Comparison of the inhibitory capacity of two groups of pure natural extract on the crystallization of two types of material compound urinary stones in vitro study

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Abstract: Urolithiasis is defined as the result of an abnormal precipitation within the urinary tract. This precipitation is most often from the normal constituents of the urine. This is a fairly common condition in the population. She is happy and recurrent etiology is often unknown if hypothetical. In Algeria, as in many countries, a large number of patients use herbal medicines in the treatment of their diseases including urolithiasis. Thus the aim of this study is the most widely used to evaluate the effectiveness of aqueous extracts of medicinal plants, in the treatment of calcium urolithiasis oxalo-and magnesium-amoniaco in vitro. The study also examines the effect of these extracts on the states of crystallization (nucleation, crystal growth, crystal aggregation), followed by photography on polarized light microscope. In this regard, we are devoted to studying the crystallization steps from oxalo-calcium and phospho-calcic prepared as artificial urine and supersaturated aqueous solutions, maintained at 37 ° C to remain close to biological conditions. Extracts of the first group of herbs: Ammodaucus leucotrichus, Ajuga iva, Globularia alypum, Atriplex halimus are studied on the crystallization calcium oxalate, we cite the Ammodaucus leucotrichus which acts on the stages of nucleation, growth and the aggregation with a total inhibition. The second group of extracts plants tested on calcium phosphate crystallization: Acacia raddiana, Citrullus colocynthis, Rhus tripartita, Pistacia lentiscus, Warionia saharae, are able to significantly reduce phosphate crystallization in vitro. It is easily proved by FTIR and optical microscope. In conclusion the results of our work allows us to confirm the use of these plants as an aqueous decoction, in the field of urolithiasis. These activities may help to strengthen the body in depressed situations.

1. Introduction

Nephrolithiasis is a complex disease that results from a combination of various factors related to both urine composition and kidney morphoanatomy. Calcium oxalate is the predominant component of renal stones (70%). Two types of calcium oxalate stones have been distinguished, composed of either calcium oxalate dihydrate (COD) or calcium oxalate monohydrate (COM). COD crystals are thermodynamically unstable and develop only under kinetically favorable conditions, such as a high degree of supersaturation (high calcium concentration >170 mg/L and/or hyperoxaluria), low concentrations of crystallization inhibitors (phytate, citrate), and urodynamically appropriate conditions (e.g., urinary stagnation). Due to thermodynamic instability, COD crystals slowly
transform to stable COM crystals mainly in contact with urine [1]. All over the world especially in developing countries, approximately 80% of population continues to use traditional medicine in primary medical problems. In the past decade, therefore, research has been focused on scientific evaluation of traditional drugs of plant origin. There is an urgent need to systematically evaluate the plants used in traditional medicine. Such research could lead to new drug discovery or advance use of indigenous herbal medicines for treatment. This revival of interest in plant derived drugs is mainly due to the current widespread belief that green medicine is safe and more dependable than the costly synthetic drugs many of which have adverse side effects. [2] Urolithiasis is a complex process that occurs from series of several physicochemical event including supersaturation, nucleation, growth, aggregation and retention within the kidneys. Calcium- containing stones, especially calcium oxalate monohydrate, calcium oxalatedihydrate and basic calcium phosphate are the most commonly occurring ones to an extent of 75-90% followed by magnesium ammonium phosphate (Struvite) to an extent of 10-15%, uric acid 3-10% and cystine 0.5-1%.Patients suffering from diseases like hyperparathyroid-ism, renal tubular acidosis, cystinuria, hypercalcuiuria, hyperoxaluria, crohn’s disease etc. are more prone to stone formation. [3] The pathogenesis of calcium oxalate stone formation is involved nucleation, crystal growth, crystal aggregation, and crystal retention in multistep process. [4] It is widely accepted that many patients become recurrent stone formers. Therefore, the prevention of recurrence (up to 60%) is crucial. Unfortunately, despite considerable progress noted in the field of medical therapy, there is no satisfactory drug to treat urolithiasis. Calcium oxalate is known in three crystalline forms:

- Calcium oxalate monohydrate (whewellite, CaC₂O₄·H₂O) crystallizes in the monoclinic system.

