the urinary calculi [1, 2, 3, 4]. The nucleation theory suggests that urinary stones originate from the crystals or foreign bodies present in supersaturated urine. There is a lithogenic tendency due to oxaluria, etc. The crystal-inhibitor theory, on the other hand, suggests that calculi form due to the absence or low concentrations of the host's natural stone inhibitors.

The growth of urinary calculi can be simulated in the laboratory by growing crystals in a silica hydro gel medium. The gel framework, though chemically inert, provides a three dimensional matrix in which the crystal nuclei are delicately held. It also provides a substratum for the gradual supply of nutrients for growth. This growth of urinary crystals in silica hydro gel can be considered as a simplified in vitro model for the highly complex growth of urinary calculi in vivo.

The growth of crystals in the gel is the simplest technique under ambient conditions which is suitable for the crystal growth of compounds sparingly soluble and which decompose at low temperatures. The gel density, pH of the reactants and the concentration of reactants are important factors influencing the growth of good quality single crystals at room temperature. The gel

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growth technique was described in detail by Henisch [5], Henisch et al. [6], as well as by Patel and Rao [7]. Diverse types of crystals having various applications have been grown by this technique [5, 6, 7, 8, 9, 10, 11, 12].

In the present investigation, calcium hydrogen phosphate dihydrate (CHPD) or brushite crystals were grown in silica hydro gel medium. The effects of the extracts of the putatively litholytic herbal medicinal plants *Tribulus terrestris* Linn and *Bergenia ligulata* were studied on the growth of these crystals. *T. terrestris* and *B. ligulata* are popularly known in India as Gokshura and Pashanbheda, respectively [13, 14]. *T. terrestris* grows wild in sandy soil and its fruits are described as having cooling, demulcent, diuretic, tonic and aphrodisiac properties. It is commonly used in India for urinary disorders and kidney stones. It is given as a formulation with the gum of *Commiphora wightii* commonly known as Goksahuradi guggulu. The roots of *B. ligulata* have been used for the therapy of urinary stones, chronic ulcers, viral hepatitis, and benign prostatic hypertrophy. In addition, *B. ligulata* has anti-inflammatory and cytoprotective properties [14]. The extracts of these plants were studied in the presence of artificial reference urine (ARU) and human urine (HU). The growth behaviors of CHPD crystals are reported, under these conditions.

Materials and methods

Growth of CHPD crystals

Glass test tubes were used as a crystallization apparatus and the single diffusion reaction technique was employed. One of the reactants, 2.5 M orthophosphoric acid, was mixed with sodium metasilicate solution having a specific gravity of 1.06, so that the pH of the mixture was maintained within the range of 4.0–6.0. After gelation took place, the supernatant solution of 1 M calcium chloride was gently poured onto the set gel in various test tubes. The experiments were repeated three times and each time three test tubes were used for the same supernatant solution. After pouring on each supernatant solution, the test tubes were capped with airtight stopples. The experiments were conducted at room temperature ($\sim 35^\circ\text{C}$). The first Liesegang ring was observed within 12 min of pouring the solution. In total, 18 Liesegang rings were observed over time. The elongated platelet shape CHPD crystals grew within the rings. In addition, platelets originated from a single point, that is, star shaped crystals were observed. Figure 1 shows a schematic diagram and Fig. 2 is a photograph showing the growth of crystals. The largest needle was 18 mm in length. The crystals were characterized by Fourier transform infrared spectroscopy, thermogravimetric analysis, scanning electron microscopy, and powder x-ray diffraction methods, and confirmed to be CHPD, which corresponds with earlier results [15].

