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Evaluation of Anti Urolithiatic Activity of *Phoenix dactyleferae* Seeds Extract in Ethylene Glycol Induced Urolithiasis in Rats

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ABSTRACT

Purpose: To evaluate the activity of anti-urolithiatic of the hydroalcoholic extracts of *Phoenix dactylifera* Linn seeds (roasted and non-roasted) in calcium oxalate urolithiasis in male albino rats. **Methods**: Lithiasis was induced by oral administration of ammonium chloride 1% and ethylene glycol (0.75 %v/v) in male albino rats for 28 days. The non-roasted and roasted extracts (500 mg/kg) were administered orally from the 15th day as a curative regimen urine analysis, serum analysis, biochemical analysis of kidney homogenate and histopathological study were performed. **Results**: The urine volume, urine magnesium and kidney GSH levels were decreased as well as calcium excretion in urine, serum creatinine and urea, kidney MDA and NO contents were increased in lithiatic group as compared to control group. Treatment with both hydroalcoholic seed extracts restored urine volume, magnesium and kidney GSH, MDA and NO levels while treatment with of non-roasted extracts reduced the elevated level of urinary calcium, serum creatinine and urea levels as compared to lithiatic group. Histopathological examination revealed tubular degeneration, dilatation, presence of CaOx crystals in the lumen of renal tubules and intense interstitial mononuclear cell infiltration in the lithiatic control group. These histopathological alterations were markedly regressed in other treated groups. **Conclusions**: The results demonstrate that the hydroalcoholic extracts of *Phoenix dactylifera* Linn seeds either in non-roasted or roasted state have potent antiurolithiatic activity against calcium oxalate urolithiasis induced by ethylene glycol in male albino rats.

INTRODUCTION

Urolithiasis is a common chronic disorder in humans; it concerned the third most common disorder of the urinary tract [1]. The worldwide incidence of urolithiasis is quite high, between 1,200 and 1,400 per 100,000 will develop urinary stones each year with a male/female ratio of 3:1. They have an estimated lifetime risk (over the world) of 2%-5% in Asia, 8%-15% in Europe and America and around 20% in the Middle East. It is associated with high rate of recurrence, which is around 10%-23% per year, 50% in 5-10 years and 75% in 20 years, due to an imbalance between promoters and inhibitors in the kidneys [2]. More than 80% of kidney stones are composed of calcium oxalate stones alone or mixed with calcium phosphate [3]. Their recurrence of urolithiasis causes a serious problem because the patient who had one stone is more likely to form another even after treatment [4].

The literatures reported that the formation of kidney stone started by accumulation of foreign substances in urinary tract, and this materials or substances may originate from blood cells or from the urinary casts [5]. The advanced treatment of kidney stone like endoscopic stone removal and extracorporeal shock wave lithotripsy (ESWL) are prohibitively costly for the common man and recurrence is quite common. However herbal medicines have been reported that they are more efficacious, less side effects and reduce the rate of recurrence when compared to modern medicines [6]. Therefore, it is valuable to use the medicinal plants as another or alternative strategy in the treatment of kidney stones and other diseases. According to the traditional medicinal systems including Ayurveda, most of the remedies were originated from plants and they were proved to be useful through the rationale behind their use is not well established by systemic pharmacological and/or clinical studies except for some composite herbal drugs. These plant products are reported to be effective in decreasing recurrence rate of urinary calculi with no side effects [7]. *Phoenix dactylifera* Linn., belonging to family Arecaceae, have been cultivated in the Middle East over at least 6000 years ago [8], and it's known in Arabic as Nakl; fruit as Balah and Tamr; their seeds as nawa.

The date has always played an important role in the economy and social life of the people of various regions of the world [9], the fruit of the date palm is composed of a fleshy pericarp and seed [10]. The date seeds represent about 15% of the date fruits total weight [11]. Reviewing the

folk medicine and literatures, the parts of date palm were used traditionally in treatment of many illnesses and also for public purposes, the date seed have been used traditionally as the animal feed or grinded into smaller size and being roasted to turn it into caffeine-free coffee substitute [12].

Literatures also presented that date seeds contain many of essential nutrient and compounds that have pharmacological activity against many diseases, such as they contain a percent of fat ranged from 5.7 to 12.7 from the total weight other than oil, also they have been composed of protein, carbohydrates, moisture and ash [13], in addition to that, many studies investigate that the date pits contain several important minerals as potassium, magnesium, calcium, sodium, phosphorus and iron [14, 15].