- Calcium oxalate dihydrate The (weddelite, CaC₂O₄·2H₂O) crystallizes in the tetragonal system

- The trihydrate calcium oxalate (CaC₂O₄·3H₂O) crystallizes in the triclinic system. This form is exceptional in urine and rarely encounter. Although CaOx is supersaturated in urine, the formation of renal calculi in healthy people is difficult because of all kinds of inhibitors in urine, including citrate, magnesium, osteopontin, and tyrosine hydroxylase [5]. Chemically speaking, CaOx calculi formation is closely related to with the following factors: high concentration of calcium and oxalate in urine, nucleation, growth and aggregation of CaOx crystals, and adhesion of calcium oxalate monohydrate (COM) to renal tubular epithelial cells. [6] CaOx calculi formation is thought to follow two main routes, (slow) formation of subepithelial plaques at papillary tips and (rapid) formation of intratubular plugs [7]. Struvite is the mineralogical name of Ammonium Magnesium Phosphate Hexahydrate [NH₄MgPO₄·6H₂O] crystals. The general chemical formula for a struvite type compound is X’Y₂’PO₄·nH₂O, where n=6-8. Potassium Magnesium Phosphate Hexahydrate [KMgPO₄·6H₂O], also known asstruvite-K, and sodium magnesium phosphate heptahydrate [NaMgPO₄·7H₂O],or struvite-Na⁺, are new struvite type compounds. Struvite Na⁺ is the sodium analog to struvite in spite of the excess of water molecule. In struvite-Na⁺ the Na⁺ cations replace the NH₄⁺ cations. Struvite has attracted considerable attention due to its common occurrence in a wide variety of environments. Struvite occurs as one of the components of the urinary calculi in humans as well as in animals [8]. Struvite and carbonate apatite are main components of the so-called infectious urinary stones, which are the result of the activity of microorganisms producing urease, mainly frome proteus species, the aggregation of precipitating particles and bacteria is suspected to be one of the primary causes for urinary stone formation. [9] Urine is normally supersaturated with most stone forming salts components, as well as contains chemicals that prevent or inhibit crystal development in urinary tract. However, the presence of certain molecules raise the level of supersaturation of salts needed to initiate crystal nucleation or reduce the rate of crystal growth or aggregation and prevents stone formation calcium oxalate stones represent up to 80% of analyzed stones. [10]. The techniques consist of exploration, in general, to study the appearance of crystals, their importance and their size in a solution which is added the inhibiting substance. The mode of action of inhibitors also often remains hypothetical, but many of them can act on various stages of crystallization (germination, growth, aggregation). Some act chemically competition, others by changing the ionic strength, others
occupant, the surface of the crystal lattice selective sites. It is not necessary to cover the whole of the crystal surface, and some are active not binding on a very small percentage of surface [11]. In Algeria, as in many countries, a large number of patients use medicinal plants to treat their illnesses including urolithiasis. The purpose of this study is to evaluate the efficacy of aqueous extracts of medicinal plants commonly used in Algeria, in the treatment of calcium-oxalate urolithiasis and amonio-magnesium in vitro. The study also covers the action of these extracts on the statements of crystallization (nucleation, crystal growth, crystal aggregation) followed by photograph polarized light microscope. In this context, we are devoted to studying the crystallization steps from calcium oxalate-aqueous solutions and phospho-calcic and supersaturated, maintained at 37 °C to remain close to biological conditions.

2. Materials and Methods

2.1 Synthetic urine

2.1.1. Crystal calcium oxalate

We chose the classical model for the study of oxalate crystallization because of its simplicity and reproducibility. Anti-calcium oxalate crystallisation activities of Algerian plants [12] Synthetic urine supersaturated with calcium oxide was prepared according to a previously described method [12] at a constant temperature of 37°C in capped vessels. Chemicals of reagent-grade purity were dissolved in deionized and redistilled water. The artificial urine was prepared immediately before use by mixing in a T-type mixing chamber. For determination of the effects of plant extracts on crystal formation, preparation of the synthetic urine was performed in their presence at various different concentrations.

