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Effects of *Cynodon dactylon* rhizomes and *Erica multiflora* flowers aqueous extracts on calcium oxalate crystallization *in vitro*

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Abstract

In Morocco, *Cynodon dactylon* L. and *Erica multiflora* L. are commonly used as phytotherapeutic agents in folk medicine for the treatment of urolithiasis. The aim of the study is to evaluate the effectiveness of their aqueous extracts on calcium oxalate (CaOx) crystallization *in vitro*. Crystallization was induced in human urine samples by adding sodium oxalate in the absence or presence of the extracts at different concentrations. The nature and the frequency of crystals were analyzed by plain and polarized light microscopy. The nucleation and aggregation of CaOx crystals were measured separately using turbidimetric spectrophotometry methods. Induction time and turbidity slope were respectively compared in the presence of the extract with that of the control. Both plant extracts promoted the formation of CaOx dihydrate rather than monohydrate crystals in urine in a dose manner. In nucleation assay, the induction time decreased in the presence of both plants extracts when compared with that of control. In aggregation assay, *Cynodon dactylon* inhibited efficiently CaOx aggregation. In contrary, we did not observe an important inhibitory effect with *Erica multiflora* extract. These data indicate that extract obtained from *Cynodon dactylon* may contain substances that promote the formation of small CaOx dihydrate crystals and inhibited aggregation. *Cynodon dactylon* aqueous extract might be beneficial for the prevention of kidney stones. However, the effect of *Erica multiflora* extract need to be further studied in order to find a possible action on other lithiasis types.

Keywords: Nephrolithiasis, Kidney stones, Urine, Medicinal plants.

Introduction

About 70-80% of kidney stones are composed of calcium usually combined with oxalate or phosphate (Coe *et al.*, 2005; Moe, 2006). Calcium oxalate (CaOx) crystallizes in three different hydrated forms, calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD) and calcium oxalate trihydrate (COT). COM and COD crystals are often found to be the constituent of urinary calculi (Pierratos *et al.*, 1994). The formation of such concretions involves several physicochemical events, e.g. nucleation, growth and aggregation, but the mechanism of these processes remains incompletely understood. Urolithiasis is largely a recurrent disease with substantial economic consequences and a great public health importance (Romero *et al.*, 2010; Knoll *et al.*, 2011). Various medical therapies are proposed but their efficacies are less convincing (Sakhaee *et al.*, 2012; Eisner *et al.*, 2013). Hence, phytotherapy with medicinal plants is widely used worldwide as an alternative primary healthcare. Indeed, there is a resurgence of using medicinal plants for the treatment of several pathologies including urinary kidney stones (Atmani, 2003; Butterweck & Khan, 2009; Miyaoka & Monga, 2009). As far as nephrolithiasis is concerned, in Morocco, there are many herbal plants that act as antilithiasis agents notably *Herniaria hirsuta* (Atmani & Khan, 2000; Atmani *et*

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al., 2003, 2004), Citrus juice (Touhami *et al.*, 2005) and others (Bellakhdar, 1997; Atmani, 2003). Among these plants, *Cynodon dactylon* (*C. dactylon*) and *Erica multiflora* (*E. multiflora*) are commonly used in Moroccan traditional medicine to treat kidney stones and as diuretic plants (Bellakhdar *et al.*, 1991; Bellakhdar, 1997). *C. dactylon* has been reported to have also antidiabetic (Singh *et al.*, 2007; Karthik & Ravikumar, 2011), antioxidant (Auddy *et al.*, 2003), anti-inflammatory (Sindhu *et al.*, 2009) as well as diuretic properties (Sadki *et al.*, 2010). Regarding *E. multiflora*, some studies have demonstrated that many species have hyperlipidaemic (Harnafi *et al.*, 2007), anti-inflammatory and antinociceptive (Akkol *et al.*, 2008), antiviral (Sassi *et al.*, 2008) as well as diuretic activity (Sadki *et al.*, 2010). Several phytochemical constituents seem to be responsible for their biological activities (Bruneton 1987; Garjani *et al.*, 2009; Karthik & Ravikumar, 2011). In the present study, the effect of the plant extracts obtained separately from *C. dactylon* and *E. multiflora* have been studied to establish the scientific validity for their antilithiasic property on calcium oxalate (CaOx) crystallization *in vitro*.