Based on the evidence that Phenolic compounds of date pits have been shown to possess such benefits as an antioxidant [16], anticarcinogenic [17], anti-inflammatory activities [18], as well as reduction of cardiovascular diseases [19]. This study was designed to evaluate the curative role of these extract against ethylene glycol induced urolithiasis.

MATERIALS AND METHODS

Plant materials:

Date fruits (*Phoenix dactylifera* L. Family Arecaceae) were obtained from the local market in Khartoum city- Sudan; the seeds were separated manually from the flesh, soaked in water to washed and remove any remaining date flesh. The seeds were then sun-dried. The dried seeds were then divided into two parts; one of them was roast until they were slightly burned while the other part remains without roasting. They were grounded into a fine powder using special mill in the center of geological research at the ministry of minerals -Republic of Sudan and kept in dark well closed container.

Animals

Adult male albino rats of Wistar strain weighing 130-150 g, provided by National research centre, Egypt, were used throughout this study. The animals had free access to food and water ad

libitum and maintained in a controlled environment under standard conditions of temperature and humidity with an alternating 12 hr light and dark cycle.

Chemicals

Ethylene glycol and ammonium chloride were purchased from Sigma-Aldrich Chemical Co. (USA).

Experimental Design

Preparation of *Phoenix dactylifera* seeds extract

About 200 g of the non-roasted and roasted powdered seeds were subjected to extraction with 70% ethanol by maceration for ten days. The concentrated extracts were obtained by evaporating the solvent, under reduced pressure in a rotary evaporator at 60-70^{0}C every 24 hours. The obtained extracts were then dried to constant weight at room temperature.

Ethylene glycol induced urolithiasis model

Ethylene glycol induced hyperoxaluria model was used to assess the antiurolithic activity of non-roasted and roasted date seed extract in albino male rats. Animals were divided into five different groups containing six animals each. Ethylene glycol (0.75% v/v) and ammonium chloride (1%) in were put in drinking water for induction of renal calculi till the 28th day except for control group [20].

Group – I: Normal control; received saline (5ml/kg) for 28 days.

Group – II: Urolithiatic control; received ethylene glycol (0.75%) and ammonium chloride (1%) for 28 days in drinking water.

Group – III: Standard treated control: Ethylene glycol (0.75%) and ammonium chloride (1%) in drinking water + Cystone (750 mg/kg) from the 15th day till the 28th day [21].

Group – IV: Ethylene glycol (0.75%) and ammonium chloride (1%) in drinking water + non-roasterd (normal) date seed extract (500 mg/kg) from the 15th day till the 28th day [22].

Group – V: Ethylene glycol (0.75%) and ammonium chloride (1%) in drinking water + roasted date seed extract (500 mg/kg) from the 15th day till the 28th day [23].

Assessment of antiurolithiatic activity

Urine collection and analysis

At the end of the 28th day, the animals were kept separately in metabolic cages for 24 hours for urine collection. Animals had free access to drinking water during the urine collection period.

The collected urine samples were measured for the following parameters:

Urine volume: was measured using the measuring cylinder and reported per ml.

Urine pH: The acidity of collected urine was measured using a pH meter

Urine calcium: was estimated by using commercially available specific diagnostic kits (Biodiagnostic, Egypt) according to per o-cresolphthalein complex one method [24].

Urine magnesium: was estimated by using commercially available specific diagnostic kits (Biodiagnostic, Egypt) according to per Calmagite method [25].

Serum biochemical analysis

Twenty four hours following the last administration, blood samples were withdrawn from rats of all groups via retro-orbital vein under light ether anesthesia. Serum was used for estimation of serum creatinine and urea using specific diagnostic kits (Biodiagnostic, Egypt).

Renal tissue biochemical analysis

Immediately after blood sampling, animals were sacrificed by cervical dislocation under ether anesthesia. No animal died prior to this experimental end point. The two kidneys from each rat were immediately dissected out and rinsed with PBS to remove excess blood. Weighed parts from both kidneys were homogenized (MPW-120 homogenizer, Med instruments, Poland) in PBS to obtain 20% homogenate then centrifuged for 5 minutes at 4000 x g using a cooling centrifuge (Sigma and laborzentrifugen, 2k15, Germany).

