

Preventive effect of *Cynodon dactylon* against ethylene glycol-induced nephrolithiasis in male rats

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Abstract

Objective: This study was carried out to investigate the preventive effects of hydroalcoholic extract of *Cynodon dactylon* (*C.dactylon*) roots on calcium oxalate calculi in rat.

Materials and Methods: 24 Wistar rats were randomly divided into 4 groups: group A received tap drinking water while, Groups B, C, and D received 1% ethylene glycol daily for 28 days. Rats in groups C and D received ethanolic extract of *C.dactylon* at doses equivalent to 3.2 mg/kg and 12.6 mg/kg of root powder, respectively in drinking water from day 0 to day 28. Urine and blood were collected on days 0 and 28 and analyzed for biochemical elements. After 28 days, the kidneys were removed and prepared for histological evaluation of calcium oxalate deposits (CaOx).

Results: The number of CaOx deposits in 10 microscopic fields of kidney slices in group B was 24.5 ± 4.40 which was significantly higher than group A ($p < 0.001$). In group C, the number of deposits was significantly lower than group B. The weight of the kidneys was increased in group B vs group A ($p < 0.05$). However, *C.dactylon* was able to decrease the weight of kidneys in group C ($p < 0.05$). Urine oxalate level decreased in nephrolithiatic rats treated with the extract.

Conclusion: This study showed that *C. dactylon* extract was able to reduce the growth of urinary stones in the rat. Therefore, the beneficial action of *C.dactylon* extract on human kidney stones may be suggested. However, further studies must clarify the mechanism.

Keywords: *Cynodon dactylon*, Kidney stone, Ethylene glycol, Calcium oxalate

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Introduction

Urinary calculi are the third prevalent disorder in the urinary system and may cause obstruction, hydronephrosis, infection and hemorrhage in the urinary system (Hadjzadeh *et al.*, 2007). Surgical operation, lithotripsy and local calculus disruption are widely used to remove the calculi; however, these procedures are expensive and the recurrence is common (Basavaraj *et al.*, 2007; KVSRR *et al.*, 2007). Various therapies including thiazide diuretics and alkali-citrate are being used in attempt to prevent recurrence but scientific evidence for their efficacy is less convincing (Bashir and Gilani, 2009).

Calcium oxalate (CaOx) and Calcium phosphate represents up to 80% of urinary stones. Kidney stone formation is a complex process involving several physicochemical events including supersaturation, nucleation, growth, aggregation and retention of salts within the renal tubules (Menon and Resnick, 2002; Coe *et al.*, 2005). Several products from medical plants have been used in treatment of urinary calculi for centuries (Grases *et al.*, 1995; Viel *et al.*, 1999; McHarg *et al.*, 2003; Atmani *et al.*, 2004; Laroubi *et al.*, 2007).

C. dactylon L, a members of Cynodonteae tribe and Chloridoideae sub-family, is a resilient and perennial grass native to the warm temperate and tropical regions (Auddy *et al.*, 2003; Bethel *et al.*, 2006). *C. dactylon* is claimed to have antidiabetic, antimicrobial (Singh *et al.*, 2007), hypolipidemic (Singh *et al.*, 2008b), anti-inflammatory, and anti-emetic (Ahmed *et al.*, 1994) properties. The plant is diuretic and used as a remedy for urinary infections, kidney stones and congestion (www.mdidea.com). The diuretic effect of *C. dactylon* was attributed to the presence of flavonoids (Satish and Mahesh, 2009). The root and rhizomes of this plant are also

used in the treatment of depression, vomiting, cough, epilepsy, and hemorrhage (Miraldi *et al.*, 2001). It was reported that the plant possesses protective effect against stroptozotocin-induced hepatic injury in rats (Singh *et al.*, 2008a). The aqueous extract of *C. dactylon* rhizomes was used as a cardiac tonic in heart failure and had a curative effect on congestive heart diseases (Garg and Khosa, 2008). The present study was carried out to investigate the preventive effects of hydroalcoholic extract of *C. dactylon* roots on kidney calculi in a rat model.