Materials and methods

Plant materials and preparation of extracts

Erica multiflora L. (*E. multiflora*) was collected in Tafoghalt, a city in the East of Morocco. A voucher specimen (13009) was deposited at the Agronomic and Veterinary Institute of Hassan II in Rabat city, Morocco. *Cynodon dactylon* L. Pers (*C. dactylon*) was collected in Oujda city in Morocco. A voucher specimen (76267) was deposited at the Scientific Institute in Rabat city, Morocco.

The extracts were prepared in a similar manner to that used by patients with some modifications. Indeed, 10 g of *E. multiflora* plant were extracted by boiling in distilled water during 10 min. For *C. dactylon*, 10 g were boiled during 2 min and this first extract was discarded then boiled again for a further 20 min. Both extracts were filtered to remove any plant particles and then dried at 45°C for overnight. The yield was about 18 and 6% respectively. The obtained powders were separately used to prepare solutions at different concentrations in distilled water.

Crystallization assay in whole urine

A 24 hours urine samples from normal subject with no history of urolithiasis were collected in the presence of some thymol crystals to prevent microbial growth. Aliquots of 2 mL of urine were added into tubes and allowed to warm up to 37°C. After that, 50 µL of each extract solution at different concentrations were added into the tubes, to make a final concentration from 0.125 to 2 mg/mL. Some tubes with no extract added were served as controls. Calcium oxalate (CaOx) crystallization was initiated in each urine sample by adding 50 µL of 0.1 M sodium oxalate solution. Tubes were incubated in water bath at 37°C for 30 min. Finally, the content of each tube was read at 620 nm and examined for crystal identification with plain and polarized light microscopy.

Nucleation assay

The method used was similar to that described previously (Hennequin et al., 1993). Solutions of sodium oxalate and calcium chloride were prepared at final concentrations of 0.5 and 3 mM respectively in a solution containing sodium acetate 10 mM and sodium chloride 0.15 M at pH of 6.5. Both solutions were filtered through 0.22 µm filter. To 950 µl calcium chloride solution, 100 µl of different concentrations of plant extract were admixed. The crystallization was obtained by the mixture of 950 µl sodium oxalate solution. The reaction was maintained under agitation at 700 rpm. OD was monitored at 620 nm every 12 seconds

during 20 min. The percentage of inhibition produced by the plant extract is calculated by the following formula:

(Ti - T0 / T0) × 100. Where Ti: induction time in the presence of inhibitor (extract) and T0: induction time in control. The average of the values is obtained after three tests.

Aggregation assay

The method used was similar to that described previously (Hess *et al.*, 1989) with some minor differences. COM crystals were used at a final concentration of 0.8 mg/mL, containing 10 mM sodium acetate and 0.15 M sodium chloride and the pH was adjusted to 6.5. Two ml of crystal solutions were put in cuvette and OD was followed at 620 nm after stopping stirring, either in the absence or the presence of different concentrations of the extract at 37°C. Experiments were run in triplicate. The rate of aggregation was estimated by comparing the turbidity slope in the presence of the extract with that of control. The percentage of inhibition produced by the addition of extract is calculated by the following formula: (S_i - S_0/ S_0) × 100. Where S_i: Turbidity slope in the presence of extract and S_0: Turbidity slope in control.

Statistical analysis

The data are expressed as mean ± standard error of mean (SEM). Student's t-test was used for statistical comparison of data. The *p* values less than 0.05 are considered significant.

