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ORIGINAL PAPER

Cynodon dactylon extract as a preventive and curative agent in experimentally induced nephrolithiasis

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Abstract *Cynodon dactylon* (Poaceae family) decoction was used in the treatment of kidney stones. However, no scientific study was undertaken so far to demonstrate the beneficial effect of the plant. Thus, the aim of the current study is to evaluate the effect of *Cynodon* aqueous extract as a preventive and curative agent in experimentally induced nephrolithiasis in a rat model. Ethylene glycol (EG) was used in the experiment to induce calcium oxalate (CaOx) deposition into kidneys. In preventive protocol, *Cynodon* decoction was administered in the same day with EG to evaluate the ability of the extract to prevent crystal deposition. However, in curative protocol, rats were first rendered nephrolithiasic and then the extract was administered to assess the ability of the plant to eliminate the pre-existing crystal deposition. In both protocols, urinary biochemical and other variables were measured during the course of the study. Crystalluria and renal histology were examined as well. The results showed that, in both protocols, all measured variables were similar for both the rat groups. Nevertheless, urinary biochemical analysis was apparently unaffected by the extract except oxalate in preventive protocol, and calcium, sodium, and potassium in curative protocol which were significantly highly excreted in treated rats compared to untreated animals. Crystalluria was characterized mostly by the presence of large quantities of CaOx monohydrate and CaOx dihydrate particles in untreated rats. However, crystalluria was mainly dominated by the presence of CaOx dihydrate particles with reduced size. The most apparent beneficial effect of *Cynodon* extract was seen in kidney tissues where reduced levels of CaOx deposition have been noticed especially in medullary and papillary sections from treated rats. We concluded that *C. dactylon* extract has beneficial effect in preventing and eliminating CaOx deposition into kidneys. Such findings provide a scientific explanation for its use in the treatment of kidneys stones.

Keywords Nephrolithiasis · Calcium oxalate · Crystallization · *Cynodon dactylon* · Rats

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Introduction

The worldwide incidence of urolithiasis is quite high and calcium oxalate (CaOx) constitutes the main compound found in analyzed urinary calculi in industrialized countries [1–5]. Remarkable advances have been made in the management of urolithiasis following the introduction of several techniques including extracorporeal lithotripsy and nephrolithotomy. However, effective protocols to prevent stone recurrence are far to be satisfied. Thus, it appears useful to look for new prophylactic measures to be used either alone or in combination with already existing methods. In this regard, phytotherapy is currently gaining space in the field of the management of urolithiasis providing, therefore, a vast scope for successful treatment [6, 7]. Actually,

medicinal plants are world widely used and we are seeing an increase of interest in studying them in order to provide a scientific explanation for their beneficial effect [8–13]. *Cynodon dactylon*, a largely distributed medicinal plant, is used in Morocco in the treatment of kidney stones and as a diuretic [14–16]. However, to the best of our knowledge, no study was undertaken to show evidence for the direct action of the plant in the treatment of kidney stones. Thus, the purpose of our current study is to assess the beneficial effect of *C. dactylon* extract as a preventive and curative agent in experimentally induced nephrolithiasis in rats.

Materials and methods

Preparation of extract

Rhizomes of *C. dactylon* (Poaceae family) were bought from a local herbalist and graciously identified by a botanist in our department (Professor A. Berrichi, Department of Biology, Faculty of Sciences, Oujda, Morocco). A voucher specimen (76267) was deposited at the Rabat Scientific Institute, Rabat, Morocco. A decoction extract was prepared from the rhizomes following the method used by patients with minor modifications. Briefly, samples (18 g in 400 ml) were boiled in distilled water for 2 min and the liquid containing the first extract was discarded. The remaining of the plant was then boiled again for 20 min. The second extract was filtered and evaporated to dryness. The powder was reconstituted with distilled water at a concentration of 50 mg/ml. The extraction yield was about 2%. The extract is kept in refrigerator during the experiment.

Experimental protocol and samples collection

To assess the effectiveness of *C. dactylon* extract, two protocols, preventive and curative protocols, were designed using male Wistar rats. The experiments were conducted in accordance to internationally accepted standard procedure for animal use.