The supernatant was removed immediately and assayed for reduced glutathione (GSH), lipid peroxides, measured as malondialdehyde (MDA) and nitric oxide (NO) contents using (Biodiagnostic, Egypt) kits.

Histopathological examination

Other parts of kidneys from all groups were fixed in 10% neutral buffered formalin for 72h at least, washed, dehydrated, and embedded in paraffin. Sections of 5µm thickness were stained with Hematoxylin and Eosin (H&E).

Statistical Analysis

All the values are presented as means ± standard error of the means (SE). Comparisons between different groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey HSD test for multiple comparisons.

RESULTS

Renal function markers

Urine parameters

Administrations of ethylene glycol showed a significant decrease in urine volume and elevate urine pH as compared with those of normal group. Treatment of rats with both non-roasted and roasted date seed extracts elevated urine volume while roasted date seed extract only decreased urine pH compared with ethylene glycol group (Table 1). Also, treatment with Cystone elevated urine volume.

Electrolyte content of urine

Results in (table 1) also showed that a significant increasing and decreasing in calcium and magnesium levels respectively after administration of ethylene glycol as compared to the results of control group. However; the treatment of rats by either roasted or non-roasted date seeds extracts in a dose of 500 mg/kg showed the highest antiurolithiatic activity by decreasing the

level of calcium and increasing magnesium level. The extract of non-roasted seeds showed higher activity than that of roasted seeds and Cystone.

Serum parameters

Administration of ethylene glycol showed a significant elevation in serum creatinine and urea compared with those of normal group. Treatment of rats with both non-roasted and roasted date seed extracts (500 mg/kg) decreased urea serum level while non-roasted date seed extract reduced the level of serum creatinine compared with ethylene glycol group. Treatment with Cystone decreased creatinine and urea serum levels (Table 2).

Oxidative stress markers

Renal GSH content was significantly reduced following ethylene glycol administration, and a significant elevation of renal MDA and NO contents were detected. On the other hand, normal levels of oxidative stress biomarkers were observed in groups treated with non-roasted (normal) and roasted date seed extracts, while treatment with Cystone decreased MDA and NO levels (fig. 1).

Histopathology

Kidneys of normal control rats showed normal renal glomeruli and tubules with no evidence of tubular degeneration, dilatation or inflammatory reaction (fig. 2a). Meanwhile, characteristic histopathological alterations were demonstrated in the urolithic control group represented by extensive vacuolar degeneration of renal tubular epithelium (fig. 2b) and presence of CaOx crystals in the lumen of renal tubules which are greatly dilated (fig. 2c). Intense peritubular and interstitial mononuclear cell infiltration (fig. 2d) were frequently demonstrated in this group in addition to presence of foci of regenerative renal tubules (fig. 2e). Marked improvement of the histopathological alterations was demonstrated in Cystone and non-roasted (normal) Phoenix dactylifera (500mg/kg) treated group manifested by absence of inflammatory reaction, mild tubular degeneration and decreased number of renal tubules containing calculi (fig. 2f & 2g, respectively). On the other hand, treatment with roasted Phoenix dactylifera (500mg/kg) revealed mild improvement represented by vacuolar degeneration of renal tubules and presence of CaOx crystals in the lumina of some renal tubules (fig. 2h).

Table 1: Effects of treatment with non-roasted and roasted Phoenix dactylifera seed extracts on urine volume and pH as well as urine Ca and Mg levels