Results

The extracts obtained from *C. dactylon* rhizomes *and E. multiflora* flowers were tested separately at different doses on CaOx crystallization in whole normal human urine (Figure 1). The addition of *C. dactylon* extract increased absorbance which became significantly higher as compared to the control tubes. There was an increase of about 44, 52, 65, 74, and 80% for 0.125, 0.25, 0.5, 1, and 2 mg/mL respectively. In contrary, the OD was irregular with *E. multiflora* aqueous extract and the increased was only between 18 and 32% at the same concentrations respectively. The examination of crystals by light microscopy showed that increasing extract doses produced a higher density of CaOx crystals and their number increased (Figure 2 and 3). These octahedral crystals have the morphological characteristic of COD crystals. In the nucleation assay (Figure 4), the results show that the extract of both plants promoted the nucleation of CaOx particles especially at the concentration exceeding 0.125 mg/ml (promotion is expressed as negative values).

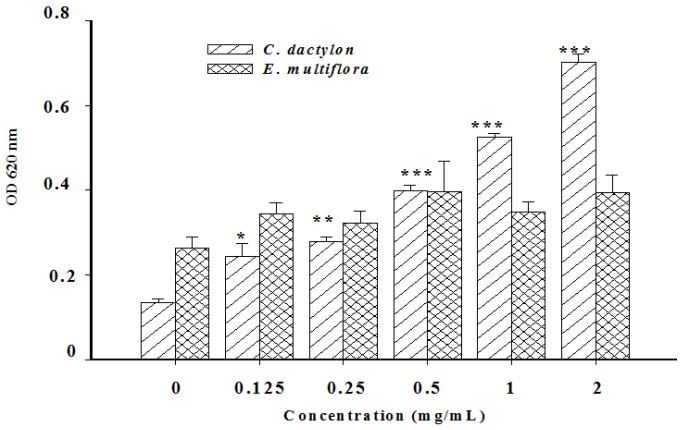

Figure 1. Evolution of the turbidity in function of different amounts of *Cynodon dactylon* rhizomes and *Erica multiflora* flowers extracts on calcium oxalate crystallization in whole normal human urine. Values are reported as mean ± SEM.*P< 0.05; **P<0.01; ***P<0.001 compared with controls using student's t-test.

In the aggregation assay, turbidity slope of CaOx crystals is related to the concentration of extracts. The response is dose-dependent showing that crystals were less aggregated (Figure 5). The inhibiting aggregation of *C. dactylon* reaches 85% at concentration of 0.25 mg/mL. For *E. multiflora*,

the inhibiting effect on the aggregation was observed only when doses from 0.0625 to 0.25 mg/mL were used with a maximum not exceeding 50% (Figure 5).