For the preventive protocol, the plant extract was studied to evaluate its ability to prevent the deposition of CaOx crystals into kidneys and their development to calculi. Thus, rats, weighing about 210 g, were housed individually in metabolic cages, fed regular chow and had free access to tap water ad libitum. They were then divided into two groups of six rats each and received ethylene glycol (EG) 0.75% in their drinking water for 3 weeks. During the experiment, one group received daily 1 ml of *C. dactylon* extract (50 mg/ml) by gavage and was used as treated group, while the other group received 1 ml of distilled water instead and used as control group. Twenty-four hour urine samples were collected for both groups 1 day before EG administration, and then on days 1, 7, 14, and 21 of treatment in presence of thymol crystals as antibacterial agent.

For the curative protocol, the plant extract was evaluated for its ability to eliminate the pre-existing CaOx deposition in kidneys. In this case, rats were first given 0.75% EG and 1% ammonium chloride for 3 days and then switched to EG alone until they reached 3 weeks. Then, rats were divided into two groups of six. Remaining on EG 0.75%, one group was given 1 ml distilled water while the other group was given 1 ml of the plant extract for 2 weeks. Urine samples were collected individually 1 day before starting the treatment and on days 1, 3, 7 and 14 of the treatment in presence of thymol crystals.

It is important to point out that we conducted a preliminary study to determine the effective dose of *Cynodon* extract. Three doses (125, 250 and 500 mg/kg of body weight) were used based on the information collected from several herbalists which were recommended for patients. The results obtained for the dose of 125 mg/kg showed little effect on CaOx deposition into kidneys, while 250 and 500 mg/kg doses were both effective. Therefore, the 250 mg/kg dose was chosen in our study.

Measurement of variables

All rats were weighted before each urine collection and placed individually in metabolic cages to determine water intake, urinary volume, pH, oxalate, calcium, magnesium [9] as well as sodium and potassium levels (Atomic Absorption Spectrophotometer, Varian).

Table 1 Body weight, water intake, urinary volume and pH from untreated and treated rats following a preventive protocol

Groups	Body weight (g)		Water intake (ml)		Urinary volume (ml)		Urinary pH	
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
Day 0	212.1 ± 8.7	216.0 ± 11.2	25.5 ± 2.6	23.0 ± 2.6	9.5 ± 1.7	11.3 ± 1.9	7.99 ± 0.01	7.83 ± 0.08
Day 1	225.4 ± 9.4	224.0 ± 12.3	9.3 ± 1.1	10.0 ± 1.3	11.7 ± 1.3	12.0 ± 1.8	6.97 ± 0.13	6.84 ± 0.15
Day 7	227.9 ± 10.6	223.3 ± 13.5	13.8 ± 2.0	17.3 ± 3.2	17.8 ± 1.4	19.5 ± 1.6	5.99 ± 0.12	5.99 ± 0.16
Day 14	230.6 ± 10.1	216.3 ± 11.7	16.8 ± 3.0	24.8 ± 4.5	21.7 ± 3.0	26.8 ± 3.3	5.80 ± 0.07	5.94 ± 0.14
Day 21	208.4 ± 13.8	213.0 ± 13.5	31.7 ± 3.8	28.2 ± 5.6	27.2 ± 2.4	25.0 ± 4.2	5.81 ± 0.08	5.95 ± 0.18

Crystalluria analysis

Crystal habit and size of each urine specimen were examined at room temperature by plain and polarized light microscopy. Twenty-four hour urine samples obtained from all rats were first mixed well and then aliquots were withdrawn and put on Malassez cell. Two different types of CaOx crystals over 2 μm were taken into our consideration: CaOx monohydrate (COM) and CaOx dihydrate (COD). A semi-quantitative analysis was performed using a score from + to ++++ according to the number of crystals counted.

Light microscopy examination of rat kidneys

At the end of the experimental study, all animals were killed by aortic puncture after anesthesia. Systematically, kidneys from each rat were harvested and weighted. The median section of the kidneys was then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer saline, dehydrated in a gradient of ethanol, embedded in paraffin and cut into 5–7 μm serial sections. Ten slides containing five sections from each kidney were deparaffinized, stained with hematoxylin and eosin and examined by microscope under plain and polarized light.

Statistical analysis and expression of results

Student's *t* test was used for statistical comparison of data between groups. *P* values less than 0.05 were considered significant. Results are presented as means ± standard errors (SE).