Material and methods

Preparation of extract

C. dactylon was collected from the campus of Imam Reza, Mashhad, Iran. The roots were dried and powdered. Then, 100 g of the powder was mixed with a sufficient volume of 96% ethanol and extracted with a soxhlet apparatus for 16 to 18 hours. The solvent was removed and the extract was dried in an oven with the temperature of 50°-60°C. The dried extract weighed 20 g; therefore, it had 20% of *C. dactylon* root powder. The extract was kept in a refrigerator and added daily to the drinking water of the rats (Houghton *et al.* 1995).

Toxicity studies

The toxic dose was determined on the basis of a pre-studied experiment which was carried out on the mice. Two doses that were less than the lethal dose (LD50) (3.2 mg/kg and 12.6 mg/ kg as lower and higher dose, respectively) were taken as effective dose in the current study.

Animals

24 male Wistar albino rats weighing 200–250 g were used for this experiment. They were randomly divided in 4 groups

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(A-D) and maintained at $25 \pm 2^\circ\text{C}$, under 12 h light/dark cycles. The animals were given standard diet. This study was performed in accordance with the guide for the care and use of laboratory animals of Mashhad University of Medical Sciences, Mashhad - Iran.

Ethylene glycol-induced urolithiasis model

Kidney calculi were induced by ethylene glycol in rats. Group A served as control and received regular rat food and drinking water ad libitum. Group B animals received 1% ethylene glycol (Merk, Germany) in drinking water for 28 days (Christina et al., 2002). In groups C and D animals received 1% ethylene glycol in drinking water along with hydroalcoholic extract of *C. dactylon* equivalent to 3.2 mg and 12.6 mg root powder/kg body weight, which was determined according to the accurate measurement of rats daily water consumption, for 28 days respectively.

Analysis of urine

24h urine samples was collected on days 0 and 28 of the experiment using individual metabolic cages. Urine oxalate and citrate were measured using commercially available assay kits (Rajagopal, 1984; Sriboonlue et al., 1998).

Serum analysis

Blood was collected from the retro-orbital sinus under anesthetized condition on day 0 and day 28 and analyzed for calcium, magnesium and potassium. Potassium level was measured by flame photometer (Hald, 1947). Calcium and magnesium levels were measured by autoanalyser (Technicon RA-1000) (Ng et al., 1985).

Kidney histopathology

At the end of experiment midline incisions were made to expose the kidneys. Kidneys were removed and weighted. The right kidney was fixed in 10% neutral buffered formalin, processed in a series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5 micrometer and stained with Haematoxylin and Eosin (H and E) for histopathological examination. The slides were examined under light microscope to study the kidney and calcium oxalate deposits. Aggregations of CaOx deposits (tubules containing CaOx deposits) were counted in 10 microscopic fields (Hadjzadeh et al., 2007).

Statistical analysis

The values were expressed as mean \pm SEM. The statistical analysis of data was done by one-way ANOVA, followed by Tukey multiple comparison tests using 5% level of significance. The statistical package used was SPSS 15.

Results

Urine oxalate and citrate levels:

Mean urinary oxalate and citrate levels of the all groups are presented in Table 1. At the baseline there was no difference between groups in urine oxalate levels. Urine oxalate levels in group D was significantly higher than group B on day 28 ($p < 0.001$). Also, there was a significant difference in urine oxalate on 0 and 28th day in group C ($p < 0.01$). Table 1 also shows that urine citrate levels were similar in all groups at the beginning of the experiment. Urinary citrate excretion decreased on day 28 after administration of extract in groups C and D compared with groups A and B, however this

difference was statistically insignificant. There was also a significant difference in urine citrate on 0 and 28th day in group C ($p < 0.01$).