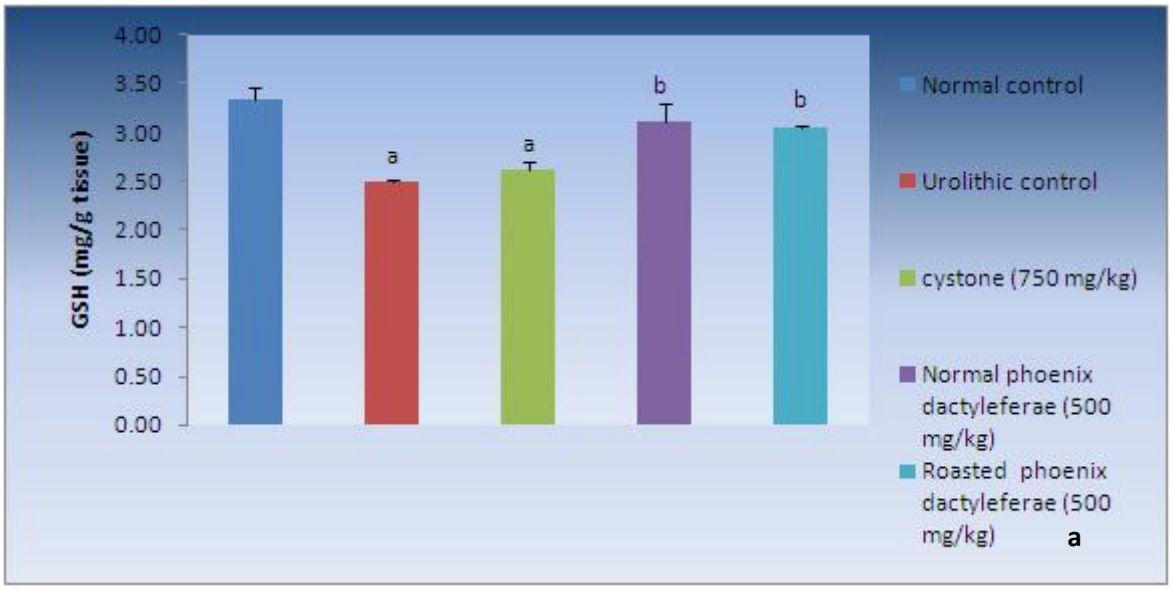
	Normal control	Urolithic control	Cystone (750 mg/kg)	Non roasted Phoenix dactyleferae (500 mg/kg)	Roasted Phoenix dactyleferae (500 mg/kg)
Urine volume (ml/24h)	8.44±0.07	3.22±0.18 [a]	4.40±0.10[ab]	8.34±0.06 [b]	5.34±0.06[ab]
Urine pH	6.64±0.02	8.64±0.02 [a]	6.20±0.16 [b]	8.44±0.30 [a]	6.24±0.06 [b]
Ca (mg/24h)	33.79±1.32	41.39±0.92 [a]	33.83±0.84 [b]	33.03±0.33 [b]	42.39±1.00 [a]
Mg (mg/24h)	36.25±0.51	29.13±1.41 [a]	34.47±0.21 [b]	38.03±0.85 [b]	36.50±0.94 [b]

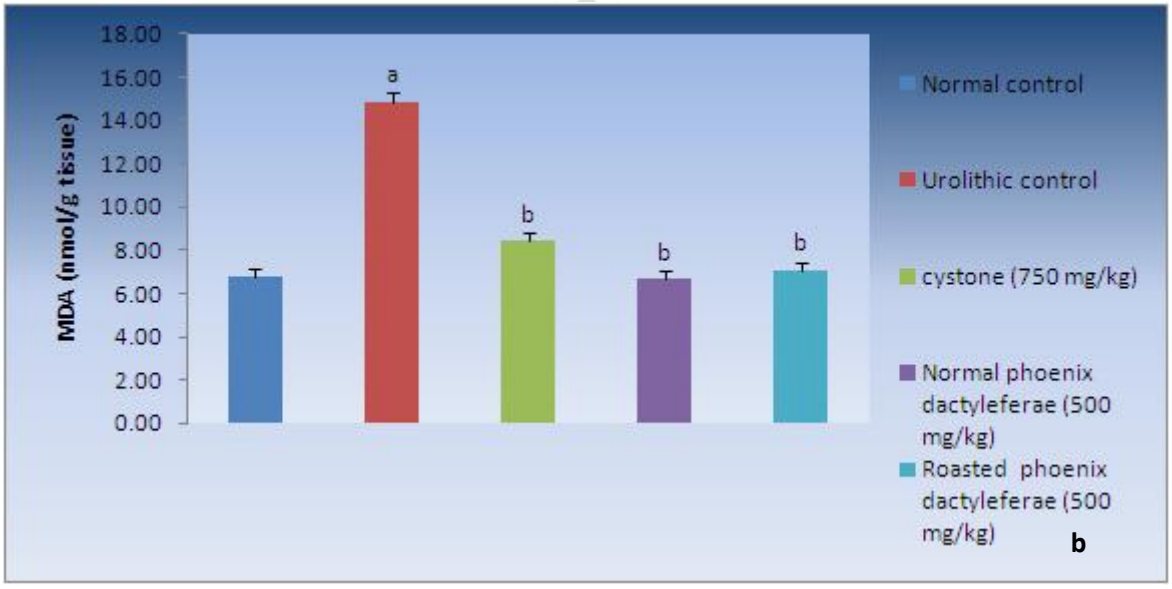
Data were expressed as mean ± SE (n=6). Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons. [a] Significantly different from normal control at P<0.05. [b] Significantly different from urolithic control at P<0.05.