Results

Preventive protocol

The values reported in Table 1 showed that there is no difference between the two groups of animals in terms of body weight, water intake and urinary volume from both untreated and treated groups. Urinary pH decreased similarly in both groups during the experiment (Table 1).

The excretion levels of oxalate were only higher in treated group on day 21 ($P = 0.0171$) (Table 2). For calcium, magnesium, sodium and potassium excretion levels, no differences were observed (Table 2).

For crystalluria, before EG administration, the main crystals observed were struvite. However, after EG administration, COM and COD particles were found in the urine of both groups of rats. During the experiment, we noticed that COD particles were more excreted in treated animals while COM crystals were remained stable (Fig. 1a, b).

Table 2 Urinary parameters from untreated and treated rats following a preventive protocol

Groups	Oxalate (mg/24 h)		Calcium (mg/24 h)		Magnesium (mg/24 h)		Sodium (mg/24 h)		Potassium (mg/24 h)	
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
Day 0	0.29 ± 0.05	0.29 ± 0.03	3.31 ± 0.55	2.82 ± 0.33	14.06 ± 0.89	13.51 ± 1.64	36.98 ± 5.65	32.82 ± 1.85	211.58 ± 18.67	200.38 ± 42.46
Day 1	0.15 ± 0.02	0.19 ± 0.03	7.07 ± 2.07	5.14 ± 1.15	16.48 ± 5.20	9.77 ± 1.23	12.43 ± 2.16	10.04 ± 3.15	67.51 ± 5.38	55.83 ± 5.82
Day 7	0.32 ± 0.03	0.35 ± 0.02	14.60 ± 1.23	11.91 ± 2.96	12.14 ± 1.47	12.52 ± 2.40	19.93 ± 3.05	13.45 ± 3.15	81.55 ± 8.70	57.57 ± 9.47
Day 14	0.38 ± 0.03	0.45 ± 0.07	11.11 ± 4.25	12.18 ± 3.51	20.64 ± 2.95	13.54 ± 2.26	16.24 ± 2.67	10.10 ± 2.91	97.23 ± 18.04	85.52 ± 17.03
Day 21	0.55 ± 0.07	1.26 ± 0.05 ($P = 0.0171$)	11.35 ± 3.59	8.38 ± 1.33	17.69 ± 3.28	20.69 ± 3.25	8.94 ± 2.48	12.31 ± 1.09	39.07 ± 9.99	48.78 ± 11.02