Simulation of the sedimentary crystal formation

Mixture agitation was maintained to prevent sedimentation. The crystal size development was monitored by polarized microscopy at different time intervals. Sample drops were examined at every five minutes by polarizing optical microscopy. Crystals were identified using a microscope of the Zeiss type with 40 x magnifying lens, equipped with a WINDER M 476079 camera. Percentage of inhibition of crystallisation (I%) was calculated as previously described [13] and based on the formula, \( I\% = \left( \frac{TSI - TAI}{TSI} \right) \times 100 \), in which TSI and TAI represent number crystals in absence and presence of inhibitors (plant extracts), respectively. Nucleation, growth and aggregation of crystals were visually assessed under the microscope.

2.1.2. Crystal phosphate calcium

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 Na2SO4.10H2O, 1.46 g/l MgSO4.7H2O, 4.64 g/l NH4Cl, 12.13 g/l KCl et 0.24 g/l Ca2+

B : 2.65 g/l NaH2PO4.2H2O, 18.82 g/l Na2HPO4.12H2O, 13.05 g/l NaCl, 1 g/l Na3C6H5O7.2H2O et 0.05 g/l C2O42-. The solution in C2O42- 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.

2.2 Preparation of medicinal plants

2.2.1. For calcium oxalate inhibition:
Extracts plants of wild Algerian plants were studied (Table 1). Different parts of the plants were harvested from natural resources in 2006 and 2007, mainly when plants were at flowering stage. Voucher specimens were deposited in the laboratory of Plant Physiology, Oran University [14]. In some cases, a species was sampled at different times. Infusions were prepared daily just before handling by suspending a weighed amount of dry plant material in boiling tap water at room temperature. The suspension was stored at room temperature for 15 min and then filtered through filter paper.

Table 1 a: Plant materials harvested from the wild in west and south of Algeria

| Plant species (family)                     | Part used               |
|------------------------------------------|-------------------------|
| mixed aerial parts                       | Ajuga iva (Lamiaceae)   |
| fruits                                   | Ammodaucus leucotrichus (Apiaceae) |
| leaves                                   | Atriplex halimus (Chenopodiaceae) |
| flowers & roots                          | Globularia alypum (Globulariaceae) |

2.2.2. For phosphate calcium inhibition:

Table 1 b: Plant materials harvested from the wild in west and south of Algeria

| Plant species (family)                     | Part used |
|------------------------------------------|-----------|
| Acacia Radiana                           | bark      |
| Citrullus Colocynthis                     | sheet     |
| Pistacia lentiscus                       | sheet     |
| warionia saharae                          | sheet     |
| Rhus tripartite                          | sheet     |

3. Results

3.1. Experimental details

3.1.1 After 5 minutes

Table 2. Evolution of the size of oxalate calcium crystals in the presence of extracts plants after 5 minutes

|                  | % Inhib [25%] | % Inhib [50%] | % Inhib [75%] | % Inhib [100%] |
|------------------|---------------|---------------|---------------|---------------|
| Globularia (bark) | 21.20         | 76.57         | 89.00         | 93.58         |
| Globularia (Sheet) | 81.41         | 89.52         | 92.27         | 95.41         |
| Ammodaucus (Sheet) | 80.20         | 88.21         | 92.27         | 95.41         |
| Atriplex halimus  | 56.80         | 66.49         | 83.76         | 92.80         |
| Ajuga iva        | 84.03         | 89.92         | 92.80         | 89.79         |
3.1.2 After 30 minutes

Table 3. Evolution of the size of oxalate calcium crystals in the presence of extracts plants after 30 minutes

| Plants          | % Inhib [25%] | % Inhib [50%] | % Inhib [75%] | % Inhib [100%] |
|-----------------|---------------|---------------|---------------|---------------|
| Globularia (bark) | 75.53         | 90.45         | 89.73         | 96.09         |
| Globularia (Sheet) | 88.78         | 95.58         | 96.77         | 95.22         |
| Ammodaucus      | 94.98         | 96.87         | 97.25         | 97.85         |
| Atriplex halimus | 63.12         | 67.18         | 91.64         | 89.26         |
| Ajuga iva       | 89.97         | 95.22         | 97.01         | 96.06         |

