Antiurolithiatic Activity of *Pinus Eldarica* Medw. Fruits Aqueous Extract in Rats

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Purpose: To evaluate the antiurolithiatic activity of *Pinus eldarica* fruit on induced calcium oxalate nephrolithiasis in rats.

Materials and Methods: Calcium oxalate nephrolithiasis in rats was induced by administering ethylene glycol 1% for 30 days via drinking water. The prophylactic and therapeutic groups received *P. eldarica* fruit extract (500 and 1000 mg/kg/day) as well for 30 days and from the 14th day through the end of the experiment, respectively. The following variables were assessed; urine volume, urinary calcium excretion, and crystalluria. Finally, rats' kidneys were histopathologically examined.

Results: The aqueous extract prophylactic treatment (500 mg/kg/day) increased urinary calcium excretion. Qualitative analysis of crystalluria and histopathologic examination showed that the administered dose of extract prevented stone formation in the kidneys significantly. The prophylactic treatment did not increase urine volume in comparison with ethylene glycol. Stone formation did not decrease in the treatment group.

Conclusion: This study indicates that *P. eldarica* fruit extract prevents calcium oxalate deposition, without producing diuresis.

Keywords: Pinus, kidney calculi, calcium oxalate, ethylene glycol

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INTRODUCTION

Urolithiasis is defined as the presence of one or more calculi in any location within the urinary tract. The disease affects 1% to 5% of the population in developed countries with a peak incidence between 20 and 50 years of age. Men are three times more likely to be affected than women and the lifetime risk of developing a calculus in a Caucasian man is nearly 20%.[1] It has been reported that 91% of the urinary calculi contain calcium in some form, while 8% and 1% are composed of uric acid and cystine, respectively. The calcium-containing calculi consist of pure or various amount of calcium components such as calcium oxalate monohydrate, apatite, calcium hydrogen phosphate, and calcium carbonate. In men, 70% to 80% of the calculi contain either calcium oxalate alone or in combination with apatite.[2]

The family Pinaceae (pine family) includes many conifers such as Cedrus, Abies, Larix, Picea, and Pinus. The Iranian Pine, *Pinus eldarica*, is famous as a Tehran pine and is a tree with wide spacing between branches and stiff long dark green needles at maturity. Scientific studies about *P. eldarica* are largely restricted to botanical research. This

plant consists of quercetin, pinene, myrcene, caryophyllene, camphene, and abietic acid. The main constituents of the leaf oil are germacrene D (26.6%), β-caryophyllene (17.1%), α-pinene (11.8%), β-pinene (7.9%), elemicin (4.3%), and α-humulene (4.2%). Major components of the fruit oil are β-caryophyllene (34.0%), α-pinene (16.3%), longifolene (10.5%), α-humulene (6.4%), δ-3-carene (6.3%), and β-pinene (3.8%).[3] The compounds of turpentine oil from title pine species are relatively stable, and the oil content of the resin has been reported to be 15% to 19%, and varies between 9.7% to 19.3%. Monoterpenes consist of α-pinene (31.5% to 64.2%), δ-3-carene (16.9% to 42.8%), and β-pinene (2.5% to 11.7%) mainly. The contents of camphene, myrcene, limonene, β-phellandrene, γ-terpinene, terpinolene, tricyclene, sabinene, and p-cymene are much smaller.[4]

In traditional medicine, it is alleged that some species of *Pinus* such as *P. gerardiana Wall* clears fluids of the kidneys and the bladder, and strengthens them to store urine. It also treats polyurea (of renal or cystic origin) and stops the formation of stones and ulcers in the bladder.[5] In this study, we evaluated the antiurolithiatic activity of *P. eldarica* fruit on induced calcium oxalate nephrolithiasis in rats.

MATERIALS AND METHODS

Plant Material. Fruits of *P. eldarica* were collected from School of Pharmacy in Mashhad, Iran. Voucher samples were preserved for reference at the Herbarium in Department of Pharmacognosy, School of Pharmacy, Mashhad.

Animals. Adult Wistar male and female rats (200 ± 10 g) were chosen for study. Animals were housed in plastic cages in an animal colony room 12/12 hours light/dark cycle at 21 ± 2 °C with food and water ad libitum. All animal experiments were carried out in accordance with Mashhad University of Medical Sciences, Ethical Committee Acts.