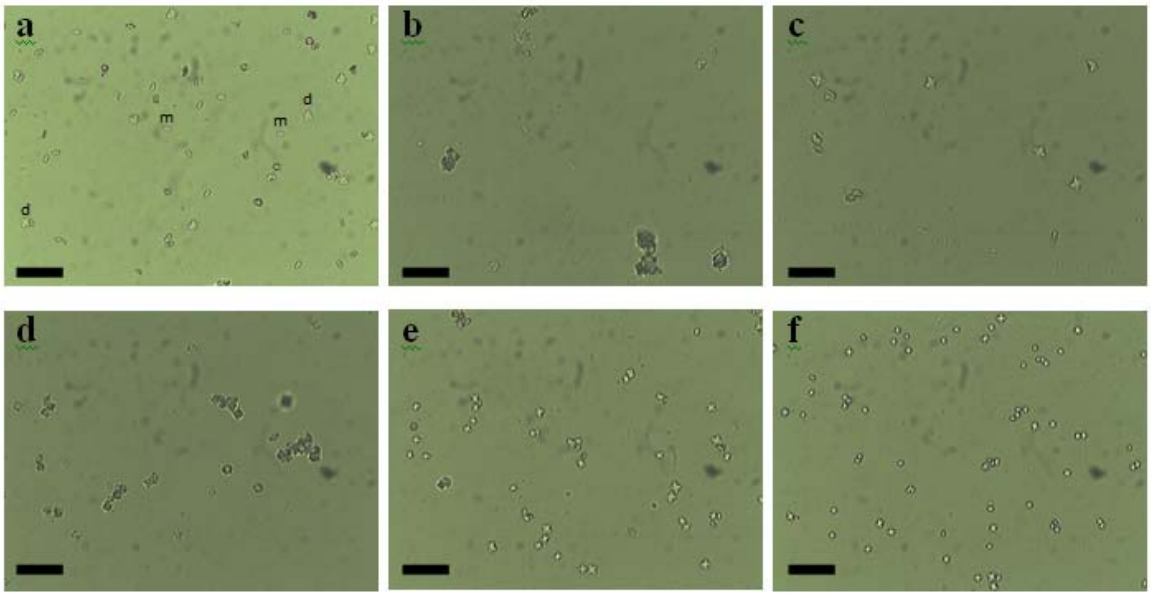

Figure 2. Light microscopy analysis of CaOx crystals induced in whole normal human urine in absence of extract (a) and in presence of 0.125 (b); 0.25 (c); 0.5 (d); 1 (e) and 2 mg/mL (f) of *Cynodon dactylon* rhizomes extract. Bar: 20 µm. (d: dihydrated CaOx crystals. m: monohydrated CaOx crystals).

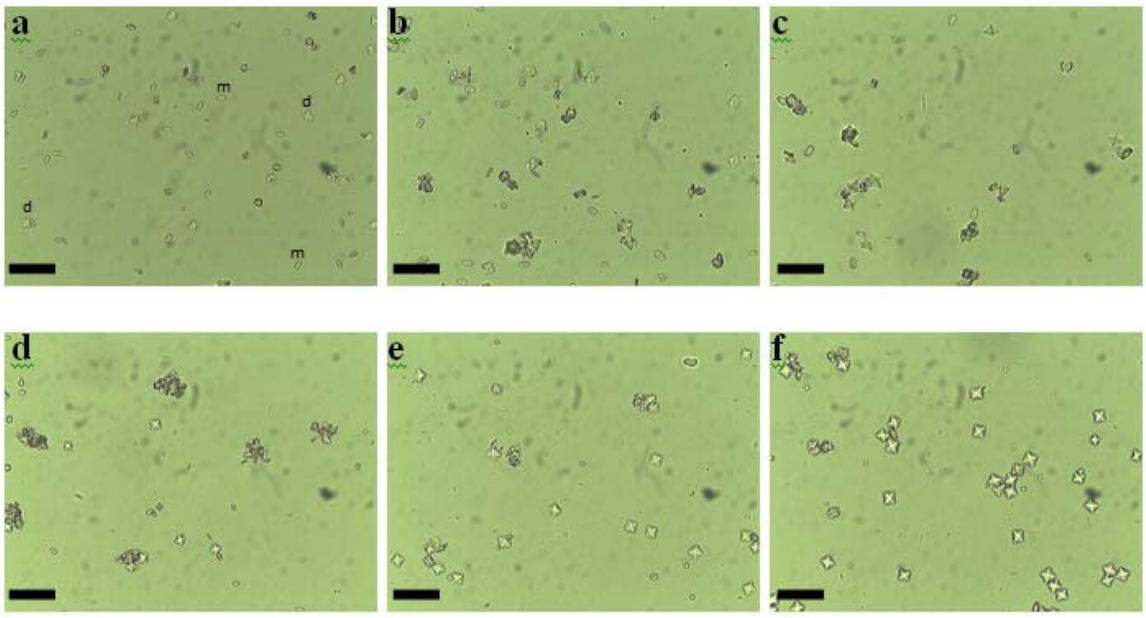

Figure 3. Light microscopy analysis of CaOx crystals induced in whole normal human urine in absence of extract (a) and in presence of 0.125 (b); 0.25 (c); 0.5 (d); 1 (e) and 2 mg/mL (f) of *Erica multiflora* flowers extract. Bar: 20 µm. (d: dihydrated CaOx crystals. m: monohydrated CaOx crystals).