3.2. Inhibition of calcium phosphate:

3.2.1 After 5 minutes

Table 4. Evolution of the size of struvite crystals in the presence of extracts plants after 5 minutes

| Temps | Evolution of the size of struvite crystals and 1% inhibition | Plants            |
|-------|-------------------------------------------------------------|-------------------|
|       | SI 1ml 1% 5ml 1% 10ml 1% 15ml 1% 20ml 1%                   |                   |
| 5     | 30 16 46.7 16 46.7 28 6.7 28 6.7 16 46.7 Acacia Radiana   |                   |
| 5     | 30 16 46.7 12 60 16 46.9 16 46.7 12 60 Citrullus Colocynt |                   |
| 5     | 30 24 20 16 46.7 16 46.7 24 20 16 46.7 Pistacia lentiscus |                   |
| 5     | 30 20 33.4 20 33.4 G - 20 33.4 20 33.4 warionia saharae    |                   |
| 5     | 30 24 20 20 33.4 28 6.7 24 20 32 Rhus tripartita         |                   |
3.2.2. After 30 minutes:

Table 5. Evolution of the size of struvite crystals in the presence of extracts plants after 30 minutes

| Temps |  | Evolution of the size of struvite crystals and 1% inhibition |  | Plants |
|-------|---|----------------------------------------------------------|--|--------|
|       | SI |  | 1ml | 5ml | 10ml | 15ml | 20ml | 28.9 | 12 | 73 |
| 30    | 45 | 28 | 37.8 | 24 | 46.7 | 40 | 11 | 32 | 28.9 | 12 | 73 |
|       | 30 | 45 | 32 | 28.9 | 28 | 37.8 | 40 | 11 | 32 | 28.9 | 32 | 28.9 |
|       | 30 | 45 | 28 | 37.8 | 20 | 55.5 | 20 | 55.5 | 28 | 33.8 | 20 | 55.5 |
|       | 30 | 45 | 28 | 37.8 | 20 | 55.5 | 28 | 37.8 | 20 | 55.5 | 32 | 28.9 |
|       | 30 | 45 | 28 | 37.8 | 24 | 46.7 | 32 | 28.9 | 20 | 55.5 | 20 | 55.5 |
|       | 30 | 45 | 32 | 28.9 | 28 | 37.8 | 40 | 11 | 32 | 28.9 | 32 | 28.9 |
|       | 30 | 45 | 32 | 28.9 | 28 | 37.8 | 40 | 11 | 32 | 28.9 | 32 | 28.9 |
|       | 30 | 45 | 32 | 28.9 | 28 | 37.8 | 40 | 11 | 32 | 28.9 | 32 | 28.9 |
|       | 30 | 45 | 32 | 28.9 | 28 | 37.8 | 40 | 11 | 32 | 28.9 | 32 | 28.9 |
|       | 30 | 45 | 32 | 28.9 | 28 | 37.8 | 40 | 11 | 32 | 28.9 | 32 | 28.9 |

4. Discussion

The work provides an update on therapy approaches needed depending on the damage type of the urinary tract and the diversity of medicinal plants answered in nature. Many of these plants are active human specialties. Most plant extracts used in our work has proved effective in tests on calcium oxalate inhibition majority component in urinary stones. In this study extracts: *Ammodaucus leucotrichus, Ajuga iva, Atriplex halimus, Globularia alypum* (roots), *Globularia alypum* (flowers), were presented to inhibit the formation of crystals of calcium oxalate monohydrate (Table 2 and 3). From extracts of plants tested, demonstrated potent inhibition higher and therefore the greatest potential Antilithic 97.85 % after 30 minutes *Ammodaucus leucotrichus* is known in Algeria as "Kammun Sofi-es" or "Hairy Cumin" also showed strong inhibition calcium oxalate crystallization.