Preparation of Extract. In decoction method, the fruits powder (100 g) was added to one liter hot distilled water for 30 minutes and then filtered through cloth. The extract was then evaporated to dryness at 50 °C (yield: 5.5 % w/w).

Experimental Protocol.[6-7] Hyperoxaluria and calcium oxalate deposition in the kidneys were induced by ethylene glycol in the drinking water to a final concentration of 1% for 30 days to induce lithiasis. Group 1 was given ethylene glycol 1% only for 30 days; group 2 received water plus normal saline; groups 3 and 4 (prophylactic groups) received ethylene glycol 1% plus the extract 500 or 1000 mg/kg/day for 30 days; groups 5 and 6 (therapeutic groups) received ethylene glycol 1% plus the extract 500 or 1000 mg/kg/day from the 14th day through the end of the experiment. Urine samples (24-hour) were collected at 0, 7, 14, and 30 days. All agents were given via drinking water.

To complete the treatment, on the 31st day, all rats were sacrificed and both kidneys of each rat were removed for histopathologic examination. Removed kidneys were fixed in 10% buffered formalin (MERK) overnight and then each was sliced longitudinally in 3 sections, including anterior, middle, and posterior parts of the kidney. Thereafter, they were automatically processed and inserted in paraffin blocks, and at least 6 sections with 5 micron thickness were obtained from each kidney and stained by Hematoxylin and Eosin.

For accurate microscopic evaluation, each stained tissue slice were divided into 8 parts encompassing the cortex and medulla, and from each part, a cortical and a medullary field were randomly studied by an Olympus microscope, type BX 40, with a magnification of 400 X (10 × 40). So for both kidneys of a rat, 192 microscopic field-areas were evaluated and undoubted stones in renal tubules were calculated. Calcium in urine was analyzed using atomic absorption spectroscopy.

Statistical Analysis. Data were presented as mean ± standard error. Statistical analysis was performed by SPSS software (Statistical Package for the Social Science, version 13.0, SPSS Inc, Chicago, Illinois, USA), using one-way ANOVA followed by Tukey-Kramer post-hoc test for multiple comparisons. *P* values less than .05 were considered statistically significant.

RESULTS

Urinary Calcium. Urinary calcium excretion was increased after 14 days by ethylene glycol treatment. The prophylactic treatment of extract (1 g/kg) increased calcium excretion during 7, 14, and 30 days of the treatment. The pretreatment effect of extract on urinary volume on day 30 is shown in figure 1.

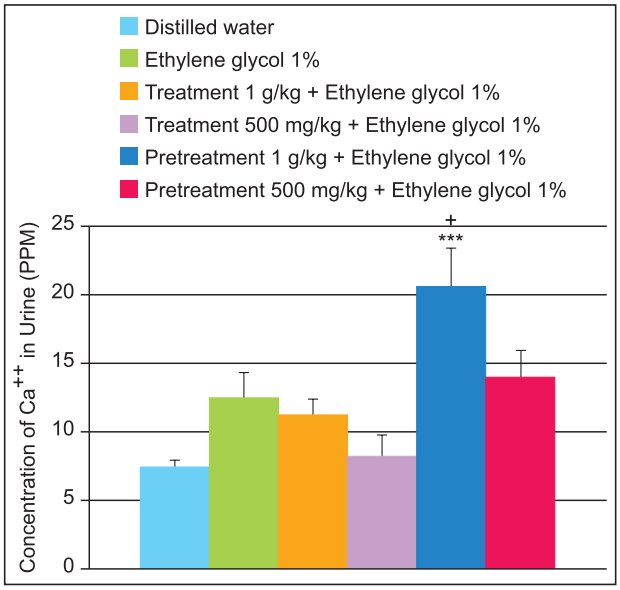

Figure 1. Effect of *P. eldarica* fruit extract on urinary concentrations of calcium in nephrolithiasis induced by ethylene glycol 1% after 30 days. Values are expressed as mean ± S.E.M. of 8 rats in each group. *** $P < .001$ compared with distilled water; + $P < .05$ compared with ethylene glycol group, Tukey-Keramer test.

