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Original Article

In vitro effect of *Citrullus Colocynthis* and *Acacia Radiana* on Phosphate Calcium Crystallization

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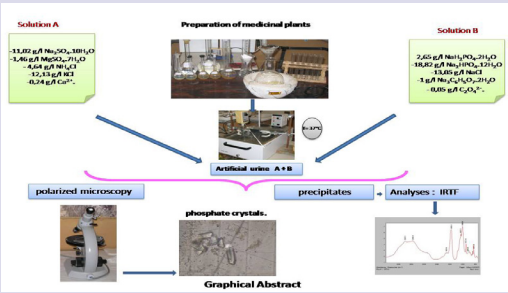
ABSTRACT

Introduction: In this work, we performed an *in vitro* crystallization study enabling the specification of kinetic and thermodynamic conditions of formation and growth of crystalline calcic phosphates species by changing the pH. We used inhibitors, which are medicinal plants which prevent, slow down or reduce crystallization phases. **Materials and Methods:** We chose the classical model for the study of phosphate crystallization without inhibitor and with it, in order to assess the inhibiting capacity of any chemical species used. The precipitation of the solid phase of phosphates from artificial urine at different initial pH values was the object of our investigation. The crystal size development was monitored by polarized microscopy at different time intervals. After crystallization time, the mixture was filtered, the recovered dried precipitates were analysed by FTIR. In the absence of inhibitor, the crystallization of phosphates at pH=8.00, led to the formation of struvite and Amorphous Carbonated Calcium Phosphates (ACCP), after 4 hours. In presence of inhibitor at pH=8.00 at lower concentrations of sage inhibition was partial. **Result and Disucssion:** The addition of 1 ml of sage to the mixture decreases the size of crystal, after 4 hours the size of crystals stabilized at 20.67 µm. The complete disappearance of brushite crystals was obtained after addition of 10mL of *Citrullus Colocynthis*, only Pentahydrated octocalcic phosphates (POP) and ACCP were formed. In the presence of *Acacia Radiana*, the inhibition of struvite growth and aggregation increased. The addition of up to a volume of 20 mL of *Acacia Radiana* resulted in total inhibition and crystalline transformation of the ACCP into carbapatite (CA). Phosphate compounds encountered in urine can be dangerous and the use of inhibitors to prevent, slow down or reduce crystallization phases might be very helpful. **Conclusion:** In this investigation, *Citrullus Colocynthis* and *Acacia Radiana* proved to be good inhibitors. Their effect increases with solution pH but they are more efficient in less acidic or neutral urine than in alkaline one.

Key words: *Acacia Radiana*, *Citrullus Colocynthis*, Crystallization, Inhibition, Struvite.

PICTORIAL ABSTRACT



Graphical Abstract

SUMMARY

- It is to prepare artificial urine and imitate the etiology of the formation of crystalls in humans.
- *Citrullus Colocynthis* and *Acacia Radiana* proved to be good inhibitors.
- The complete disappearance of brushite crystals was obtained after addition of 10 mL of *Citrullus Colocynthis*, only Pentahydrated octocalcic phosphates (POP) and ACCP were formed.
- Mechanisms of action of these inhibitors of the crystallization of phosphate during steps: nucleation, growth and aggregation.

Abbreviations used: FTIR: Fourier transform infrared spectroscopy, ACCP : Amorphous carbonated calcium phosphates, POP: Pentahydrated octocalcic phosphates, CA : Carbapatite.