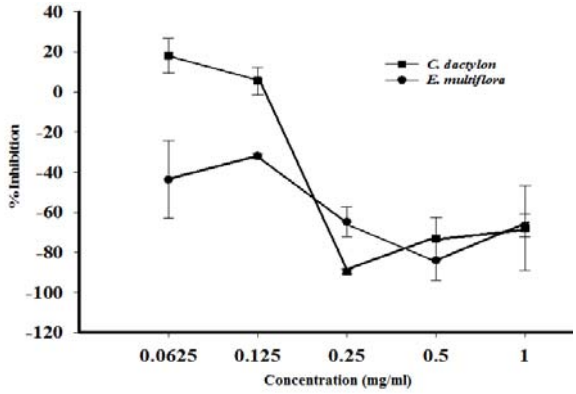

Figure 4. Evolution of inhibition percentage of *Cynodon dactylon* rhizomes and *Erica multiflora* flowers extracts at various concentrations on CaOx crystal nucleation.

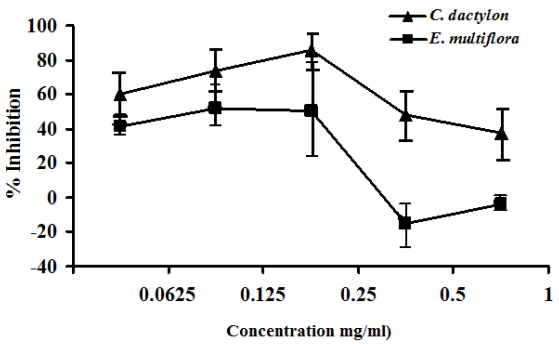

Figure 5. Evolution of inhibition percentage of *Cynodon dactylon* rhizomes and *Erica multiflora* flowers extracts at various concentrations on CaOx crystal aggregation.

Discussion

In our present work, *C. dactylon* and *E. multiflora* extracts were studied to evaluate their antiurolithiasic potential using *in vitro* models. The main findings of the present study were that extracts obtained from *C. dactylon* and *E. multiflora* promoted the crystallization of CaOx in whole urine (Figure 1), similarly to the results obtained with *Herniaria hirsuta,* a well studied plant on CaOx crystallization[11] (Atmani & Khan, 2000). The addition of *C. dactylon* extract increased OD which became significantly higher than in the control tubes. The increase of the turbidity is mainly due to an increase in the particles number with increasing concentrations of extract.

It is well established that, for crystals formation, urine must be supersaturated with respect to the stone forming minerals, especially to calcium oxalate, at a level that exceeds their solubility. Thus, one approach to prevent CaOx stone formation is to stop or at least lower urinary supersaturation (Khan, 2006). For this point, our result seems to be controversial since *E. multiflora*, and especially *C. dactylon* extract, promoted crystal formation rather than inhibiting it. As a matter of fact, crystalluria may occur similarly in both healthy and stone forming subjects where the latter tend to excrete and eliminate larger and aggregated particles than the former ones. The limiting factors in kidney stone formation could be those processes that modulate the size of the particles.

We believe that this phenomenon could be advantageous in preventing urinary stones formation by excreting and eliminating small particles from urinary tract and reducing thereby their chance to be retained. Furthermore, as we demonstrated in our earlier study, both plant extracts have diuretic activity which helps to reduce urinary supersaturation and quickly eliminates crystals out off urinary tract (Sadki *et al.*, 2010).

Another finding of this study is that COD crystals are largely predominating in treated urine, while COM crystals prevail in untreated urine (Figure 2 and 3). Moreover, several micromolecular inhibitors such as citrate, pyrophosphate and magnesium facilitate the formation and the stability of COD crystals (Berg *et al.*, 1976; Ackermann *et al.*, 1989; Yuzawa *et al.*, 1998).