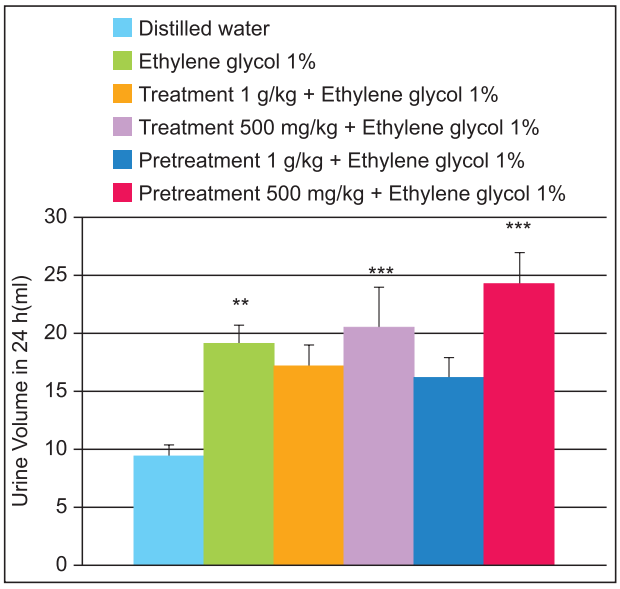

Figure 2. Diuretic activity of *P. eldarica* fruit extract in rats on day 30. Values are expressed as mean ± S.E.M. of 8 rats in each group. ** $P < .01$; *** $P < .001$ compared with distilled water, Tukey-Keramer test.

Diuretic Activity. After 7 days, a low dose of extract (prophylactic group) increased mildly the urine volume. Ethylene glycol increased urine volume after 30 days of the treatment. Practically, the extracts (treatment and prophylactic treatment) did not show diuretic activity compared with ethylene glycol group. The pretreatment effect of extract on urinary volume on day 30 is shown in figure 2.

Histopathologic Findings. Induced urinary stones by ethylene glycol were presented as well-formed crystalloid materials and more predominantly in cortical than medullary renal tubules (Figure 3). Also in severe nephrolithiasis, stones were observable in renal calyces. Ethylene glycol dramatically induced stone formation in renal tubules. There was a considerable

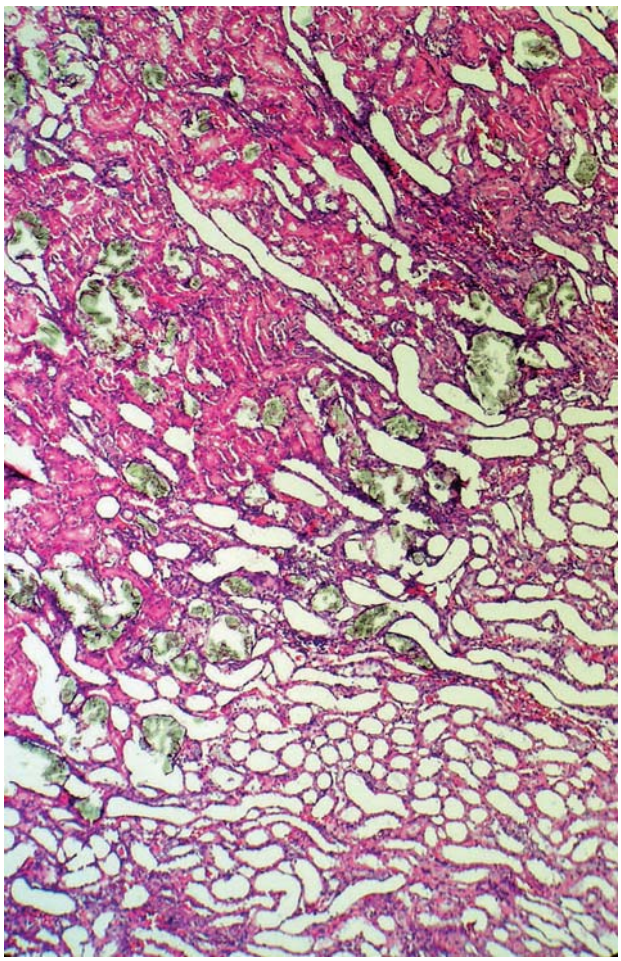

Figure 3. Histopathologic appearances of many stones in an ethylene glycol-treated rat (X 100, Hematoxylin and Eosin).

reduction in stone formation by the kidney with prophylactic dose of extract (1 g/kg) (Figure 4). The extract did not decrease the deposition of crystal in the treatment group (Figure 5).

