Effect of *Cymbopogon proximus* (Mahareb) on ethylene glycol-induced nephrolithiasis in rats

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Nephrolithiasis is a common, painful, costly and recurrent disease, the management of which remains to be an enigma. Phytotherapeutics could be useful as either alternative or complementary therapies in the management of nephrolithiasis. This study was designed to test the efficacy of *Cymbopogon proximus* (CP), a traditional Sudanese plant commonly known as “Mahareb”, in preventing ethylene glycol-induced nephrolithiasis in rats. Thirty male Wistar albino rats were divided randomly into 3 groups of 10. Group 1 was the normal control. Group 2 (stone group) had free access to drinking water containing 0.75% ethylene glycol (EG) and 2% ammonium chloride (AC). Group 3 (test group) was treated as group 2 and was simultaneously injected with *C. proximus* 5% aqueous extract at a dose of 1.5 ml/100 g body weight/day for 10 days. At the end of the treatment period, serum levels of creatinine, blood urea nitrogen (BUN), calcium and phosphorous were determined; measurements of kidney calcium levels were also performed and kidney histopathological examinations were done. The stone group had the highest levels of serum calcium and BUN as well as the highest kidney calcium level. Large crystal deposits were also seen in this group. The CP treated group showed significantly lower levels of serum calcium, serum BUN and kidney calcium (p<0.01); crystal deposits were not observed in this group. The results obtained suggest that CP has a significant protective effect against ethylene glycol-induced nephrolithiasis in rats.

Key words: Sudanese herbal medicine, *Cymbopogon proximus*, nephrolithiasis, calcium oxalate, ethylene glycol, ammonium chloride, aqueous extract.

INTRODUCTION

Kidney stone disease or nephrolithiasis is a common disease with an increasing incidence (Hiatt and Friedman, 1982). It is characterized by a high rate of recurrence (Uribarri et al., 1989), thus prevention is widely recommended. Calcium oxalate (CaOx) represents up to 80% of analyzed stones (Khan, 1997; Jihong et al., 2007). Currently available options for the treatment or prevention of nephrolithiasis are costly, ineffective in all patients or not without side effects (Atmani et al., 2003; Johri et al., 2010). Alternative treatments have been sought especially from herbal medicines, in conjunction with the resurgence of interest in phytotherapy and medicinal plants, as sources of effective, safe, cheap, and socially accepted treatments (Atmani et al., 2003; Johnston, 1997; Bennett and Jand Brown, 2000). In addition, there are several antiurolithic herbal remedies provided by many

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MATERIALS AND METHODS

Plant

The plants of *C. proximus* (Hochst. ex A. Rich.) Stapf, were collected and botanically authenticated by an expert in taxonomy and voucher specimens were deposited at the National Herbarium, Medicinal and Aromatic Plants Research Institute, National Centre for Researches, Khartoum, Sudan.

Preparation of plant extract

The whole plants were washed, air dried and then milled to a fine powder. The powder was suspended in distilled water at a concentration of 5%. The suspension was kept at continuous mixing for 24 h. After settlement, the supernatant (water soluble extract) was used for animals’ administration.

Animals

Thirty male albino Wistar rats weighing 150 ± 5 g were allocated into three groups (I, II, and III) of ten animals for each, they were housed in standard cages at standard laboratory conditions (temperature: 25 ± 2°C), and maintained under a controlled 12 h light/dark cycle. All animals had *ad libitum* access to normal drinking water and standard rat diet. Prior to experiment commencement, animals were left one week for an acclimatization period. Experiments were conducted in accordance with internationally accepted standard guidelines for the use of animals.

Reagents

Ethylene glycol (EG; VEB Laborchemie Apolda, Germany), was used to produce hyperoxaluria and consequently the deposition of CaOx crystals in rat kidneys. It was added in drinking water to a final concentration of 0.75% V/V. Ammonium chloride (AC; Elnasr Pharmaceutical Chemistry Co., Egypt) was combined with EG to enhance crystal deposition. It was also added in drinking water to a final concentration of 2% W/V.

Experimental design

Animals in group I served as the normal or negative control group and received no treatment throughout the experimental period. Group II animals (positive control group) received the crystal inducing chemicals, 0.75% EG and 2% AC in their drinking water for ten days. Animals in group III (test or preventive regimen group) were treated as group II and in addition they were given simultaneously 5% *C. proximus* aqueous extract daily by gavage at a dose of 1.5 ml/100 g body weight (Fan et al., 1999).