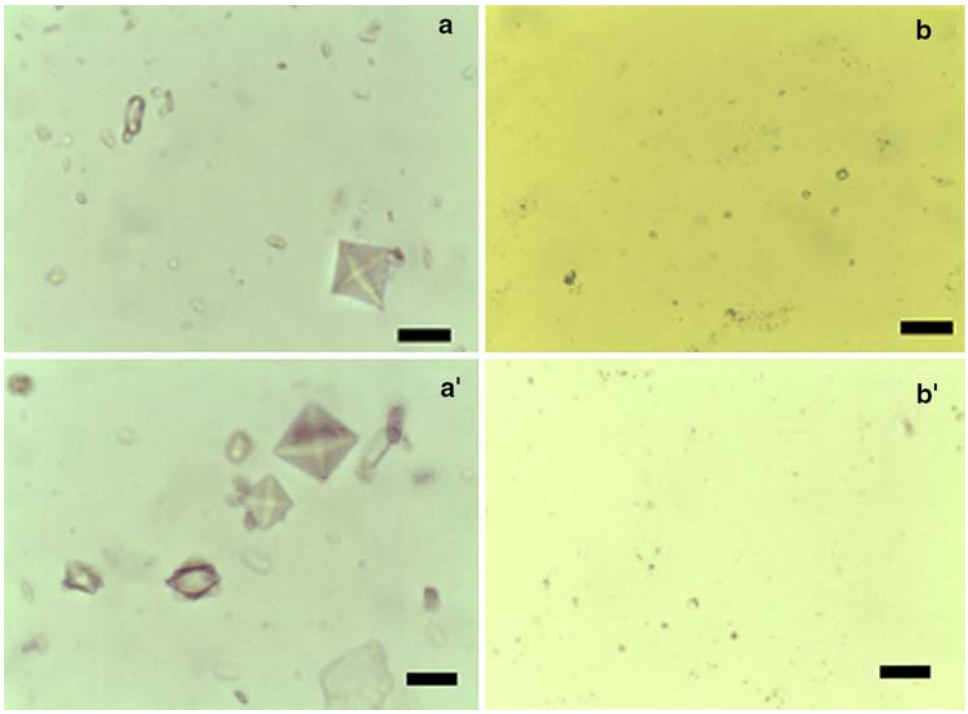

Fig. 1 Representative microscopic examination of urinary crystals excreted by **a**, **a′** untreated rats receiving 0.75% EG alone in drinking water. Large oval COM and pyramidal COD crystals can be seen, and **b**, **b′** treated rats receiving 0.75% EG in drinking water and administered *Cynodon* extract by gavage. Few oval COM and large amount of small pyramidal COD crystals can be seen. **a** and **b** are crystalluria derived from preventive protocol, while **a′** and **b′** are those derived from curative protocol. *The bar* represents 20 μm

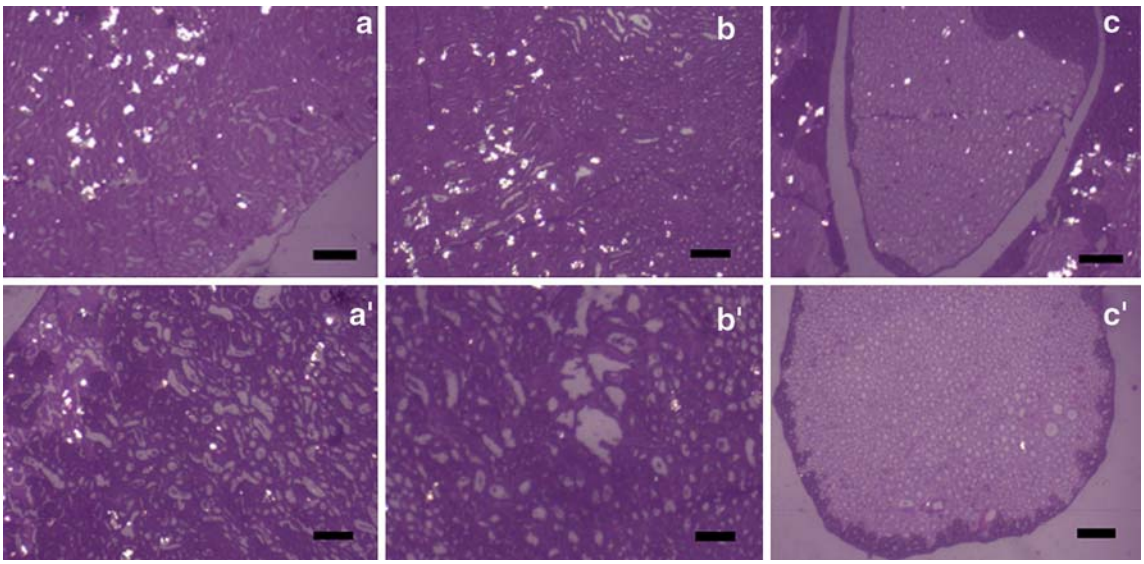

Fig. 2 Representative microscopic examination under polarized light of a median kidney section derived from a rat that received 0.75% EG alone for 3 weeks (preventive protocol) where numerous CaOx deposition were seen in **a** cortex, **b** medulla, **c** papilla. A kidney median section from a rat that received EG 0.75% in drinking water and herb extract for 3 weeks. Reduced CaOx crystals were seen in **a′** cortex, **b′** medulla, **c′** papilla. *The bar* represents 0.2 mm (200 μm)

Furthermore, on day 21, COD crystals showed a reduced size in treated rats when compared to untreated ones (Fig. 1a, b).

At the time of the killing of rats, kidneys' weight showed no significant difference between untreated and treated groups (2.77 ± 0.11 vs. 2.65 ± 0.21, $P = 0.6240$, values in g). The examination of kidney paraffin sections revealed the presence of CaOx crystals deposition on all parts of the kidney sections (Fig. 2). While crystals were randomly distributed on both rat groups' cortex, a reduced deposition levels were noticed in treated rats compared to untreated animals (Fig. 2a, a′). Deposition of crystals was also present as COM aggregates in renal medulla and papilla in untreated rats (Fig. 2b, c). However, a significant crystal deposition decrease was observed in treated rats compared to untreated animals (Fig. 2b′, c′).

