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Effects of *Atriplex halimus* and *Ajuga iva* L. Schreb on Calcium Oxalate Urolithiasis Risk *in vitro*

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In this work, an *in vitro* crystallization study enabling the specification of kinetic and thermodynamic conditions for the formation and growth of crystalline calcium oxalate has been performed. Some medicinal plants were used as inhibitors, thus preventing, slowing down or reducing crystallization phases. A classical model was chosen for the study of oxalate crystallization because of its simplicity and satisfying reproducibility. This model involves the crystallization study with and without inhibitor, in order to assess the inhibition capacity of any used chemical specie. Two solutions of dihydrated calcium chloride (40 mM/L) and sodium oxalate (4 mM/L) are prepared in presence of an amount sufficient enough of sodium chlorite. The oxalate crystal development was monitored by polarization microscopy at different time intervals. Thus, in the absence of inhibitor, crystallization of calcium oxalate led to the formation of a calcium oxalate crystal after 0.5 h. In presence of inhibitors at lower concentrations however, inhibition was partial. The addition of *Atriplex halimus* L. or *Ajuga iva* (L.) Schreber acted on the phase of growth crystallization. The rates of inhibition capacity at a 75 % concentration of *Atriplex halimus* and *Ajuga iva* were 97.82 and 97.01 %, respectively. These inhibitors developed an important inhibition capability at low concentrations. Calcium oxalate monohydrate compounds encountered in urine could be dangerous and thus using inhibitors to prevent, slow down or reduce crystallization phases might be very helpful. In this study, *Atriplex halimus* and *Ajuga iva* are shown to be good inhibitors.

Key Words: Oxalate, Inhibition, *Atriplex halimus* L., *Ajuga iva* (L.) Schreber, Crystallization.

INTRODUCTION

Urolithiasis is a very painful disease that has afflicted a wide number of human beings since ancient times[1]. The mechanism of kidney stones formation has attracted the attention of medical scientists because of its widespread clinical occurrence and the difficulty of treatment. Hyperoxaluria is one of the main risk factors of

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human idiopathic calcium oxalate disease. Oxalate, the major stone-forming constituent, is known to induce lipid peroxidation which causes disruption of the cellular membrane integrity[2,3]. Lipid peroxidation is a free radical induced process leading to oxidative deterioration of polyunsaturated lipids. This alters the membrane fluidity, permeability and thereby affects the ion transport across the cellular organelle[4,5].

The effects of *Atriplex halimus* and *Ajuga iva* on the calcium oxalate urolithiasis urinary risk factors, in herb infusion forms, have been studied *in vitro*. Calcium oxalate is one of the main constituents of deposits in urinary tract. Crystallization of calcium oxalate is of particular interest not only from the theoretical point of view but also because of its biological importance.

The exact mechanism of the initiation of the calcium oxalate stone formation is not completely understood. Factors leading to the nucleation, crystal growth and aggregation of various hydrates of calcium oxalate depend not only on the excess of calcium and oxalate concentrations but also on the presence of various foreign substances.

A number of studies have been carried out to understand the effect of various additives, on inhibition of calcium oxalate crystallization, such as metallic ions and their complexes[6], sodium dodecyl sulphate[7], α-keto glutaric acid[8] (a normal physiological constituent of urine), plant extracts[9], maleic acid copolymers[10] and a protein from human kidney[11]. This protein plays a physiologically significant role in inhibiting the stone formation in acidic urine. Inhibitory activity was found to increase with increasing concentration of the protein.

In the present paper, a systematic investigation on the inhibition and dissolution of calcium oxalate by *Atriplex halimus* and *Ajuga iva* have been reported. Both plants have multifunctional properties. They are useful in the treatment of stones, abdomen, kidney and other chronic pains.