Assessment of antiurolithic activity

At the end of the 10 days treatment period, animals were mildly anaesthetized using light ether; blood samples were collected from the retro-orbital region in non-heparinized tubes, centrifuged at 3000 rpm for 15 min. to obtain serum. Samples were then analyzed for creatinine, blood urea nitrogen (BUN), calcium and phosphorous levels. After the collection of blood samples, animals were sacrificed, abdomens opened and kidneys removed. The left kidneys were dried in an oven at 100°C. Each kidney was then weighed and subsequently minced (homogenized) in a beaker containing 7 ml of 0.5 N nitric acid, the mixture was then heated until dissolution was affected and a transparent liquid was obtained, from which the kidney calcium level was determined using flame spectroscopy. The amount of calcium was expressed as µg/g dry kidney (Touhami et al., 2007). The right kidneys were fixed with 10% neutral buffered formalin and subsequently embedded in paraffin, cut into 4 µm thick sections for slides, and stained with hematoxylin and eosin. The sections were examined under light microscopy by a histopathologist (Figure 1).

Statistical analysis

Data were presented as the mean ± standard deviation (SD). One way analysis of variance (ANOVA) was used for comparisons among different groups followed by the post hoc test, Fisher's least significant difference (LSD) for multiple comparisons. P value < 0.05 was considered to indicate a significant difference. Statistical Package for Social Sciences (SPSS) version 18 was used for this analysis.

RESULTS

Serum analysis

Serum levels of creatinine and BUN were significantly
Table 1. Serum creatinine, BUN, calcium, phosphorous and kidney calcium in the three groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative control group</th>
<th>Positive control group</th>
<th>Test group</th>
</tr>
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<tbody>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.72 ± 0.02</td>
<td>1.44 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.85 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>BUN (mg/dl)</td>
<td>31.83 ± 1.23</td>
<td>47.15 ± 0.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.41 ± 1.62&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>Calcium (mg/dl)</td>
<td>8.54 ± 0.17</td>
<td>6.30 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.06 ± 0.06&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phosphorous (mg/dl)</td>
<td>8.01 ± 0.04</td>
<td>8.88 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.22 ± 0.09&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney homogenate calcium (µg/g)</td>
<td>7.68 ± 0.13</td>
<td>28.52 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.72 ± 1.94&lt;sup&gt;ab&lt;/sup&gt;</td>
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<sup>a</sup>Comparisons are made with group I; <sup>b</sup>Comparisons are made with group II; <sup>*</sup>Statistically significant at P < 0.001.

Elevated in the positive control group as compared to the normal control group; whereas the test group showed lower levels of both creatinine and BUN, approximating normal values. Calcium levels were reduced in the positive control group (6.23 mg/dl) in relation to the negative control group (8.62 mg/dl), while the test group (EG/AC/CP treated) revealed a significant (P < 0.001) elevation in calcium levels (7.03 mg/dl) as compared to the positive control group. Serum levels of phosphorous were the highest in the positive control group (8.94 mg/dl), while the test group had the lowest levels (7.25 mg/dl). Differences were significant (P < 0.001) among the three groups (Table 1).

Calcium levels in the kidneys

Calcium levels in the kidneys of rats in the positive control group, were remarkably increased (28.47 µg/dl) compared to levels produced by the negative control rats (7.69 µg/dl). Simultaneous treatment of rats in the test group with the herbal extract significantly reduced kidney calcium contents (9.33 µg/dl) with a probability of chance occurrence (P < 0.001).

Histological examination

Renal cortexes and medullas of animals in the positive control group showed marked tubular cell necrosis, tubular atrophy, intraluminal deposits, diffuse interstitial edema, interstitial inflammatory cell infiltrate, and focal fibrosis (Figure 2). Glomerular morphology remained unchanged except that few collapsed glomeruli were seen. Plant treated group showed almost normal histological features, no calcium deposits were seen (Figure 3). These morphological findings were consistent with the data of the left kidney calcium level.