Table 3 Body weight, water intake, urinary volume and pH from untreated and treated rats following a curative protocol

Groups	Body weight (g)		Water intake (ml)		Urinary volume (ml)		Urinary pH	
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
Day 0	259.7 ± 17.0	260.2 ± 12.6	10.3 ± 0.3	12.0 ± 1.6	15.7 ± 1.2	16.0 ± 0.7	6.08 ± 0.08	6.18 ± 0.06
Day 1	249.8 ± 9.1	251.1 ± 13.6	12.0 ± 1.9	10.0 ± 0.5	14.2 ± 0.6	14.2 ± 0.7	5.96 ± 0.08	6.05 ± 0.12
Day 3	246.6 ± 19.4	247.3 ± 13.5	5.5 ± 0.9	3.2 ± 1.1	9.7 ± 0.8	10.3 ± 0.7	6.20 ± 0.09	6.18 ± 0.13
Day 7	248.1 ± 14.2	247.8 ± 14.0	16.3 ± 2.7	13.2 ± 1.2	15.8 ± 1.6	15.6 ± 1.2	5.87 ± 0.07	6.05 ± 0.09
Day 14	258.4 ± 18.0	256.2 ± 6.4	17.8 ± 0.8	17.0 ± 2.3	19.0 ± 0.8	21.0 ± 1.7	5.76 ± 0.06	5.84 ± 0.11

Curative protocol

As shown in Table 3, there was no difference observed for body weight, water intake, urinary output and urinary pH throughout the study.

Concentrations of urinary biochemical analytes determined in the two groups of rats are shown in Table 4. No significant difference was observed for oxalate and magnesium between both groups. However, for calcium, the values were mostly similar except on day 7 which was higher in treated rats ($P = 0.0187$). Values of urinary sodium and potassium were not significant except on day 7 for sodium ($P = 0.0463$) and on day 14 for potassium ($P = 0.0102$) in treated group.

For crystalluria, on day 1, there was no difference between both groups of rats. The main crystals found excreted were COM and COD particles. However, there were more COM and COD crystals excreted in the urine of treated rats on days 3, 7 and 14 while crystalluria remained unchanged in untreated rats (Fig. 1a′, b′).

On the day of killing, the weight of kidneys showed no significant difference between the two groups (3.74 ± 0.17 vs. 3.75 ± 0.19, $P = 0.9674$, values in g). Examination of kidney sections showed large deposition of crystals, as mini stones aggregate in the intercellular spaces of the tubular epithelial, inside the epithelial cells and the interstitium (Fig. 3a–c). However, compared to untreated animals, the treated rats had generally less aggregated crystals' deposition and the residual aggregates found were restricted to the renal cortex (Fig. 3a′–c′).

Discussion

In recent years, there has been a resurgence of interest in studying medicinal plants that are used in the treatment of several human diseases including urolithiasis [8–13]. A wide range of plants are used traditionally for their purported effect on the elimination of urinary calculi. Among these plants, *C. dactylon* is used in Morocco by a large number of lithiasis patients by drinking the decoction obtained from the rhizomes. So far, no scientific data are available to indicate the beneficial effect of this plant for patients in urolithiasis. Therefore, we used an experimentally induced lithiasic rats to study the effect of *C. dactylon* extract as a preventive and curative agent for the treatment of kidney stones.