Some other considerable histopathologic findings such as focal renal tubular dilatation

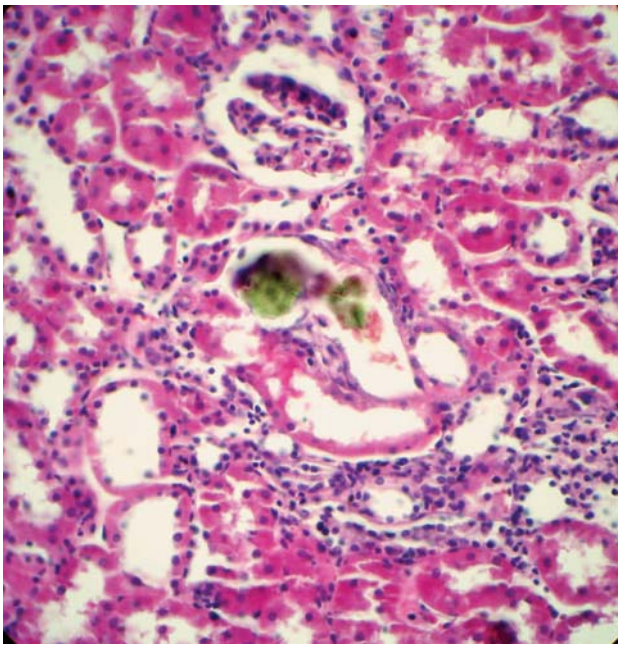

Figure 4. Histopathologic appearances of one cortical tubule in a *Pinus eldarica* extract-pretreated rat (X 200, Hematoxylin and Eosin).

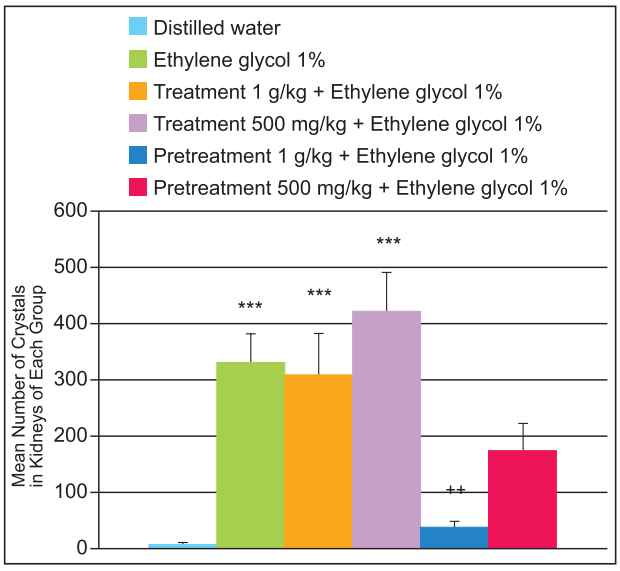

Figure 5. Effect of *P. eldarica* fruit extract on number of crystal deposited in the kidney of rats. Values are expressed as mean ± S.E.M. of 8 rats in each group. *** $P < .001$ compared with distilled water; ++$P < .01$ compared with ethylene glycol group, Tukey-Keramer test.