DISCUSSION

At present, antiurolithic treatments provided by conventional medicine are costly, ineffective in all patients, not well tolerated, or not without side effects (Atmani et al., 2003; Johri et al., 2010). In addition, recurrence is a central problem in urolithiasis with quite high rates (Uribarri et al., 1989; Ruml et al., 1997) and thus prophylactic treatment is highly recommended. Various pharmacological and non pharmacological treatments have been applied for this aim. Although, these preventive measures have improved the medical management of nephrolithiasis and lead to reduction in the rates of stone formation and prolongation of the post surgical stone-free periods (Ruml et al., 1997), stone recurrence can be reduced by only half, and there is so far no satisfactory drug for the treatment of nephrolithiasis, especially for the prevention or the recurrence. Due to the complications of nephrolithiasis treatment along with the worldwide resurgence of interest in medicinal plants and phytotherapy (Johnston, 1997; Bennett and Jand Brown, 2000), there has been a trend to look for alternative antiurolithic treatments from natural sources and to test the antilithic remedies that are found in many traditional systems of medicine all over the world (Atmani et al., 2003; Atmani, 2003; Butterweck and Khan, 2009).

This study was designed to prove the possible antiurolithic property of one of the herbs that are used traditionally for kidney stones, <i>C. proximus</i>. The preventive effects of the plant extract on a calcium oxalate urolithic rat model induced by the ingestion of EG in conjunction with AC were particularly studied. By combining two urinary stone risk factors (hyperoxaluria and hypocitraturia), EG/AC combination is an accelerated model of nephrolithiasis in which animals are rendered nephrolithic by the seventh day of treatment (Khan, 1997; Fan et al., 1999).

Assessment of serum creatinine and blood urea nitrogen (BUN) was carried out to test the renal function, and as markers of glomerular and tubular damage (Thamilselvan and Menon, 2005). Results revealed significantly higher levels of creatinine and BUN in the EG/AC treated group when compared with the normal control group (P < 0.001); in agreement with the results of other studies (Jihong et al., 2007; Touhami, 2007; Thamilselvan and Menon, 2005; Al-Attar, 2010; Bahuguna et al., 2009). In nephrolithiasis, the glomerular filtration rate (GFR) is reduced due to obstruction of the urinary outflow with stones; as a result waste products particularly nitrogenous compounds such as creatinine,
uric acid and urea accumulate in the blood. In addition, exposure to high levels of oxalate and calcium oxalate crystals is known to induce lipid peroxidation in renal tubular epithelial cells which is associated with the production of free radicals; this will cause damage to the renal tubular membrane surface and is considered relying upon recent evidence- to be a prerequisite for the nucleation, attachment, and development of CaOx kidney stones (Thamilselvan and Menon, 2005; Thamilselvan et al., 2000). In this study, renal damage was indicated by the elevated creatinine and BUN (Touhami et al., 2007; Bahuguna et al., 2009).

Two more serum parameters were assessed, calcium and phosphorous. Results revealed that the EG/AC treated group had a lower calcium and a higher phosphorus level in serum as compared to the normal group (P < 0.001). The reduced serum calcium level correlates with an increased urinary calcium excretion and a subsequent deposition of calcium oxalate in the kidney as a consequence of treatment with EG. Similar results were obtained from other studies (Al-Attar, 2010; Rajagopal, 1977; Hiramaya et al., 1993). In contrast, a study by Touhami et al. (2007) showed an elevated serum calcium in the EG/AC treated group; the cause of which was not explained (Touhami, 2007). With regard to phosphorous, the group treated with EG/AC showed a significantly higher serum level in agreement with the findings of other studies; and is explained as being the result of the nephrotoxic effect of glycol (Touhami, 2007; Rajagopal, 1977; Patel et al., 2011). On the other hand, studies by Tsai et al. (2008) and Tsai et al. (2009), have shown a lower level of serum phosphorous, in EG fed rats (Tsai et al., 2008; Tsai, 2009), whereas Al-Attar (2010) obtained insignificant changes.

Levels of calcium in the kidneys were also determined as an evidence of the presence of calcium deposits. Results showed that the EG/AC treated group had about four folds higher renal tissue calcium level than the normal control group; in consistence with previous findings (Jihong et al., 2007; Touhami et al., 2007; Fan et al., 1999; Bahuguna et al, 2009; Mitra et al., 1998; Soundararajan et al., 2006).

For further confirmation, histopathological examinations were performed. Results revealed that rats fed with 0.75% EG plus 2% AC in drinking water for 10 days (positive control group), successfully produced renal deposition of CaOx crystals. Intratubular crystal deposits were seen in both cortex and medulla; in addition, there was marked tubular cell necrosis, tubular atrophy, diffuse interstitial edema, interstitial inflammatory cell infiltrates...
and focal fibrosis; few collapsed glomeruli were also observed (Figure 2). These findings are consistent with the findings of Fan et al. (1999), Jihong et al. (2007) and Touhami et al. (2007) and they provide extra validation to the use of combined EG/AC rat urolithiasis model.