In the preventive protocol, two groups of rats were rendered nephrolithiasic by getting 0.75% EG in drinking water. One group of rats from the two received the *Cynodon* extract in the same time to evaluate its effect on preventing CaOx deposition into kidneys. Our results showed the amount of water used, the urinary output, as well as the urinary pH was similar in both groups of animals (Table 1). Also, in this experimental condition, there was no significant diuresis noticed in the treated group. It is worthwhile to mention that *C. dactylon* is described as a diuretic plant [14–16]. Such effect was not observed in our current study due to the fact that the extract of the plant was administered to rats that have received EG. Due to its sweetness taste, we believe that rats drunk EG in a large amount which could have masked the diuresis effect of the plant. The diuretic effect of the plant needs to be undertaken in normal rats, a study that is underway in our laboratory. At the biochemistry levels (Table 2), there was no significant difference between the two groups except for oxalate which was highly excreted in the treated rats at the third week (the end of the experiment) versus untreated animals. In relation to the excretion, crystalluria was followed throughout the duration of the experiment. COM and COD particles were observed in the urine from the first day of EG usage. Mostly, there was no significant difference between the two treated groups of animals except on the day 21 (third week) where treated rats excreted more COD crystals of a reduced size than COM (Fig. 1). In regards to urolithiasis, it is important to emphasize that the crystal structure changes in treated rats have several positive virtues. First, it shows that substances from the plant end up in the urine and excerpt their action directly or indirectly on crystals. Second, the apparition of more COD than COM particles is advantageous since COM crystals have high adhesion affinity to renal epithelial cells when compared to COD particles. In

Table 4 Urinary parameters from untreated and treated rats following a curative protocol

Groups	Oxalate (mg/24 h)		Calcium (mg/24 h)		Magnesium (mg/24 h)		Sodium (mg/24 h)		Potassium (mg/24 h)	
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
Day 0	0.21 ± 0.01	0.20 ± 0.02	0.88 ± 0.16	1.15 ± 0.88	4.72 ± 0.57	4.63 ± 0.38	15.02 ± 2.16	15.11 ± 1.36	73.72 ± 6.37	84.95 ± 5.65
Day 1	0.24 ± 0.01	0.26 ± 0.04	1.13 ± 0.16	0.86 ± 0.19	3.34 ± 0.48	3.74 ± 0.18	3.20 ± 1.05	4.32 ± 1.23	69.63 ± 10.54	68.49 ± 4.53
Day 3	0.19 ± 0.02	0.26 ± 0.02	0.64 ± 0.10	0.61 ± 0.11	3.65 ± 0.29	4.21 ± 0.55	4.32 ± 1.23	4.39 ± 0.97	68.09 ± 2.74	78.89 ± 6.56
Day 7	0.22 ± 0.01	0.26 ± 0.06	0.62 ± 0.03	1.53 ± 0.28 ($P = 0.0187$)	4.68 ± 0.34	4.93 ± 0.35	7.08 ± 0.81	10.03 ± 0.54 ($P = 0.0463$)	58.35 ± 4.48	74.84 ± 6.76
Day 14	0.38 ± 0.02	0.38 ± 0.08	0.94 ± 0.29	0.87 ± 0.18	5.41 ± 0.72	6.27 ± 0.67	12.37 ± 0.91	13.54 ± 0.97	69.04 ± 3.34	92.04 ± 6.47 ($P = 0.0102$)

fact, Wesson et al. [17, 18] have shown that renal tubule in cells' culture bound 50% more to COM than COD crystals and naturally occurring macromolecules in urine, known as inhibitors of crystallization, were found to favor the formation of COD particles. Thus, it appears that extract compounds excerpt their action similarly than natural urinary inhibitors. Such action can be explained by the change in urinary supersaturation [19] or the binding of substances onto crystals growth sites leading therefore to the change of the crystal shape [20, 21]. Moreover, several studies reported that both oxalate ions and COM crystals are injurious to renal epithelial cells by providing substrates for nucleation of crystals, promoting crystals aggregation, and exposing sites for the attachment and retention of crystals leading to its adhesion to the epithelial cells and therefore to the development of calculi [22–28]. It seems that naturally occurring urinary macromolecule inhibitors protect renal epithelial cells from crystal attachment and thereby reducing cell injury [29, 30]. Thus, the plant extract seems to play similar role by protecting renal epithelial cells at least in part by reducing cell damage by preventing crystal adhesion as demonstrated for Urocalun, an herbal medicine prepared from an extract of *Quercus salicina* Blume/*Quercus stenophylla* Makino [31]. Such action needs to be proven by undertaking more experiments such as those used for *Herniaria hirsuta* [32]. All these beneficial effects were confirmed by microscopic examination with polarized light of kidney sections which revealed less CaOx deposition in treated animal when compared to untreated ones (Fig. 2).