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INTRODUCTION

Struvites form in humans as a result of urinary tract infection with ureolithic micro organisms that split urea and cause persistently alkaline urine.[1-2] Struvite, ammonium magnesium phosphate hexahydrate {(AMPH)-(NH4)MgPO4·6(H2O)}, is a biomineral and occurs as crystallites in urine and as a type of kidney stone. It is also known as triple phosphate stone, infection stone or urase stone. Worldwide, they compose 30% of all kidney stones.[3] The struvite has been studied from different viewpoints and its chemical and physical properties were described. Amongst others, pH and the solution composition were measured during the struvite precipitation and dissolution, also in recent years.[4-5] Plants remain the basis for a large proportion of the medications used today for the treatment of variety of diseases. A number of researchers have documented the use of traditional medicinal plants in India[6] Although generally, there has been no indication of the type of calculus being treated. During recent decades, studies of the antilithiasic effect of herbal extracts have been reported, but in the majority of these reports, the effect did not seem to be mediated by urinary biochemical changes.[7] Urine of a healthy person is under-saturated with regard to Struvite, but because of the conditions provoked by urease-producing microorganisms and the urine complex composition, the precipitation of struvite can occur. Under such conditions struvite often precipitates together with apatites and the sediment can easily be attached to the particles of organic matter formed as a consequence of the infection. This mechanism favors the crystal deposition and aggregation, so that struvite stones grow rather quickly. Struvite stones may grow rapidly over a period of weeks to months and, if not adequately treated, can develop into a Staghorn or branched calculus that involves the entire renal pelvis and calyces. Patients with infected Staghorn calculi who receive no treatment have about a 50% chance of losing the kidney.[8-9] The aim of this study was to evaluate the effect of extract plants : *Citrullus Colocynthis* and *Acacia Radiana* on struvite formation by artificial urine crystal formation, using deferent concentration of inhibitors growth stages on crystal formation by optic microscope and FTIR.

MATERIALS AND METHODS

Synthetic urine

Artificial urine is the classical model for the study of phosphate crystallization because of its simplicity and satisfactory reproducibility. This model includes the study of crystallization without inhibitor and with it, in order to assess the inhibiting capacity of any chemical species used. Two solutions of following composition were mixed:

A : 11.02 g/l $Na_2SO_4.10H_2O$, 1.46 g/l $MgSO_4.7H_2O$, 4.64 g/l NH_4Cl, 12.13 g/l KCl et 0.24 g/l Ca^{2+}

B: 2.65 g/l $NaH_2PO_4.2H_2O$, 18.82 g/l $Na_2HPO_4.12H_2O$, 13.05 g/l NaCl, 1 g/l $Na_3C_6H_5O_7.2H_2O$ et 0.05 g/l C_2O_{42-}. The solution in C_2O_{42-} is prepared from oxalic acid 0.05 g.

The precipitation of the solid phase of phosphates from artificial urine at different initial pH value (pH=8) was the object of our investigation. Artificial urine is prepared by mixing and stirring two equal volumes of 50 ml of solutions A and B at constant temperature (37°C) in capped vessels to give final artificial urine. The pH of solution B was adjusted to required value by adding either HCl or NaOH as appropriate. Mixture agitation was maintained to prevent sedimentation. The crystal size development was monitored by polarized microscopy at different time intervals by proceeding as follows: Sample drops were examined every five minutes by polarising optical microscopy. Crystals were identified with x 40 magnifying lens. After crystallization time, the mixture was filtered, the recovered dried precipitates were analyzed by FTIR spectroscopy, electronic microscopy scanning analysis.

Preparation of medicinal plants

Acacia radiana was collected in spring near to Bechar and *Citrullus Colocynthis* from Ghardaia region (Sahara of Algeria). The extract was prepared according to a similar procedure used often by patients. Fresh herb was dried at 45°C overnight, boiled in distilled water. The powder was reconstituted to prepare a solution of 15g/l in distilled water.

Formula of inhibition

Percentage of inhibition of crystallization (I%) was calculated as previously described[10] and based on the formula, I%=[(TSI-TAI) / TSI] x 100, in which TSI and TAI represent numbers of struvite crystals in absence and presence of inhibitors (plant extracts), respectively. Nucleation, growth and aggregation of crystals were visually assessed under the microscope.

RESULTS

Acacia raddiana (sheet)

The effects of the plant *Acacia raddiana* (sheet) on the size of crystals and aggregates as well as the percentage of inhibition are shown by the (Figures 1 and 2).

The study by optical microscopy shows that at 20 ml, the size of the crystalline species, struvite was partially reduced.