The Marrubium deserti and Ammodaucus leucotrichus L. an Algerian endemic species, has several applications in traditional medicine for example as a remedy for asthma and diabetes, and was found to have antibacterial properties. In this work, an antioxidant and antimicrobial activities was performed on phenolic extracts of Marrubium deserti, *Ammodaucus leucotrichus* plants,[15]. Calcium interferes with the crystallization of struvite by blocking active growth sites and competing for phosphate to form calcium phosphate. As in the case of urinary calculi associated with metabolic disorders, the formation of infectious urolithiasis is a result of many physical and chemical processes. Environment of urine is just as important as bacterial factors in the development of the urolithiasis development, which emphasizes the importance of proper diet in the therapy. This diet should not only involve the reduction of Ca2+, Mg2+ and phosphate. Previously, it was shown that the intensity of struvite crystallization is positively correlated with an increase in the pH level.[16]. Behind this second experiment the idea was to know the role of plant extract in dissolving the already formed stones nucleus in renal system. For this artificial calcium oxalate crystal were prepare in the laboratory by standard method.[17] The first step in the formation of urinary stones is the nucleation of urinary minerals from supersaturated urine. The formed nucleus (generally less than 10 nm) grows and/or
aggregates to a pathological size (several tens of microns). These crystallites are then retained in the urinary tract or fixed by urinary tract organization, forming urinary stones (millimeters to several centimeters) [18]. *Ammodaucus leucotrichus* was found to potently inhibit nucleation, growth and aggregation. The results for the inhibitory effect of extracts of plants on calcium phosphate crystallization as given in Tables 4 and 5 are identical to the work Chauhan and all have shown that the decrease in size of struvite crystals in the presence of an extract diffusa plant Boerhaavia 13.33 and 30.37% inhibition for 05 and 1% concentration of extract [19]. Our results also with all the plants we studied expresses this inhibition on the sizes of the crystals, for example: the effect of the bark of the *acacia radiana* 60% from its value without inhibitor. *Pistacia lentiscu* a decrease of 46% from its value without inhibitor. Kidney stones are hard, solid particles that form in the urinary tract. In many cases, the stones are very small and can pass out of the body without any problems. However, if a stone (even a small one) blocks the flow of urine, excruciating pain may result, and prompt medical treatment may be needed.. [20]. Medicinal plants have played as significant role in various ancient traditional system of medication. Even today, plants provide a cheap source of drugs for majority of world’s population. Several pharmacological investigations on the medicinal plants used in traditional antiurolithiatic therapy have revealed their therapeutic potential in the *in-vitro* or *in-vivo* models.[21]. The same procedure was followed as an example: the Rustipentila plant, has an inhibitory effect only on the size of the crystals, and aggregates. The addition of 10 mL of plant volume provides an inhibition of 28.9% compared to its value without inhibitor. Moreover the minimum crystal size after 4 hours at 15 mL reaches 20 microns or a percentage inhibition of 55.5%. The same parameters (size and number of crystals, the crystallization time) were also studied. Medicinal plants are able to significantly reduce the phosphate crystallization in vitro, can be clearly seen by optical microscope that aqueous extracts of: *Acacia radiana*, *Citrullus colocynthis*, *Rhus tripartita*, *Pistacia lentiscu*, *Warionia saharae*, produce a significant amount of growth inhibition phosphate crystals. This in vitro study provides useful information for the in vivo studies.

**Conclusion**

The result of our work does not allow us to confirm the use of these plants in the field of urolithiasis. However, all our extracts gave an inhibitory activity with the aqueous decoction. These activities may help to strengthen the body in depressed situations. In addition, a detailed study of the toxicity would be necessary to achieve determine doses. The plant extracts are generally used in the rough. This is why we utilize high doses for our tests. To overcome this, it is best to isolate the active principles of different plant extracts and present them in an acceptable dosage form. Awareness is still needed on the appropriate use of traditional medicine and the importance of the environment. Through this work, we hope to bring our modest contribution to the promotion of traditional medicine to reach place at the disposal of the population based drugs effective medicinal plants and accessible.

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