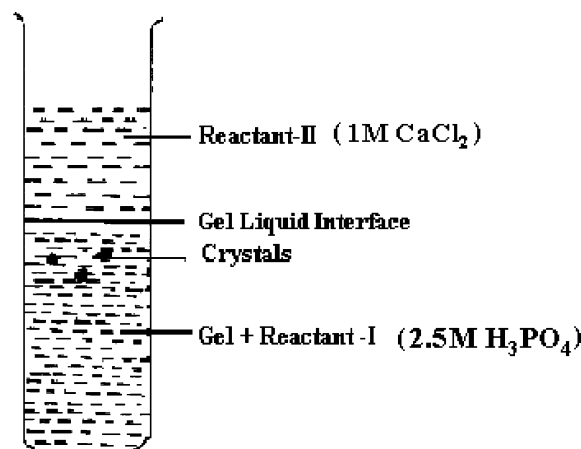


Fig. 1 Schematic diagram for crystal growth

The apparent length (cm) of growing crystals in test tubes at different times was measured using a traveling microscope with a lowest differentiation of 0.001 cm. The mean apparent length was obtained from the data for each time. The crystal grow within the Liesegang rings, which are not visible in Figs. 3 and 4, could be measured using a traveling microscope for aqueous extracts of *T. terrestris* and *B. ligulata*.

Preparation of different additive solutions

Different additive solutions were prepared by the following methods. These were studied along with the supernatant solutions, in different gel growth sets, so that their effects could be examined.

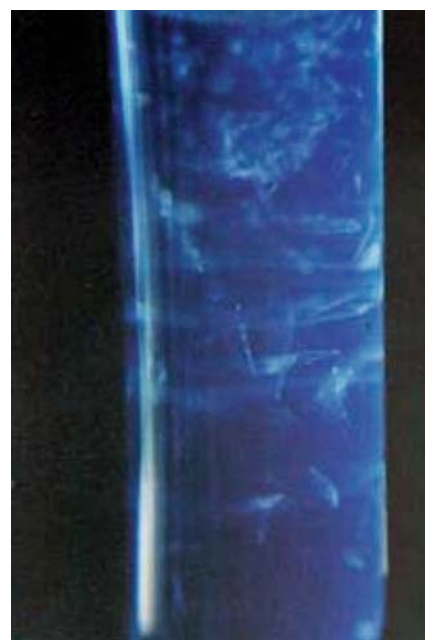


Fig. 2 Photograph of grown crystals within test tube for SS-A

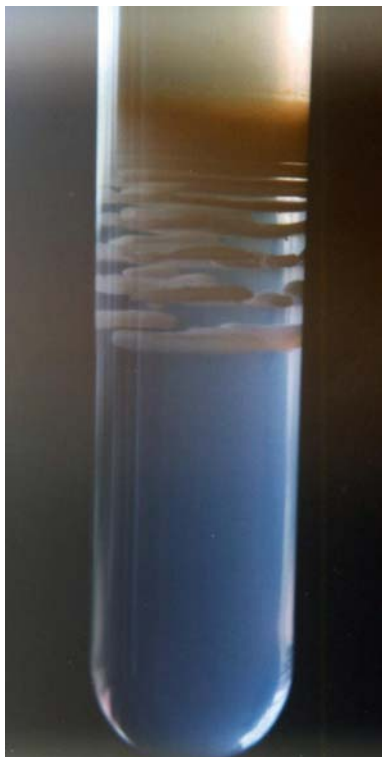


Fig. 3 Photograph of the type of the Liesegang rings for SS-F

Artificial reference urine

ARU was prepared by mixing equal amounts of solutions containing 0.1055 M sodium chloride, 0.0323 M sodium dihydrogen phosphate, 0.00321 M sodium

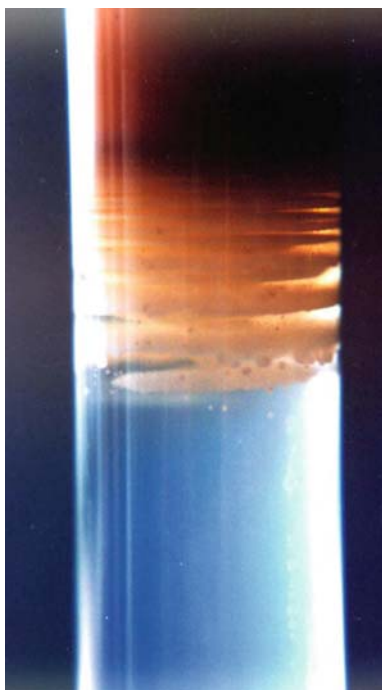


Fig. 4 Photograph of development of Liesegang rings for SS-G

citrate, 0.00385 M magnesium sulphate, 0.01695 M sodium sulphate, and 0.0637 M potassium chloride. The pH was adjusted to 6.5 using ammonium hydroxide or hydrochloric acid as per requirements [16].