EXPERIMENTAL

Commercial dry herb from *Atriplex halimus* L. (Chenopodiaceae) and *Ajuga iva* (L.) Schreber (Labiatea) were purchased from a specialized market (Oran Co., Algeria) and identified by the plant taxonomy unit of Oran University. Infusions were prepared daily just before handling by suspending a weighed amount of dry plant material in boiling tap water. The suspension was kept at room temperature for 15 min and then filtered through filter paper. The infusion was subsequently used at room temperature.

Synthetic urine: We chose the classical model for the study of oxalate crystallization because of its simplicity and satisfying reproducibility. This model includes the crystallization study with and without inhibitor, in order to assess the inhibiting capacity of any used chemical specie.

The synthetic urine was prepared immediately before use by mixing an equal volume of solutions A and B in a T-type mixing chamber. Both solutions were prepared by dissolving chemicals of reagent-grade purity in deionized then redistilled water (A: $Na_2C_2O_4$ (2 mM/L); B: $CaCl_2.2H_2O$ (10 mM/L)).

The two solutions were prepared in presence of NaCl to obtain an ionic force equivalent to that of the human organism.

Simulation of the sedimentary crystal formation: Artificial urine is easily prepared by mixing and stirring up two equal volumes of 50 mL of A and B solutions at constant temperature (37 °C) in capped vessels. Calcium oxalate crystallization is does not depend on pH[12].

Mixture agitation was maintained to prevent sedimentation. The crystal size development was monitored every five minutes by submitting some sample drops to a polarized optical microscope of the Zeiss type equipped with a WINDER M 476079 camera. Crystals were identified with a 'x 40' magnifying lens.

RESULTS AND DISCUSSION

Study of the oxalate crystallization without inhibitors: The kidney oxalate stone is the result of urine supersaturation with certain urinary salts such as calcium oxalate. Since oxalate species are pH-independent, the oxalate crystallization in the absence of inhibitor led to the formation of calcium oxalate monohydrate (COM), as detected by polarized light microscopy. The process of calcium oxalate crystallization in control solutions, *i.e.* without the addition of inhibitors is shown below (Table-1 and Fig. 1).

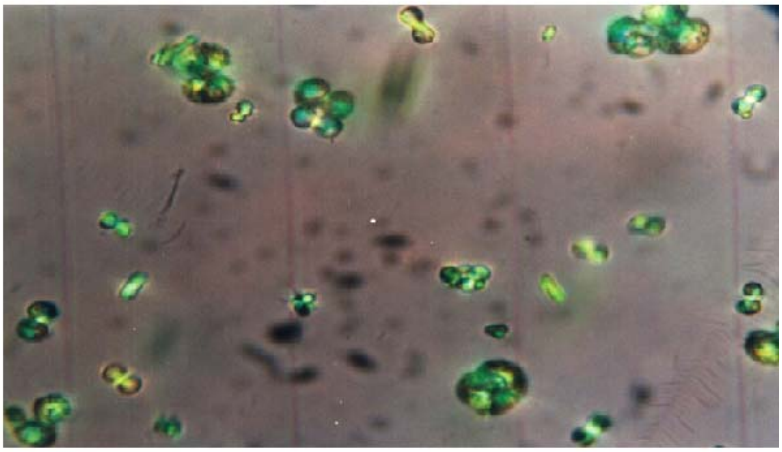

Fig. 1. Calcium oxalate monohydrate crystals grown without inhibitors

TABLE-1
STUDY OF THE CALCIUM OXALATE MONOHYDRATE (COM) CRYSTALLIZATION WITHOUT INHIBITORS

Time (min)	5	10	15	20	25	30
Number of (COM)/mm^3	663	725	840	704	690	762
(COM) aggregation/mm^3	101	104	115	114	87	76
Total	764	829	955	818	777	838