The presence of granules, we note that in general the crystallization occurs rapidly at all different volumes although we recorded a gradual decrease in the size of the crystals based on volumes of plant extract and time. The effect of the leaves of *Acacia raddiana* on struvite crystals at pH=8 is not important. After 4 hours, the crystal size reaches a minimum value of 24 microns with a volume of 15 ml, a decrease of 57.02% from its value without inhibitor. Also, we note that the crystal size reaches a maximum rating decrease from 240 minutes at a volume of 20 ml is almost 60% inhibition Comparing the average crystal thickness in (Figure 1 and 2) is more representative and shows that the limitation of crystallization by adding extracts could play some role in the growth of independent struvite crystals concentrations or the duration Cooling equipment of. The crystals formed are reduced to Inhibit 55%, but substantially more visible, obvious differences were observed in the aggregates of the crystals produced in (Figure 2) could therefore equally important inhibition that reaches to 96% after one hour in a mixture of 15 ml of extract, the inhibition could be improved in experiments aggregates its figures are intended to be representative.

We observe that 1 ml few aggregates appeared from 1 to 2 per field, against 15 ml the number increases by 3 to 4 per field. Addition of 1 and 5 ml volumes delay the onset of the aggregates to 10 minutes. The minimum size of the aggregates is 52 microns or a percentage inhibition of 69%.

Citrullus colocynthis (L.) Schrad

In Algeria, traditional medicine uses *Citrullus colocynthis* L. fruit for the treatment of diabetes, cancer and hemorrhoids. It should be noted that

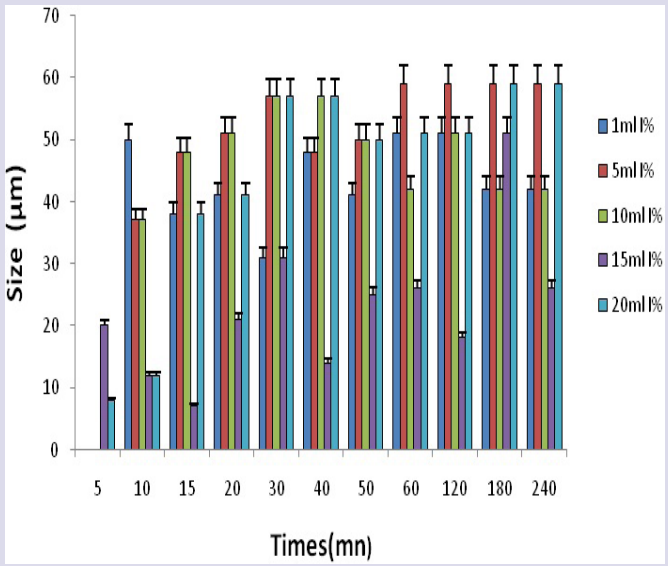

Figure 1: Evolution of the size of struvite crystals in the presence *Acacia raddiana* (sheet)

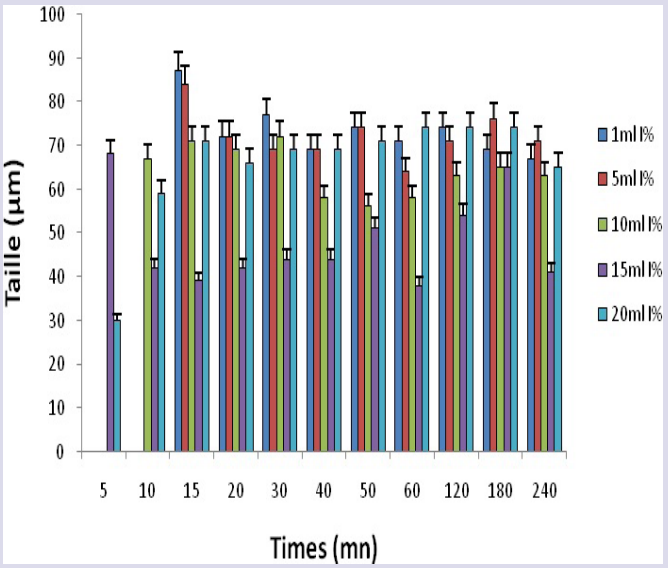

Figure 2: Evolution of struvite size aggregates in the presence *Acacia raddiana* (sheet).