Human urine

Urine from a healthy, adult volunteer was collected and filtered twice before being used as an additive to the supernatant solutions. This urine was analyzed in the pathology laboratory and nothing abnormal was detected. A fresh urine sample were used.

Aqueous extraction of T. terrestrisAE

The aqueous extract (AE) of *T. terrestris* was prepared with 25 g of the fine powder of the fruits of *T. terrestris* boiled in 400 ml distilled water for 30 min, and thereafter filtered twice.

Aqueous extraction of B. ligulataAE

The AE of *B. ligulata* was prepared in the same way as for *T. terrestris* by taking 25 g fine powder of the roots of *B. ligulata*, boiling it in 400 ml distilled water for 30 min, and filtering it twice.

The nomenclature of different additive solutions on the growth of CHPD crystals

An attempt was made to investigate the putative activity of the plant extracts and solutions as inhibitors of CHPD crystal formation. The supernatant solutions (Table 1) were added to the set gels and the results were noted. The experiments were repeated three times.

Results

As the number of Liesegang rings increased, the initial rings tended to diffuse. The distance between two consecutive rings increased on moving towards the bottom of the test tubes. The CHPD crystals grew within the Liesegang rings. The pH of SS-A was 7.4, whereas for SS-F and SS-G it was 3.36 and 3.64, respectively. The number of Liesegang rings was less in the case of SS-F and SS-G than for SS-A.

Figure 5 shows the plots of average apparent length versus time for SS-A, SS-D, SS-E, SS-F, SS-F1 and SS-F2. The pure calcium chloride containing SS-A led to the nucleation of crystals within 24 h of pouring in the supernatant solutions. This led to the maximum length crystals. In SS-D, SS-E and SS-F nucleation was delayed and crystals were observed after 96 h of pouring in the supernatant solutions. Moreover, all of these three supernatant solutions exhibited an inhibitive effect compared to SS-A, and a minimum apparent

Table 1 The supernatant solutions which were added to the set gels. AE indicates aqueous extract, ARU artificial reference urine, and HU human urine

No.	Supernatant solution (SS)	Composition
1	A	12.5 ml, 1 M CaCl ₂
2	D	7 ml 1.0 M CaCl ₂ +7 ml ARU
3	E	7 ml 1.0 M CaCl ₂ +7 ml HU
4	F	7 ml 1.0 M CaCl ₂ +7 ml AE/ <i>Tribulus terrestris</i>
5	F1	7 ml 1.0 M CaCl ₂ +7 ml ARU+7 ml AE/ <i>T. terrestris</i>
6	F2	7 ml 1.0 M CaCl ₂ +7 ml HU+7 ml AE/ <i>T. terrestris</i>
7	G	7 ml 1.0 M CaCl ₂ +7 ml A.E./ <i>Bergenia ligulata</i>
8	G1	7 ml 1.0 M CaCl ₂ +7 ml ARU+7 ml AE/ <i>B. ligulata</i>
9	G2	7 ml 1.0 M CaCl ₂ +7 ml HU+7 ml AE/ <i>B. ligulata</i>

length of growing crystals was observed in SS-E. The plots of SS-A, SS-D, SS-E and SS-F are the basic reference plots for comparing the apparent length of crystals in the case of SS-F1 and SS-F2. For solutions SS-F1 and SS-F2, the average apparent length of growing crystals was large in comparison with SS-A, which is an indication of the reverse effect. Figure 6 shows the plots of average apparent length of growing crystals versus time for SS-A, SS-D, SS-E, SS-G, SS-G1 and SS-G2. The similar nature of the plots is that observed in Fig. 5, but the inhibitive effect is more in the case of SS-G and SS-G1.

The ANOVA analysis is given in Table 2 for the plots of Figs. 5 and 6. The analysis for both figures shows highly significant differences ($P \leq 0.001$, Table 2).