Study of phosphate crystallization in the presence of inhibitors: Same experimental procedure for the study of crystallization in the presence of inhibitors has been followed. In order to assess the inhibitory effect of some substances on oxalate and to understand their mechanism of action on the crystallization steps (nucleation, growth and aggregation), the effectiveness of the *Atriplex halimus* and *Ajuga iva* medicinal plants have been tested. The same procedure as before *i.e.* absence of inhibitor was followed. However, we added in the same time the inhibitors in an amount sufficient enough to obtain the physiological concentration to one of the two (A and B) solutions before mixing at the same temperature (37 °C). The plots of crystal size development as a function of time follow the same pattern as without inhibitors. The same parameters (size and number of crystals, time of crystallization) were also investigated. Thus, by knowing the size of crystals that are formed in the absence of inhibitor, it becomes easy to compare and to assess the role of the two investigated inhibitors. A series of experiments corresponding to the physiological concentrations of 25, 50, 75 and 100 % of *Atriplex halimus* and *Ajuga iva*, respectively were carried out in order to cover the physiological excretion range. The monitoring of the crystal size development by polarized light microscopy was carried out at time intervals corresponding to 5, 10, 20, 25 and 30 min of crystals formation. Determination of the inhibitory percentage (I %) was based on the formula[13]:

$$I\ \% = [(TsI - TAI)/T_SI] \times 100$$

with TsI representing the number of calcium oxalate monohydrate crystals without inhibitors and TAI representing that of calcium oxalate monohydrate crystals after inhibitor addition.

***Atriplex halimus*:** Same experimental procedure for the study of crystallization in the presence of the inhibitor has been followed. We focussed on crystallization because it may be the nucleating agent for many stones[14]. As shown below, the *Atriplex halimus* inhibitory effect is not dose-dependent. The best inhibitory concentrations (97.82 and 95.81 %) are obtained at [75 %] and [100 %], respectively of the extract concentrations. The inhibitory effect of *Atriplex halimus* plant involved growth crystal phases (Table-2 and Fig. 2).

The results given in Table-2 show the addition of 1 mL of *Atriplex halimus* extract to the mixture.

TABLE-2
INHIBITION OF CALCIUM OXALATE MONOHYDRATE CRYSTAL AFTER ADDITION OF *Atriplex halimus* EXTRACT

Time (min)	5	10	15	20	25	30
% Inhibition [25 %]	56.80	67.51	78.21	62.22	73.23	63.12
% Inhibition [50 %]	66.49	66.82	68.79	64.54	62.67	67.18
% Inhibition [75 %]	83.76	97.82	89.84	88.14	89.57	91.64
% Inhibition [100 %]	92.80	91.55	95.81	87.04	88.41	89.26

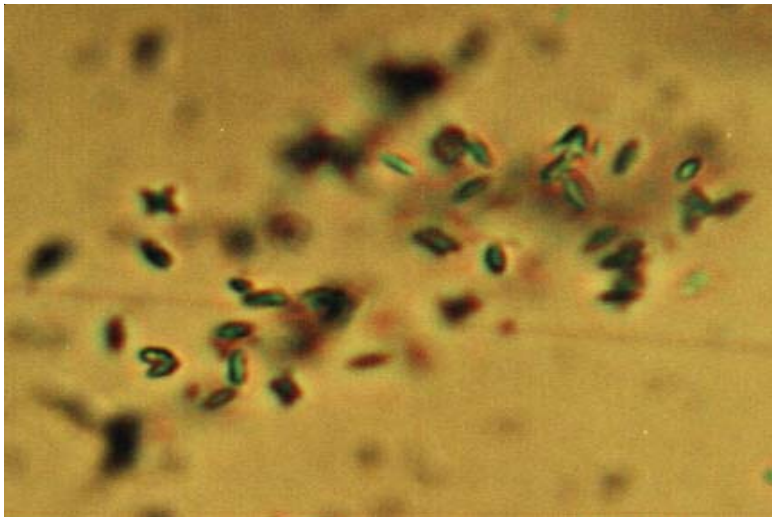

Fig. 2. Calcium oxalate monohydrate crystals after addition of *Atriplex halimus* extract

***Ajuga iva*:** The precipitation of calcium oxalate was carried out in the absence then in presence of *Ajuga iva*. Results show that crystal morphology depends considerably on the concentration of the medicinal plant extract. The crystal size was found to decrease with the increase in the medicinal plant extract concentration. Subsequently, complete crystal disintegration was observed. The crystals size as a function of time is given in Table-3, along with the corresponding picture (Fig. 3). The addition *Ajuga iva* worked strictly on the nucleation phase of calcium oxalate crystallization without affecting the other stages. The best inhibitory concentrations (97.01 and 96.06 %) were encountered at [75 %] and [100 %] respectively of extracts concentrations after 0.5 h.