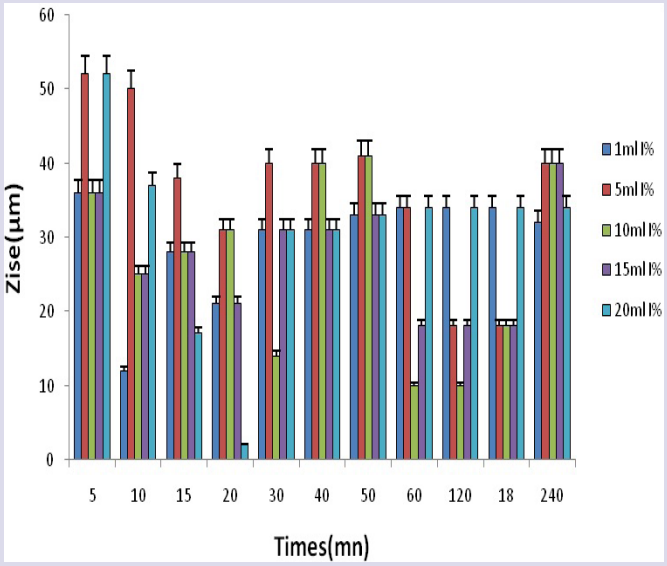

Figure 3: Evolution size struvite crystals in the presence *Citrullus colocynthis* (L.)

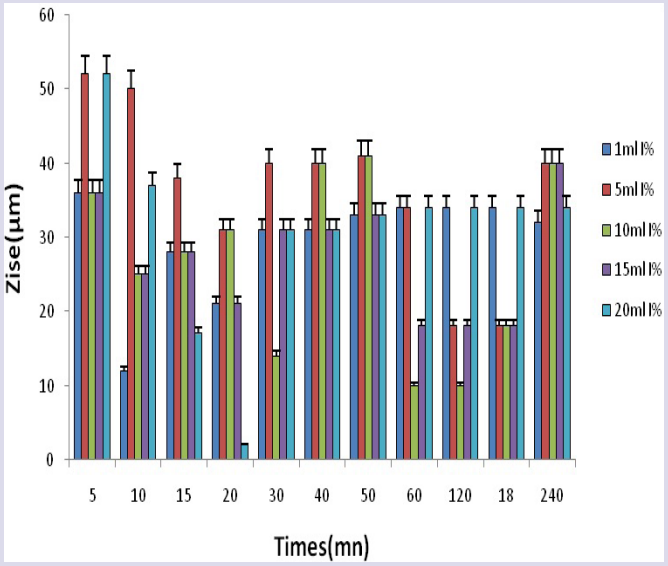

Figure 4: Evolution of the size struvite aggregates in the presence *Citrullus colocynthis* (L.)

the extract of *Citrullus colocynthis* shows a low and variable immunostimulant activity.[11] The (Figure 3) includes crystal size under the effect of the fruit of *Citrullus colocynthis* (L.) Schrad., and (% I) inhibition.

We noted during the various observations, the effect of the plant *Citrullus colocynthis* (L.) Schrad. is not as important as Acacia raddiana. After 4 hours, the minimum size of crystals reached a value of 40 Mu for volumes 1 and 5 ml, down 11% from its value without inhibitor. Despite the addition of a volume of 20 ml struvite crystals persist. However the effect of the plant *Citrullus colocynthis* (L.) Schrad. is important as it is presented to the histogram 3.

The effect of the plant *Citrullus colocynthis* (L.) Schrad. the aggregates is enough interest that its effect on the crystals.

The results are given in the (Figure 4).

The addition of 15 ml of *Citrullus colocynthis* (L.) Schrad. delays the appearance of aggregates up to 40 minutes. After 4 hours, the size of the aggregates is recorded of 40 microns to a volume of 10 ml, a decrease of 78% relative to its value without inhibitor. The number of struvite crystals at the end of the experiment is less (about 10 mm 3 crystals artificial urine). This decrease is probably due to a conversion carbapatite Amorphous Carbonated Calcium Phosphate (PACC). The formed granules were identified by optical microscopy in polarized light and confirmed by FTIR (Figure 5) and Table 1 as the amorphous carbonated calcium phosphate (PACC). The precipitate collected after 4 hours is essentially consisting of brushite predominates, with struvite and PACC.