In the group treated with *C. proximus*, 5% aqueous extract simultaneously with EG/AC stone induction, there was a significant (P < 0.001) reduction in serum levels of creatinine and BUN as compared to the EG/AC treated group indicating an improved or almost normal renal function. Serum calcium levels were markedly elevated in this group approximating the value of the normal group, in contrast to the reduced calcium level in the EG/AC group; this suggests that the plant administration caused a reduction in the urinary excretion of calcium which is usually increased after EG ingestion and is eventually followed by the deposition of CaOx crystals in renal tissue, thus the plant by this way may prevent CaOx stone formation. Phosphorus level which was elevated in the EG/AC group, was significantly reduced in the plant treated group even to a lower level than that of the normal group; this indicates a protective action of the plant against glycol nephrotoxicity.

Kidney calcium contents in the CP treated group were significantly reduced (P < 0.001) as compared to calcium levels in the EG/AC only treated group indicating none or minor calcium crystal deposition in the plant treated group.

Histopathology findings in the CP treated group showed almost normal renal architecture. There was no tubular cell necrosis, no tubular lumen dilation, no intraluminal stone, nor diffuse interstitial edema. Only mild interstitial inflammatory cell infiltrates were observed, while the glomerular morphology remained unchanged (Figure 3). These findings when compared with the EG/AC only treated group suggest a potent protective effect of the plant extract against EG/AC induced nephrolithiasis and nephrotoxicity.

From the results of this study, it was supposed *C. proximus* to have a preventive effect against CaOx nephrolithiasis that could be attributed to one or more actions of the plant. First, the plant has a well known, highly reputed diuretic action (Khalid et al., 2010; Eltohami, 2012; El Tahir and Abdel Kader, 2008; El-Askary et al., 2003; Selim, 2011), and as far as renal stones are concerned, an increased urine output is desirable.
so that the concentration of stone forming constituents is kept below the level of supersaturation which is the starting event in the process of stone formation. Secondly, an antilithic effect of the plant can be solely explained in terms of its antioxidant property, due to the increasingly growing body of evidence that suggests a decisive role of hyperoxaluria induced oxidative stress and renal cell injury in the pathogenesis of stone disease (Thamilselvan and Menon, 2005; Thamilselvan et al., 2000). Indeed many antioxidants and free radical scavengers have been studied in this context and were shown to inhibit CaOx crystal deposition by way of improving the tissue antioxidant status (Thamilselvan and Menon, 2005; Selvam, 2002; Grases et al., 2009). In addition, the antilithic effect of some herbals was claimed in recent studies to be due to their antioxidant activity rather than a diuretic effect or a change in urinary chemistry (Grases et al., 2009; Itoh et al., 2005; Sailaja et al., 2011; Ashok et al., 2010; Dodoala et al., 2010; Bashir et al., 2010). *C. proximus* was shown in a study by Selim (2011) to possess a strong antioxidant activity; the total antioxidant capacity of *C. proximus*, expressed as the number of equivalents of ascorbic acid, was found to be 48.66±3.1. Methanolic extracts of *C. proximus* also showed a highly effective free radical scavenging in the DPPH assay (Selim, 2011). The plant contains the flavonoids rutin and quercetin (Heiba and Rizk, 1986) which are well known antioxidants, both of which have been explored for antilithic activity and were proved to be effective (Park et al., 2008; Ghodasara et al., 2010). Besides the diuretic and antioxidant effects, the plant has a unique antispasmodic property as it produces smooth muscle relaxation while preserving the propulsive movement of the tissue (El-Askary et al., 2003; Selim, 2011), thus it has a further beneficial effect of relieving renal colic pain which is commonly associated with kidney stones, and at the same time aiding in the propulsion of stones especially ureteric ones. The antispasmodic principle of the plant "proximadiol" has been isolated and is already traded for this purpose (Abdel-Azim et al., 2011). Moreover, the potent antibacterial effect of the plant against multiantibiotic-resistant organisms (Selim, 2011) may be useful, as kidney stones are sometimes complicated with urinary tract infections especially if there is obstruction; and conversely infections can be a cause of certain types of stones such as struvite stones and even calcium stones.

Recent evidence suggests a role of intracellular
REFERENCES