In the curative protocol, rats were first rendered nephrolithiasic and then treated to assess the ability of the plant extract to displace prebound crystals to renal epithelial cells. In this experiment, as in preventive protocol, we did not observe any significant changes regarding all parameters except calcium, sodium and potassium levels (Table 4). The important change worth to be mentioned concerning crystalluria which was characterized by abundant excretion of crystals in treated rats indicating an elimination of crystals already deposited and attached to renal lining cells when compared to untreated ones (Fig. 1). This beneficial effect is confirmed by kidneys' histological analysis. Indeed, kidney sections of untreated rat showed abundant crystal depositions aggregated in mini stones (Fig. 3a–c). Furthermore, renal epithelial cells had more dilatation shown by large spaces in the tissue. In treated rats, less CaOx crystal depositions were seen compared to untreated animals and the necrosis as well as the tubule dilatation was very limited (Fig. 3a′–c′). At this stage, it is important to point out the relevance of our finding which demonstrated, as we showed before for *Herniaria* [32] that *Cynodon* extract has the ability to dislodge a significant amount of prebound crystals from renal epithelial cells.

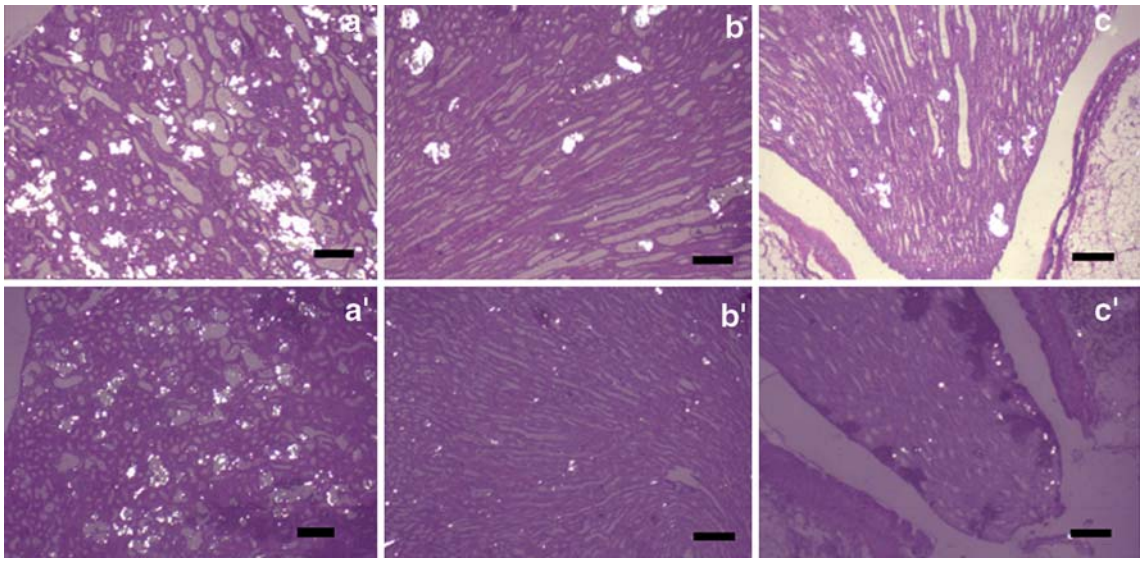

Fig. 3 Representative microscopic examination under polarized light of a median kidney section derived from a rat following a curative protocol (see "Materials and methods" for details). Large aggregated crystals in mini stones are seen in **a** cortex, **b** medulla, **c** papilla of untreated rat. Reduced crystals deposition and disaggregated stones in herbal treated rat are seen in **a′** cortex, **b′** medulla, **c′** papilla. *The bar* represents 0.2 mm (200 μm)

Overall, our findings present scientific data supporting the beneficial effect of *C. dactylon* extract as a preventive and curative agent and justify its use in traditional medicine for the treatment of kidney stones. This beneficial effect could be due to the presence of one or several active principles, probably before or after transformation(s) in vivo by drug metabolizing enzymes, and interactions with each other lead to a beneficial drug effect presented in this study. Thus, further research is needed to identify these active principles from *C. dactylon* to assess their biological activity, the efficient dosage, the quality control as well as the search for new metabolites relative to the active principles family of molecule but with more potent and efficient activities for the treatment of kidney stones. Such studies are underway in our laboratory.

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