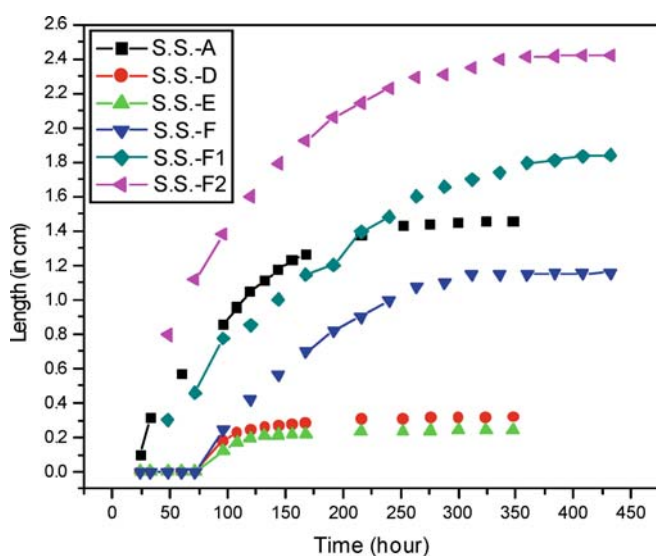


Fig. 5 Plot of apparent length of growing crystals versus time for *T. terrestris*

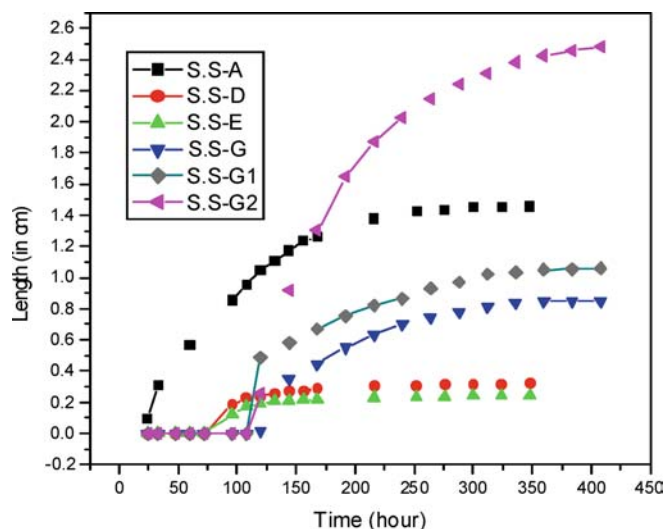


Fig. 6 Plot of apparent length of growing crystals versus time for *B. ligulata*

Discussion

Silica hydro gel is prepared by mixing a solution of sodium metasilicate into a diluted acid. In this solution, monosilicic acid is formed and becomes polymerized by the liberation of water; this process leads to a three dimensional matrix network of Si-O links with pores of different sizes, forming the gel. The effective pore diameter of silica hydro gel is of the order of 5–10 nm [5, 17, 18, 19, 20]. Diffusion of the reactant available in the supernatant solution into the gel and reaction with the other reactants already present in the gel leads to the growth of the crystals; at the critical concentration, as the diffusion increases, a few nuclei are formed. Subsequently, the supply of nutrients leads to crystal growth [5]. The growth of crystals, as well as the formation of Liesegang rings, can be explained on the basis of Fick's diffusion law equations for reagents in the gel as a function of time [5, 21, 22].

Table 2 Single factor ANOVA applied to crystal length data from the different extracts containing (a) *T. terrestris*, (b) *B. ligulata*. For (a) degrees of freedom = 5, 127, for (b) 5, 133

	Treatments	Mean
a	For <i>T. terrestris</i>	
	SS-A	1.075875
	SS-D	0.200889
	SS-E	0.154111
	SS-F	0.68715
	SS-F1	1.329294
	SS-F2	2.004176
b	For <i>B. ligulata</i>	
	SS-A	1.075875
	SS-D	0.200889
	SS-E	0.154111
	SS-G	0.42175
	SS-G1	0.56555
	SS-G2	1.22315

Recently, Joseph and Joshi [23] reported the formation of Liesegang rings in the growth of CHPD crystals and discussed the effects of various parameters on these rings. The reduction of the length of crystals and the number of Liesegang rings are due to the presence of an inhibitive solution containing AE/*T. terrestris* (SS-F) and AE/*B. ligulata* (SS-G). This can be verified from Fig. 3, 4, 5, 6 in comparison with SS-A, i.e., containing pure calcium chloride solution. The addition of different inhibitive solutions in the supernatant solution reduces the number of Liesegang rings and the average apparent length of CHPD crystals grown, which is due to the changes in the kinetics and diffusion processes.