The extracts of *C. dactylon* and *E. multiflora* might have favored the formation of COD crystals and play the same role as low molecular inhibitors. Crystals are not stones, while the formation of calculi must involve crystallization, this is not sufficient to account for stone formation as is common in both healthy

subjects and stone former ones (Werness *et al.*, 1981). COM crystals are more commonly found in stones, while COD particles are frequently observed in asymptomatic crystalluria (Robertson *et al.*, 1969). Accordingly, the aqueous extract might contain substances that promote nucleation of small COD crystals and inhibits significantly the formation of COM particles. These results are interesting since the formation of COD particles in preference to COM crystals is advantageous because it protects against stone disease by reducing the attachment of crystals to renal tubule cells (Wesson *et al.*, 1998). Indeed, in their later work, Wesson and colleagues demonstrated that COM crystals have a greater affinity to adhere onto renal epithelial cells and the presence of urinary macromolecules inhibitors favored the formation of COD particles rather than COM counterparts. Similarly, the presence of extract, especially the one obtained from *C. dactylon*, inhibited the formation of COM crystals and favored COD once contributing, therefore, to the reduction of crystal cell adhesion.

In the nucleation assay (Figure 4), the results showed that the extract of both plants promoted the nucleation of CaOx particles especially at the concentration exceeding 0.125 mg/mL lowering therefore the supersaturation. In this study, more importantly, *C. dactylon* inhibited CaOx crystal aggregation (Figure 5) similarly as citrate, a well known inhibitor of aggregation (Grases *et al.*, 1989). Citrate principals' effect is based on its capacity to complex calcium and to lower the quantity of calcium available to react with oxalate or phosphate and consequently leads to a reduction in urinary supersaturation. Crystal agglomeration has been recognized for long as the most important process leading to crystal retention with reduced ability of urine from patients with recurrent calcium stones to inhibit crystal aggregation than non stone formers (Robertson *et al.*, 1969; Finlayson & Reid, 1978). The results obtained in the present study suggest that *C. dactylon* inhibited the aggregation of CaOx crystal. Furthermore, the rate of aggregation is governed by the chance of crystals meeting each other, so the more crystals there are, the more likely is aggregation. As stated by Hess and Kok, the aggregation of crystals in solution is an extremely complex phenomenon governed by numerous interacting chemical-physical factors (Hess & Kok, 1996). We believe that *C. dactylon* extract may contain substances that inhibit the growth of COM crystals and inhibit CaOx crystal aggregation but we did not observe an important inhibitory effect of *E. multiflora* since the aqueous extracts are very complex and contain a multitude of compounds, they may act in different ways. Therefore, there is a possible therapeutic strategy for the prevention of recurrent stone disease by using medicinal plants including *C. dactylon.* These *in vitro* results are recently confirmed by an *in vivo* study using *C. dactylon* (Atmani *et al.*, 2009).

It is agreed that one or several constituents of the extract obtained from both plants may be responsible for their biological activities. Based on our current results (Data not shown), bioactive compounds contained in both extract have a polar property since they are soluble in water. In this regard, phytochemical analysis of hydroalcoholic extract obtained from *C. dactylon* rhizomes has shown the presence of sugars, flavonoids, sterols and steroid saponins (Garjani *et al.*, 2009). According to the work of Bruneto (Bruneton, 1987), it seems that *E. multiflora* plant is rich in tannins, proanthocyanidins and flavonoids substances that may account for its biological activity.

The composition and the nature of substances contained in the aqueous extract of both plants are actually under investigation in our laboratory. We are aiming to study their effects on the treatment of kidney stone formation. The

observed effect with *E. multiflora* extract could be explained by a possible action on the other lithiasis type. This possibility will be carried out in the future.

Conclusion

The results showed that extract obtained from *Cynodon dactylon* may contain substances that promoted the formation of small calcium oxalate dihydrate crystals and inhibited aggregation. *Cynodon dactylon* aqueous extract might be beneficial for the prevention of kidney stones. However, the effect of *Erica multiflora* extract was less efficient than *C. Dactylon* and should be further studied in order to find a possible action on other lithiasis types.

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