Serum magnesium, calcium and potassium levels

No difference was evident between groups in Serum magnesium levels at the baseline Table 2. Serum magnesium levels in group B was higher compared to group

A on day 28 ($p < 0.001$). Serum magnesium concentration in groups C and D was significantly lower than group B on day 28 ($p < 0.001$). There was also a significant difference in serum magnesium concentration at 0 and 28th days in group B ($p < 0.001$). No significant difference was observed in serum calcium and potassium concentration on days 0 and 28 among groups (data not shown).

Table 1. Urine concentration of oxalate and citrate.

Groups	Oxalate (mg/dl)				Citrate (mg/dl)			
	A	B	C	D	A	B	C	D
Day 0	0.33± 0.04	0.35± 0.06	0.80± 0.16	0.24± 0.02	1.20± 0.28	1.89± 0.22	1.94± 0.30	1.41± 0.19
Day 28	0.11± 0.02	0.03± 0.01	0.10± 0.004 b,**	0.18± 0.02 a,***	1.15± 0.21	1.29± 0.26	0.58± 0.15 b,**	0.94± 0.20

A, control group; B, Ethylene glycol-induced urolithiasis; C and D, treated groups with *C. dactylon* at 3.2 and 12.6 mg/kg, respectively. The values are expressed as mean ± S.E.M. (n=6 animals/group). ^a Comparisons are made with Group B. ^b Comparisons are made with day 0 in Group C. ^{**} Statistically significant at $p < 0.01$. ^{***} Statistically significant at $p < 0.001$.

Table 2. Serum concentration of magnesium in normal, ethylene glycol-fed and herbal treated rats.

Groups	Magnesium (mg/dl)			
	A	B	C	D
Day 0	3.28± 0.61	2.34± 0.06	2.10± 0.12	2.22± 0.24
Day 28	2.18± 0.08	3.64± 0.15 a .c:***	2.48± 0.10 b,***	2.03± 0.06 b,***

A, control group; B, Ethylene glycol-induced urolithiasis; C, treated group with *C. dactylon* (3.2 mg/kg); D, treated group with *C. dactylon* (12.6 mg/kg). The values are expressed as mean ± S.E.M. (n = 6 animals /group). ^a Comparisons are made with Group A. ^b Comparisons are made with Group B. ^c Comparisons are made with day 0 in Group B. ^{***} Statistically significant at $p < 0.001$.

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Histopathological analysis

Histopathological features of the kidneys of control rats showed normal features with prominent cortical tubules and Bowman's capsule and there were no calcium oxalate deposits or other abnormalities in the nephron segments either (Figures 1, 2). The number of CaOx deposits in 10 microscopic fields in the kidney slides of group B was 24.5 ± 4.40 which was significantly higher than group A ($p < 0.001$). In group B, deposits were composed of 3 to 4 large polygonal

crystals in different segments of the renal tubules (Figures 3 to 5). In group C, the number of deposits was 2.625 ± 1.179 which was significantly lower than group B ($p < 0.001$) (Figure 6). Calcium oxalate crystals in different parts of the renal tubules in group C were clearly smaller in comparison with group B. Interestingly, similar to control group there was no oxalate crystal in the nephron segments of group D ($p < 0.001$) (Figure 6).

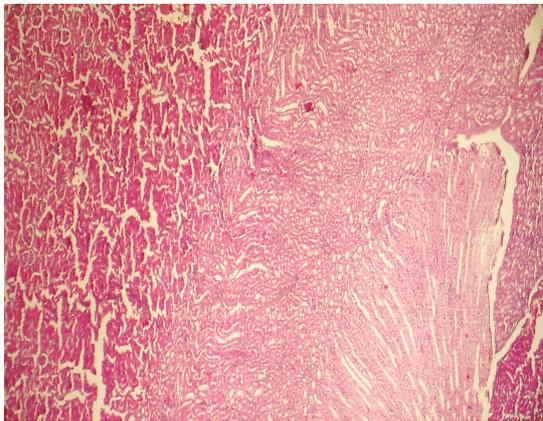


Figure 1. Normal medullary and papillary tubules in the kidney of a wistar rat in group A (hematoxylin–eosin $\times 100$).