The results given in Table-3 show the addition of 1 mL of *Ajuga iva* extract to the mixture.

TABLE-3
INHIBITION OF CALCIUM OXALATE MONOHYDRATE CRYSTAL AFTER ADDITION OF *Ajuga iva* EXTRACT

Time (min)	5	10	15	20	25	30
% Inhibition [25 %]	84.03	86.12	97.90	88.01	88.28	89.97
% Inhibition [50 %]	89.92	87.69	90.57	93.64	94.20	95.22
% Inhibition [75 %]	92.80	93.72	94.97	95.35	95.88	97.01
% Inhibition [100 %]	89.79	85.51	93.19	94.25	93.56	96.06

The present work was performed in order to assess *in vitro*, the inhibitory effects of certain medicinal plants *i.e.*, extract of *Atriplex halimus* and *Ajuga iva* on calcium oxalate crystallization. Using polarized light photography, the various phases of crystallization *viz.*, nucleation, growth and aggregation which eventually lead to

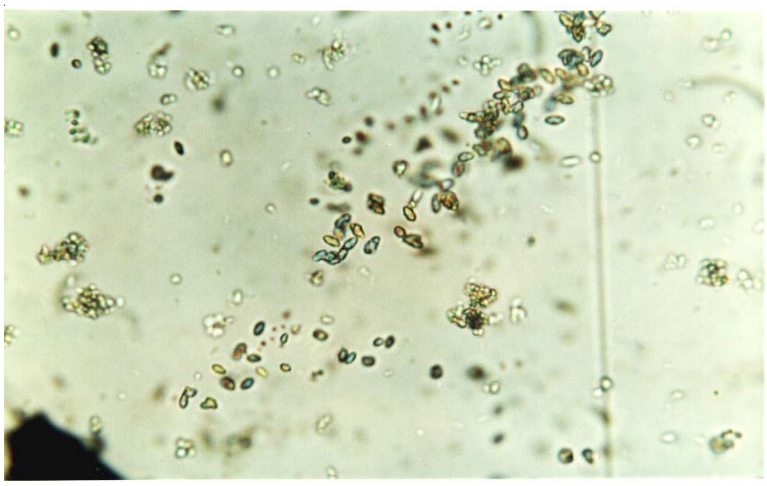

Fig. 3. Calcium oxalate monohydrate crystals after addition of *Ajuga iva* extract

urolithiasis have been clearly demonstrated. Calcium stone formation involves different phases of increasing accumulation of CaOx-nucleation, crystal growth, crystal aggregation and crystal retention. On the other hand, *in vitro* studies showed that *Ajuga iva* inhibited both the nucleation phases with a maximal inhibitory effect of 97.01 %. Nucleation is the formation of a solid crystal phase in a solution. It is an essential step in renal stone formation[15,16]. The present study have also shown the impact of *Atriplex halimus* on the crystal growth with a maximum inhibitory effect of 97.82 %. After nucleation, crystal growth is the next major step of stone formation. The driving force for crystallization is a reduction in the potential energy of the atoms or molecules when they form bonds with each other. The crystal growth process starts with the nucleation stage. Several atoms or molecules in a super saturated liquid start forming clusters. The bulk free energy of the cluster is less than that of the liquid[17]. Finally, the inhibitory effect of *Atriplex halimus* and *Ajuga iva* on the nucleation and growth processes in the case of calcium oxalate monohydrate appear to overlap during the initial lag-phase of precipitation.

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