There are various compounds exhibiting inhibitory actions on the growth of urinary stones and crystals; for example, tartrates are good inhibitors of stones in natural and artificial urine [24]. Nevertheless, urine holds more solute in the solution than pure water, hence, the concentration of a substance reaches a point at which crystallization occurs in water but does not take place in urine [1]. To study the effect of various inhibitors present in urine on the precipitation of urinary type calcium oxalate crystals, different authors have used natural urine [25] and ARU [16]. In the present study, both HU and ARU were used to study the growth behavior of CHPD crystals. ARU and HU inhibit the growth of CHPD crystals. This can be verified from the results of SS-D and SS-E, which correspond to the results obtained on calcium oxalate crystals.

Recently, Joshi and Joshi [26] reported the inhibition of CHPD crystals in the presence of the citric acid and lemon juice along with HU and ARU. In urine, inhibitors have been identified for the calcium phosphate and calcium oxalate crystal systems. Magnesium, citrate, pyrophosphate and nephrocalcein are the inhibitors in the calcium phosphate crystal system [27, 28]. Achilles et al. [29] reported the in vitro formation of urinary stones and the generation of spherulites of calcium phosphate in gels, as well as the overgrowth with calcium oxalate using a new flow model of crystallization.

The effects of the Ayurvedic drug Sveta Parpati with *B. ligulata* and *T. terrestris* in the management of urolithiasis (mutrasmari) are reported [30]. A purified fraction of ethanolic extract of *T. terrestris* fruits exhibited protection against uroliths induced by glass bead implantation in rats [31]. In another study, the administration of a drug containing *T. terrestris* to sodium glycolate fed rats produced a significant decrease in urinary oxalate excretion and a significant increase in urinary glyoxylate excretion [32].

The major constituents of *T. terrestris* include steroidal saponins [33], for example the terrestrosins A, B, C, D, and E, desgalactotigonin, F-gitonin, desglucol-anatigonin, gitonin, etc.; hydrolysed products include diosgenin, hecogenin and neotigogenin, etc. [35, 36, 37, 38]. The minor constituents of *T. terrestris* also include alkaloids [38], common phytosterols, viz. b-sitosterol, stigmasterol [34], a cinnamic amide derivative, terrestriamide and 7-methylhydroindanonone [39]. The consti-

tuents of *T. terrestris* and *B. ligulata* are mostly reported in the herbal pharmacopia [40].

However, the AE/*T. terrestris* in the supernatant solution, that is, SS-F, produced significant amounts of inhibition in comparison with SS-A. This can be verified from Fig. 5). But in the case of ARU and HU added to AE/*T. terrestris*, it was found that the apparent length of the growing crystals was more than that of SS-A. This may be due to the formation of typical chemical complexes in ARU and HU, which do not hamper the movement of calcium ions in comparison with pure AE/*T. terrestris* in supernatant solution. This may be due to their diuretic properties. They are popular as an Ayurvedic herbal remedy in India, but do not show clear inhibition in either ARU and HU in vitro.

Similarly, the AE/*B. ligulata* containing SS-G exhibited significant inhibition to the growth of CHPD crystals in comparison with SS-A. When ARU and HU are added to AE/*B. ligulata*, it was found that AE/*B. ligulata* with ARU led to an inhibitive action but AE/*B. ligulata* with HU did not exhibit any inhibition (Fig. 6). However, AE/*B. ligulata* with HU and ARU exhibited delayed nucleation. AE/*B. ligulata* with HU did not show any inhibition but along with ARU showed inhibition in comparison with SS-A. This suggests that once nucleation occurs, AE/*B. ligulata* with ARU and HU does not show inhibition but delays the nucleation significantly.