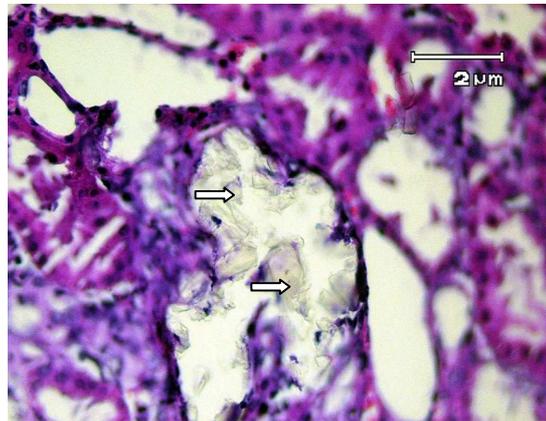


Figure 3. Calcium oxalate crystals (thick arrows) in the renal tubule of group B (hematoxylin–eosin $\times 400$).

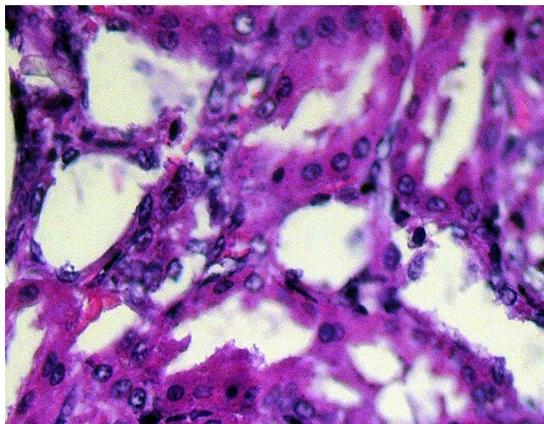


Figure 2. Normal tubules and collecting ducts in group A (hematoxylin–eosin $\times 400$).

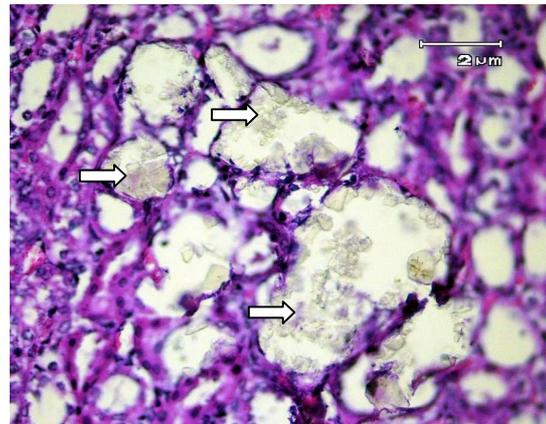


Figure 4. Multiple tubular calculi (thick arrows) in an ethylene glycol-treated rat (group B). (hematoxylin–eosin $\times 400$).

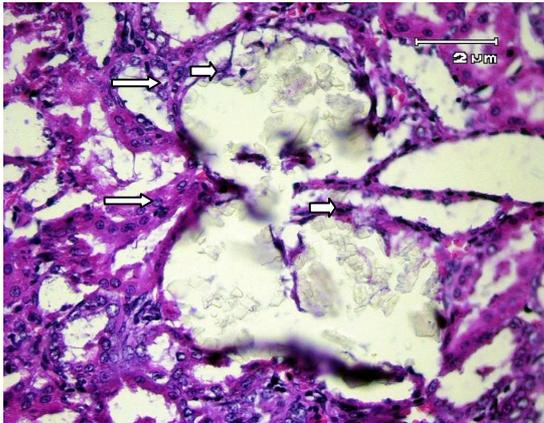


Figure 5. Secondary renal tubular dilation with epithelial damage (thick arrows) and leukocyte reaction (thin arrows) in group B (hematoxylin-eosin $\times 400$).