Table 2: Effects of treatment with non-roasted and roasted Phoenix dactylifera on serum creatinine and urea levels

	Normal control	Urolithic control	Cystone (750 mg/kg)	Non-roasted Phoenix dactyleferae (500 mg/kg)	Roasted Phoenix dactyleferae (500 mg/kg)
creatinine (mg/dl)	1.54±0.15	2.31±0.05 [a]	1.82±0.05 [b]	1.85±0.04 [b]	2.05±0.07 [a]
Urea (mg/dl)	62.74±2.09	232.80±3.10 [a]	71.66±2.55 [b]	112.42±2.23[ab]	167.52±2.36[ab]

Data were expressed as mean ± SE (n=6). Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons. [a] Significantly different from normal control at P<0.05. [b] Significantly different from urolithic control at P<0.05.





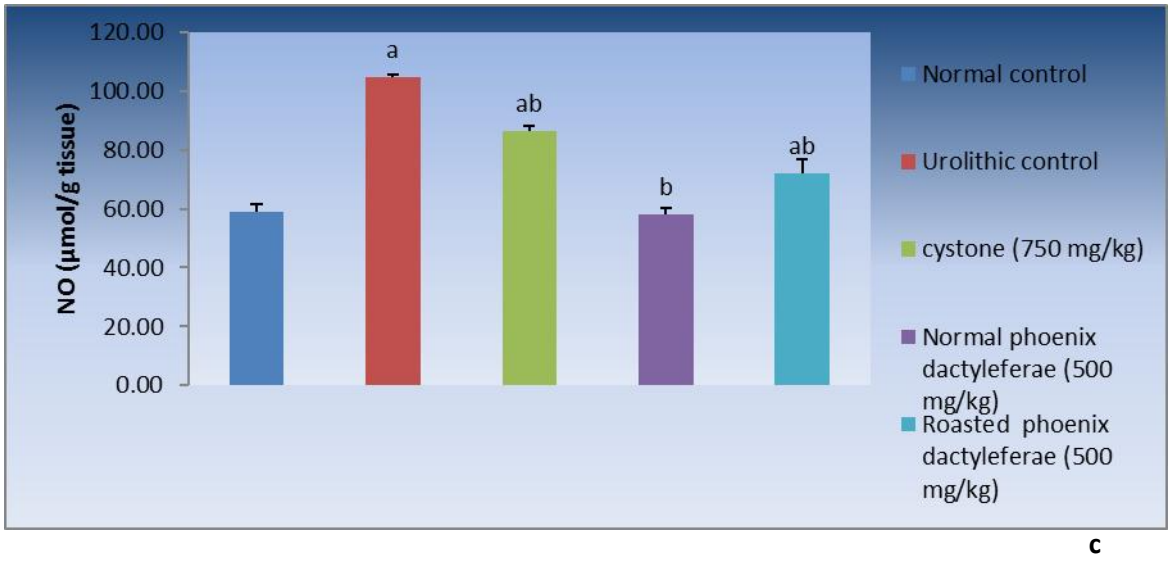


Figure 1: Effects of treatment with non-roasted and roasted Phoenix dactylifera on renal (a) GSH, (b) MDA and (c) NO contents.

Data were expressed as mean ± SE (n=6). Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons.[a] Significantly different from normal control at P<0.05. [b] Significantly different from urolithic control at P<0.05.