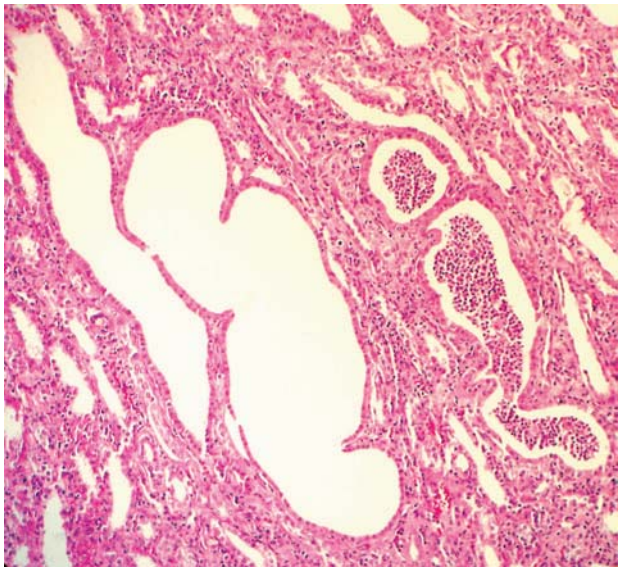

Figure 6. Histopathologic appearances of tubular dilatation, interstitial inflammation, and leukocyte-cast formation in an ethylene glycol-treated rat (X 200, Hematoxylin and Eosin).

(due to tubular obstruction by the stones), focal tubular epithelial necrosis, and focal interstitial inflammation were also noted in some rats, but were not included in results (Figure 6).

DISCUSSION

The prophylactic administration of *P. eldarica* fruit extract significantly inhibited the formation of calculi without diuretic activity. Consistent with other studies,[7-9] ethylene glycol administration resulted in development of persistent crystal formation in the kidney in all the rats. Histopathologic study also confirmed the deposition of calcium oxalate crystals and the protective effect of the prophylactic administration of *P. eldarica* fruit extract.

Ethylene glycol elevated the urinary concentration of calcium; thereby, contributed to renal stone formation like other experiments. Unlike rats with urolithiasis, the level of calcium oxalate in the kidney tissue significantly reduced in rats that were pretreated with Pinus extract. This is in contrast to the urine level of calcium, which was found to be higher than those with hyperoxaluria, demonstrating the presence of insignificant crystalluria. This effect has also been reported with green tea, which had inhibitory effect on calcium oxalate urolithiasis.[10] Urinary calcium excretion increased gradually

after administration of green tea and it was significantly higher than those in control group at 21 days after administration. The inhibitory effect of green tea on calcium oxalate urolithiasis is most likely due to antioxidative effects.[9]

The extract probably prevents urinary stone formation by excretion of small particles from the kidney and reducing the chance of them being retained in the urinary tract. Therefore, we suggested that the *P. eldarica* fruit's extract can maintain calcium oxalate particles dispersed in the solution and thus, allows them to be eliminated easily from the kidney. There was no apparent diuretic affect of the plant extract. The calcium excretion remained higher in treated than untreated rats; however, the reason is unclear and further analysis is needed to clarify this issue. The analysis of crystals in the kidney showed that rats in prophylactic group had less urolithiasis in the kidney. Rats pretreated with the extract had limited calcium oxalate deposition. The therapeutic administration of the extract did not affect the stone formation; hence, it can be stated that the plant can not dissolve pre-existing particles.

There is *in vivo* evidence that hyperoxaluria-induced peroxidative damage to the renal tubular membrane surface provides a favorable environment for individual calcium oxalate crystal attachment and subsequent development of the kidney stones. In a study, vitamin E administration completely prevented calcium oxalate crystal deposition in the kidney, by preventing hyperoxaluria-induced lipid peroxidation and tissue antioxidant imbalance.[11] There are many reports about antioxidant activity of Pinus species.[12-14] Thus, it is possible that the extract prevents stone formation via antioxidant effect.

In another study, the rate of stone formation decreased markedly in those patients who were treated with non-steroidal anti-inflammatory drugs.[15,16] As some Pinus species have anti-inflammatory activity,[17] this effect may be a contributory factor in antiurolithiatic activity of *P. eldarica* extract as well.

Tubular epithelium damage by any process such as infectious agents and mineral forming nanobacteria, may trigger the stone formation cycle.[18] Many Pinus species have antibacterial activity and this effect may prevent mineral forming nanobacteria growth and stone formation.[19-22]

CONCLUSION

We concluded that *P. eldrica* fruit extract has a potent prophylactic effect on calcium oxalate stone formation, confirming the folklore about its antiurolithiasis activity, which is apparently unrelated to diuretic effect. The mechanism underlying the dissolvable effect of the extract remains unclear, and further studies are needed to clarify this issue.

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CONFLICT OF INTEREST

None declared.

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