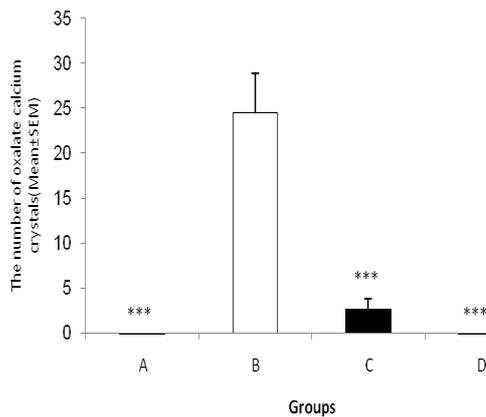


Figure 6. Number of oxalate calcium crystals in 10 microscopic fields. A, control group; B, Ethylene glycol-induced urolithiasis; C, treated group with *C. dactylon* (3.2 mg/kg); D, treated group with *C. dactylon* (12.6 mg/kg). The values are expressed as mean \pm S.E.M. (n=6 animals /group). *** $p < 0.001$ vs. group B.

Relative weight of the kidneys

At the end of the study, the weight of the kidneys was higher in group B compared with group A ($p < 0.05$). The administration of *C. dactylon* at equivalent dose of 3.2 mg/kg (group C) decreased the weight of kidneys in this group ($p < 0.05$) (Figure 7).

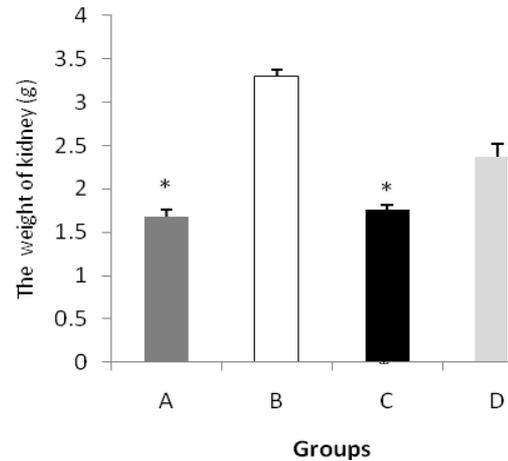


Figure 7. Weight of kidneys in different groups. A, control; B, Ethylene glycol-induced urolithiasis; C and D, treated groups with equivalent of (3.2 mg/kg) and D (12.6 mg/kg) *C. dactylon* powder. The values are expressed as mean \pm S.E.M. (n=6 animals/group). * $p < 0.05$ vs. group B.

Discussion

Urinary stone disease is mainly the result of supersaturation of urine with certain urinary salts such as CaOx, which is the most common constituent of kidney stones (Daudon et al., 1993). Several in vivo models have been developed to ascertain the effects of various therapeutic agents on development and progression of the disease (De Bruijn et al., 1993). Rat is the most frequent used animal to induce kidney CaOx deposition. Accordingly, we evaluated the effectiveness of *C. dactylon*, on rats rendered nephrolithiasis by administration of ethylene glycol.

In general, the crystallization of stone-forming salts is due to an abnormal urinary composition that is either higher in crystallization promoters (e.g. calcium, oxalate, uric acid) or lower in inhibitors (e.g. citrate, glycosaminoglycans, kidney proteins such as nephrocalcin, Tamm-Horsfall mucoprotein uropontin), or both (Barbas et al., 2002). Although the analysis