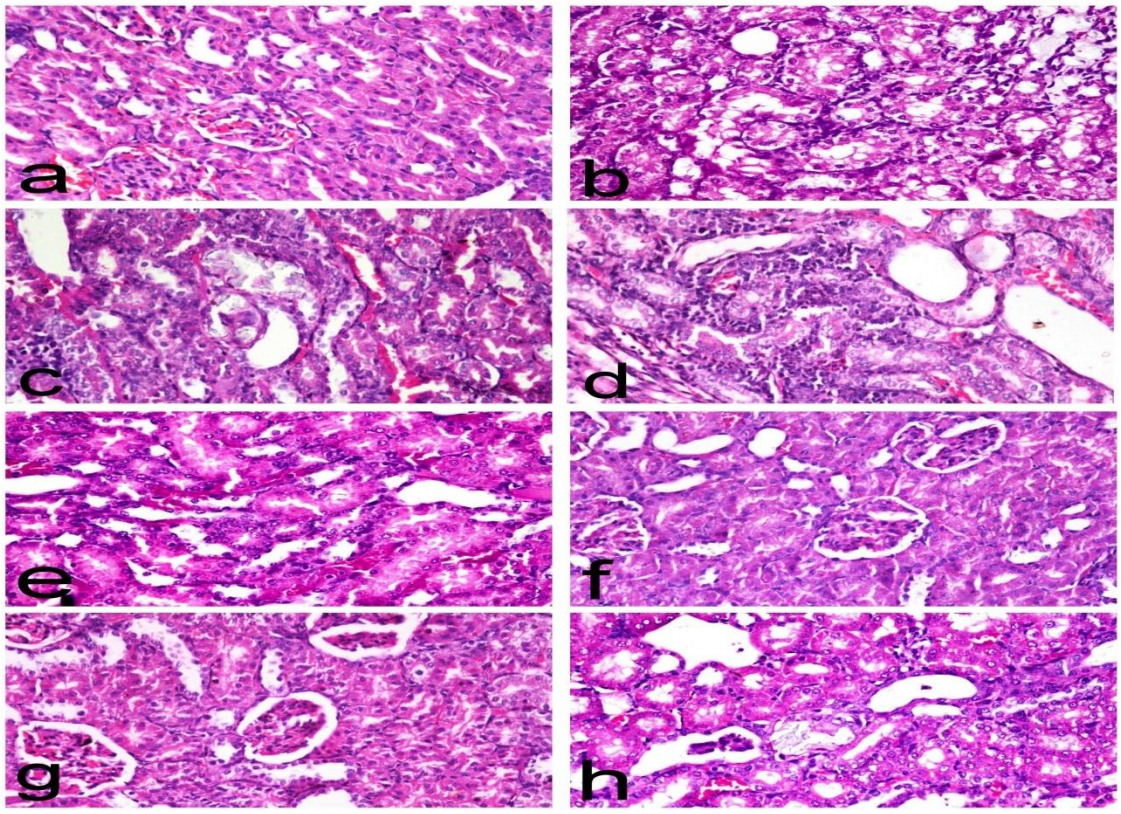


Figure 2: Photomicrograph of kidney tissue from (a) normal control rats showing normal renal glomeruli and tubules, (b, c, d & e) urolithic control rats showing (b) extensive vacuolar degeneration of renal tubular epithelium, (c) presence of CaOx crystals in the lumen of dilated renal tubules, (d) Intense peritubular and interstitial mononuclear cell infiltration & (e) regenerative renal tubules, (f) Cystone treated rats showing mild tubular dilatation, (g) Non-roasted Phoenix dactylifera (500mg/kg) treated rats showing mild vacuolar degeneration of renal tubular epithelial cells, and (h) Roasted Phoenix dactylifera (500mg/kg) treated rats showing vacuolar degeneration of renal tubules and presence of CaOx crystals in the lumina of some renal tubules. (H & E, X400).

DISCUSSION

Urolithiasis is a complex process which is a consequence of disturbance between promoters and inhibitors in the urinary tract. Excretion of oxalate in normal individuals is harmless because they are natural byproduct of metabolism. However, oxalate can be toxic when excreted in urine by large amounts due to they may crystallize at physiological pH and produce calcium oxalate [26]. It is agreed that the mechanisms of urinary stones formation involve multi-complex steps leading to crystal nucleation, aggregation and growth of insoluble deposits, although the full mechanisms are not fully understood [27].

Several compounds, such as ammonium chloride, ethylene glycol, glycolic acid, and glycine can produce oxalic acid by metabolism *in vivo*. Ethylene glycol and ammonium chloride always used as urolithiasis induction agents because they induce CaOx crystalluria without severe renal damage in rats and they mimic the etiology of stone formation in human. Ammonium chloride causes acidification for urine consequently decreases the citrate excretion in urine. This, in turn, increased the deposition of renal CaOx crystal and accelerated the lithiaisis [28]. In the present study, male albino rats were selected to induce urolithiasis because the similarity between urinary system in male rats and the human's in addition to that earlier studies have also reported that a number of precipitates in female rats was significantly less [29].