The growth of CHPD crystals occurred due to the reaction between HPO_4^{-2} and Ca^{+2} ions in the gel medium, and was followed by nucleation and the growth of crystals. The dilution of CaCl_2 in the supernatant solution did not seem to play a significant role in the present experiment, since the dilution of CaCl_2 decreased in SS-F1, SS-F2, and SS-G2, and at the same time the apparent length of crystals increased to that of the pure CaCl_2 solution.

When the HU, ARU and aqueous extracts of *T. terrestris* and *B. ligulata* were added separately to CaCl_2 solution in the supernatant solution, the average apparent length of the crystals decreased in comparison with pure CaCl_2 containing supernatant solution. This was due to the presence of natural inhibitors in these solutions, which inhibit the growth of crystals by forming soluble complexes. On the other hand, when aqueous extracts of *T. terrestris* and *B. ligulata* were added to the HU and ARU containing supernatant solution, the average apparent length of the growing crystals was more than the pure calcium chloride containing supernatant solution. This was an unexpected result, since HU, ARU, and aqueous extracts with CaCl_2 exhibited an appreciable amount of inhibition but the expected additional inhibition was not seen when all three were mixed together. This may be due to the formation of complexes between ARU or HU and the aqueous extracts of *T. terrestris* or *B. ligulata* in such a way that they do not inhibit the growth of crystals but promote it. Because natural urine and aqueous extracts contain many organic molecules and salts, it is likely

that they form a complex which does not inhibit the growth of crystals but promotes it. However, in normal remedies, *B. ligulata* and *T. terrestris* are used for urinary stone problems; perhaps their diuretic properties are more helpful than their inhibitive properties in urine. Both of these herbal extracts are known for their diuretic effects [14].

The present investigation throws light on the inhibitive processes occurring in two selected aqueous extracts of Ayurvedic medicinal plants on the growth of CHPD crystals. Single factor ANOVA performed with treated and untreated crystal growth data showed high significance ($P < 0.001$, Table 1). Although the stone formation process occurring in the human body is quite complex and takes place in a dynamic environment, the present study provided basic information, under laboratory conditions, which led us to identify new inhibiting solutions of stone growth. Further investigations in vivo and in vitro may provide useful information on new urinary calculi inhibitors, which may be useful either for the treatment of urinary calculi or their prevention. Further work is in progress.

Conclusions

Pure calcium chloride containing supernatant solution produced Liesegang rings in the gel, and needle, platelet and star-shape CHPD crystals grew within the rings. The addition of aqueous extracts of *T. terrestris* and *B. ligulata* to the calcium chloride in the supernatant solutions modified the diffusion process and hence the periodic precipitation and the number of Liesegang rings. The maximum length of the crystals was reduced due to inhibition produced by the addition of aqueous extracts of *B. ligulata* and *T. terrestris*.

HU and ARU exhibited inhibition of different orders in comparison with pure calcium chloride containing supernatant solution. Similarly, the AE/*T. terrestris* and AE/*B. ligulata* exhibited inhibition to the growth of CHPD crystals.

In the case of adding ARU or HU to AE/*T. terrestris* or AE/*B. ligulata*, HU promoted the growth of CHPD crystals for both AE/*T. terrestris* and AE/*B. ligulata*, but for ARU the AE/*T. terrestris* promoted and the AE/*B. ligulata* inhibited the growth of CHPD crystals. The AE/*B. ligulata*, along with HU and ARU, delayed the nucleation of crystals. The HU, AE/*T. terrestris* and AE/*B. ligulata* contained a large number of salts and organic molecules, and their complex formation may have promoted the effect on the growth of CHPD crystals, but when they are added separately to CaCl_2 they inhibit the growth of crystals. This suggests that these solutions separately inhibit the growth of crystals in in vitro conditions, but mixing with HU changes their behavior markedly. The diuretic nature of AE/*T. terrestris* and AE/*B. ligulata* seems to be important in the remedy rather than their inhibitive natures.

The formation of different chemical complexes may be very important in the presence of HU and ARU, which affect the motion of calcium ions in different amounts to form the CHPD crystals. This requires further attention.

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