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of urine elements seemed to be not convincing and it is difficult to draw a clear and definite conclusion, considering the current study, data can underline two important points. First, oxalate level was lower in the urine of calculi-induced animals (Group B) that is in contrast with stone formation. Also, urine oxalate concentration decreased in nephrolithiatic rats treated with the plant extract. Second, data show that ethylene glycol doesn't have any significant effect on the urinary citrate levels; also, drinking *C. dactylon* decreased urinary citrate while it was not statistically significant. Since oxalate is a key risk factor in CaOx stone formation and citrate is universally regarded as an effective inhibitor of CaOx stone formation (Bihl and Meyers, 2001), it is important to point out that medicinal plant used in this study for the treatment of urolithiasis seemed to have little effect on urinary chemistry. Further investigations are highly recommended to reach a conclusive result. An increase in serum magnesium concentration was observed in calculi- induced rats (Group B) and treatment of *C. dactylon* had no significant action on serum magnesium level compared with control rats. Magnesium has several ways for inhibiting of stone formation. For example, it is able to reduce the reabsorption of citrate in kidney tubules (Reungjui et al., 2002) and also diminishes the oxalate absorption from intestine (Massey, 2005). Thus, reducing the risk of stone formation by *C. dactylon* in this investigation would not be explained by inhibitory effect of magnesium on the kidney stone disease.

The weight of the kidneys increased in group B; this may be due to water retention or inflammation of the nephrons' epithelia that is mainly resulted from CaOx deposition. The ethanolic extract was able to decrease significantly the weight of kidneys in group C, which in part may be

due to anti-inflammatory effect of *C. dactylon* (Ahmed et al., 1994).

Microscopic examination of kidney sections derived from nephrolithiatic rats showed that crystal deposits were composed of 3 to 4 large polygonal crystals in different segments of the renal tubules. The presence of such deposits is an evidence of adhesion and retention of particles within renal tubules. However, nephrolithiatic rats treated with lower dose of *C. dactylon* extract had limited CaOx deposition. In group D, similar to control group there was no oxalate crystal in the nephron segments. It is postulated that the plant inhibits the formation of particles in kidney tubules. Thus, the plant extract may interfere directly with the inhibition of crystal adhesion to the epithelium by blocking the attachment sites located either on the cell surfaces or on the surface of the crystals themselves. It may be suggested that the extract contains substances that coat crystals, thereby blocking their adhesion to the cell surface. In another study by Atmani, et al, it has been established that *C. dactylon* extract has beneficial effect in preventing and eliminating CaOx deposition in the kidneys (Atmani et al., 2009). Also, the same results were reported with *Nigella sativa* seeds which its ethanolic extract reduced the number of calcium oxalate deposits in rats (Hadjzadeh et al., 2007).

Calcium oxalate crystals and high oxalate levels in nephrons can induce damage in the epithelial cells, and consequently, the cells may produce some products, as well as free radicals, inducing heterogenous crystal nucleation and cause aggregation of crystals (Khan and Thamilselvan, 2000). Since the crude extract was used in this study, the exact mechanisms involved in the effect of *C. dactylon* on CaOx calculi are not clear. Phytochemical analysis of hydroalcoholic extract obtained from *C. dactylon* rhizomes has demonstrated that the rhizomes contain

sugar, flavonoids, sterols and steroidal saponins (Fazly Bazzaz *et al.*, 1997; Garjani *et al.*, 2009). Plant flavonoids are antioxidant and scavenge oxygen free radicals. Therefore, it can be speculated that the role of the *C. dactylon* ethanolic extract in preventing formation of CaOx calculi or tissue disruption as seen in the present study, is in part due to the anti-inflammatory and antioxidant effects of the different compounds of the *C. dactylon* (Comalada *et al.*, 2006). The other main components of the extract are steroidal saponins. Steroid saponins have been found to have some biological and pharmacological activities including cardiac tonic, diuretic, antibacterial, anti-inflammatory and hypocholesteremic effects (Francis *et al.*, 2002). These effects may also contribute in anti-nephrolithiatic action of *C. dactylon* extract.

In conclusion, the present data indicate that preventive administration of the hydroalcoholic extract of *C. dactylon* to rats with urolithiasis reduced growth of urinary stones, supporting folk information regarding the plant antiurolithiatic activity. Therefore, it may be suggested that ethanolic extract or other products of the *C. dactylon* be used for prevention and perhaps treatment of CaOx calculi in human; further studies are necessary to clarify the mechanism.

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