After administrations of ethylene glycol showed a significant decrease in urine volume and significant elevate in urine pH as compared with those of normal group. Treatment of rats with both normal and roasted date seed extracts elevated urine volume and while roasted date seed

extract only decreased urine pH compared with ethylene glycol group (lithiatic group), these results agree with the results of previous study, which stated that this reinforces the plant (*Boerhaavia diffusa*) having diuretic activities which may be advantage in lithiatic condition. As increased urine output is recommended to reduce the possibility of stone formation [30]. In addition, the increase of urine output will dilute the concentration of urinary electrolytes which reduce the chances of precipitation of calcium and phosphorus.

The level of calcium in urine was increased significantly after administration of ethylene glycol, inducing the nucleation and precipitation of calcium oxalate. But on administration of roasted and normal seed extracts to the animals, the amounts of calcium in the urine were reduced significantly; and the normal seed extract showed highly significant effect compared to lithiatic group. It has been stated that high concentration of calcium in urine and serum initiate the precipitation process of calcium oxalate and calcium phosphate crystal [31]. In general the antiurolithiatic activity of date seeds extracts may due to the chemical composition of seeds which recorded in previous studies such as carbohydrates, and/or glycosides, sterols and/or triterpenes, tannins, flavonoids, alkaloids and/or nitrogenous bases that may have either diuretic effects or stone dissolving effects and/or as antioxidants that established in previous study [21].

Supersaturation is the step that occurs due to the presence of substances producing the kidney stones in high concentration in the urine following the decrement of urine volume and the concentration of chemicals that inhibit stone formation. In our study the magnesium level in urine showed a significant decrease in urolithiatc group upon administration of ethylene glycol when compared to control group, the increase in urine magnesium level was recovered in animals that treated by either normal or roasted seed extracts, as a consequence of the decrease of growth rate and nucleation of calcium crystals when compared to Cystone group.

Low urinary contents of crystallization inhibitors as citrate, magnesium, phosphate are a common feature in stone formation [32] and [33], because of magnesium complexes with oxalate, thus reducing CaOx supersaturation in urine [34], as a consequence decrease the growth and nucleation rates of calcium crystals [35]. Seeds of *Phoenix dactylifera* Linn. caused diuresis more than Cystone as shown in our results and facilitated the process of dissolving the preformed crystal deposits, improved the renal function by facilitating the removal of nitrogenous waste

product and decreasing the excretion of oxalate probably by interfering with metabolism.

In urolithiasis, there is a decrease in the glomerular filtration due to the obstruction of urine flow by stones in urinary system [36]. This causes impairment of renal function resulting in decreased excretion of waste products, particularly nitrogenous substances such as urea, creatinine, and uric acid with concurrent accumulation in blood [37]. In the present study administration of ethylene glycol with ammonium chloride showed a significant elevation in serum creatinine and urea compared with those of normal group which indicates the marked renal damage [38]. However, treatment of rats with both normal and roasted date seed extracts decreased urea serum level while normal date seed extract reduced the level of serum creatinine compared with ethylene glycol group which as in previous study suggested to prevents impairment of renal function [39]. These results supported by our histopathological study that showed extensive vacuolar degeneration of renal tubular epithelium with presence of CaOx crystals in the lumen of dilated renal tubules in ethylene glycol group while the treatment of rats with non-roasted date seed extracts reduced histopathological alterations.

Stone inducing treatment caused hypertrophy and extensive calcium oxalate crystal deposition in kidneys, oxidative damage as reflected from increased MDA and decreased activities of antioxidant enzymes like GSH in kidneys and deteriorated renal functions [40]. Increased lipid peroxidation and decreased levels of antioxidant potential have been reported in the kidneys of rats supplemented with ethylene glycol [41]. In our present investigation, renal GSH content was significantly reduced following ethylene glycol administration, and a significant elevation of renal MDA and NO contents were detected. On the other hand, normal levels of oxidative stress biomarkers were observed in groups treated with normal and roasted date seed extracts as compared to ethylene glycol and Cystone groups.

CONCLUSION

The present results suggest the antiurolithiatic effects of the roasted and normal *Phoenix dactylifera* seeds extract to be mediated through a combination of calcium oxalate crystal inhibition, as well as via diuretic, antioxidant, renal epithelial cell protective, and hyper-magneseuric effects. These effects may be attributed to the presence of carbohydrates, alkaloids,

triterpenoids and flavonoids etc. Further studies are recommended to determine the pointed compounds and their toxicity on human.

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