DISCUSSION

Many *in vitro* models have been developed to understand the mechanisms involved in the formation of urinary stones and determine the effects of various therapeutic agents on the development and progression of the disease. The strategy, in our work, is the one most commonly used by the research teams. It is to prepare artificial urine and imitate the etiology of the formation of crystalls in humans. On the basis of all the tests conducted during our study, we conclude that the inhibitory effects of plant extracts selected on the calcium phosphate crystallization are very close or even identical to those reported in the work of Vaidya and al, showed significant decreases in struvite crystal sizes in the presence of *Boerhaavia diffusa*, estimated at 13.33% inhibition in 5% extract and 30.37% inhibi-

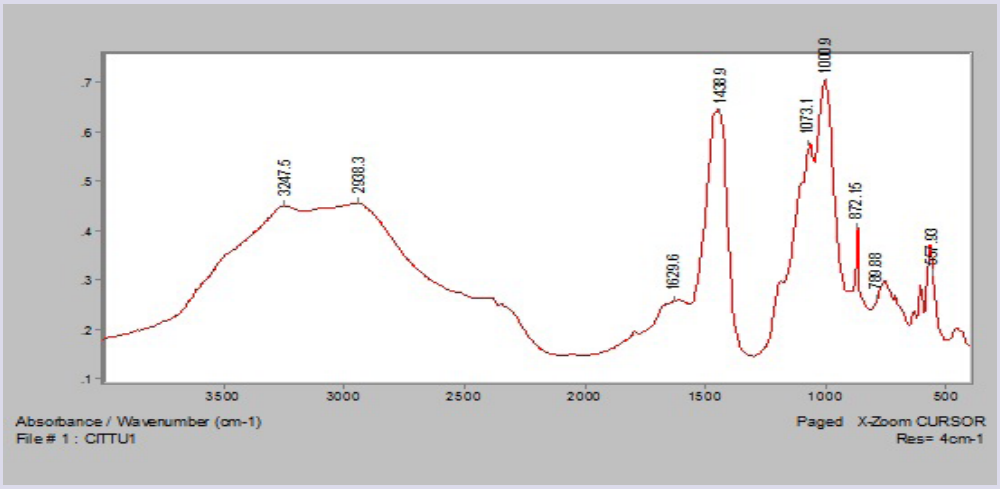

Figure 5: FTIR spectrum in the presence of 10 ml of *Citrullus colocynthis* (L.) Schrad

Table 1: Frequency bands characteristic of the constituents

Frequency (cm^{-1})	Imputation	Constituent
(TF) : 1000.9 **(m) : 1438.9 ; 2350**	Band phosphate Vibration NH4 + (deformation) Plateau molecule H_2O + ion NH_4+, décrochant à 2346 cm^{-1}	Struvite $MgNHPO_4.6H_2O$
(F) : 1073.1 **(m) : 557.93**	Band phosphate, rounded and asymmetrical Secondary vibration des CO_3^{2-} No thin strip to 600 cm^{-1}	P.A.C.C. (trace)

tion at 1% concentration of extract.[12] Our results show also that for the majority of the plants used, the inhibitory effect results in a reduction in size of the crystals. It is the case in particular of: - The effect of *Acacia raddiana* (a decrease of 66.6% relative to its value without inhibitor); - The effect of *Citrullus colocynthis* on aggregates is more interesting than its effect on the crystals (a decrease of 76% from its value without inhibitor).[13] evaluated the potential of inhibiting substances for phosphates and established the mechanisms of action of these inhibitors of the crystallization of phosphate during steps: nucleation, growth and aggregation. In addition, they have tested the effectiveness of medicinal plants such as camomile and sage on the crystallization of calcium phosphates. Medicinal plants are able to significantly reduce the phosphate crystallization *in vitro*. It is easily, shown by FTIR and optical microscope, the aqueous extracts of *Acacia raddiana, Citrullus colocynthis,* produce a significant amount of inhibition of the growth of the phosphate crystals.

CONCLUSION

Calcium Phosphate compounds found in urine can be dangerous. The use of Inhibitor to prevent, slow down or reduce crystallization stages can be very useful. Medical plant are able to significantly reduce the phosphate crystallization *in vitro.* It is easily, shown by FTIR and optical microscope, the aqueous extracts of *Citrullus Colocynthis* and *Acacia Radiana* produce a significant amount of inhibition of the growth of the phosphate crystal. This *in vitro* study provided useful information for the *in vivo*.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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