

# Effect of *Prunus Mahaleb* L. Seed Extract on Ethylene glycol- and Ammonium Chloride-Induced Urolithiasis in BALB/c Mice

Fatemeh Akbari<sup>1</sup>, PharmD;  Mohammad Azadbakht<sup>2</sup>, PhD;  Ayat Dashti<sup>3</sup>, PhD; Lale Vahedi<sup>4</sup>, MD; Ali Davoodi<sup>2</sup>, PharmD

<sup>1</sup>Student Research Committee, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran;

<sup>2</sup>Department of Pharmacognosy, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran;

<sup>3</sup>Department of Toxicology, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran;

<sup>4</sup>Department of Pathology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

## Correspondence:

Mohammed Azadbakht, PhD;  
17<sup>th</sup> Kilometer of Farahabad Street,  
Department of Pharmacognosy, School of Pharmacy, Mazandaran University of Medical Sciences, Postal code: 47178-43611, Sari, Iran

Tel/Fax: +98 11 33543728  
+98 11 33543084

Email: azadbakhtm@hotmail.com

Received: 20 August 2018

Revised: 24 September 2018

Accepted: 07 October 2018

## What's Known

- *Prunus mahaleb* L. seed extract is used in Iranian traditional medicine. Its seeds contain fatty unsaturated glycoside-free compounds, flavonoids, salicylic acid, and amygdalin.
- The seed extract is shown to have anti-oxidant and anti-bacterial properties to treat kidney stones and respiratory tract infections.

## What's New

- The results of the acute toxicity test and serum analysis confirmed the effectiveness of *Prunus mahaleb* L. in preventing the formation of kidney stones.
- Clinical trials are recommended to validate the findings of the present study.

## Abstract

**Background:** Kidney stone disease can be quite painful, recurrent, and affects many people. Despite advances in drug therapy, there is still a need to find effective drugs with fewer complications for long-term treatment of kidney stones and to prevent its recurrence. The present study aimed to evaluate the effect of *Prunus mahaleb* L. seed extract on ethylene glycol- and ammonium chloride-induced urolithiasis in BALB/c mice.

**Methods:** The *Prunus mahaleb* L. seeds were collected in Mashhad (Iran) in June 2017. Urolithiasis was induced in male BALB/c mice by adding ethylene glycol (EG) 0.75% (v/v) and ammonium chloride (AC) 2% (w/v) to their drinking water for 21 consecutive days. A total of 72 animals were randomly divided into six groups of twelve animals each. Group 1 received purified water as control; group 2 received EG+AC in drinking water; groups 3-5 received the extracts by gavage in dosages of 100, 300, 500 mg/kg body weight, respectively; and group 6 received 888 mg/kg Sankol by gavage. Note that urolithiasis was induced in groups 3-6 in the same manner as in group 2. The data were analyzed using GraphPad Prism Software (version 5.01).

**Results:** The group receiving *Prunus mahaleb* L. extract in a 500 mg/kg dose responded better to the treatment and less damage to the kidney tissue was observed. The serum parameters remarkably decreased in the calculi-induced animals. Besides, the acute toxicity test showed that the use of the extract was safe in animals.

**Conclusion:** The results showed that the use of *Prunus mahaleb* L. extract effectively prevented the formation of kidney stones.

Please cite this article as: Akbari F, Azadbakht M, Dashti A, Vahedi L, Davoodi A. Effect of *Prunus Mahaleb* L. Seed Extract on Ethylene glycol- and Ammonium Chloride-Induced Urolithiasis in BALB/c Mice. Iran J Med Sci. 2020;45(2):134-139. doi: 10.30476/IJMS.2019.45774.

**Keywords** • *Prunus mahaleb* L. • Ethylene glycol • Ammonium chloride • Kidney calculi • Urolithiasis

## Introduction

*Prunus mahaleb* L., commonly known as mahaleb, belongs to the family Rosaceae and is a tree indigenous to Iran. The tree has alternate and simple leaves, white flowers, and small oval shaped, fleshy cherry-like fruits with a bitter taste.<sup>1,2</sup> The seeds of *Prunus mahaleb* L. contain a fatty unsaturated glycoside-free compound (dihydrocoumarin) as well as other components such as flavonoids, salicylic acid, and amygdalin.<sup>3</sup> *Prunus mahaleb* L. is shown to have anti-oxidant and anti-bacterial properties to

treat respiratory tract infections.<sup>4-6</sup> Its use is also recommended in traditional medicine for the treatment of kidney stones.<sup>7</sup>

Kidney stone disease can be quite painful, recurrent, and affects about one in every 1000 people.<sup>8</sup> The stones are formed when substances in the urine are oversaturated and their accumulation, over time, in the urinary tract cause urolithiasis.<sup>9</sup> Factors that affect kidney stone formation are dietary lifestyles and an animal protein-rich diet,<sup>10</sup> low urine volume, bacteria,<sup>11, 12</sup> genetic factors, and obesity.<sup>13</sup> Epidemiological studies have shown that calcium oxalate (CaOx) formation in the kidney and the urinary tract is the main cause of kidney stones.<sup>14</sup> Surgery is the best treatment modality although not recommended for small kidney stones. Drug therapy, alone or in combination with surgery, is an effective method to treat the disease.<sup>15</sup> However, despite advances in drug therapy, there is still a need to find effective drugs with fewer complications for the long-term medical treatment of kidney stones and to prevent its recurrence.<sup>16</sup> Hence, the present study aimed to evaluate the effect of *Prunus mahaleb* L. seed extract on ethylene glycol- and ammonium chloride-induced urolithiasis in BALB/c mice.

## Materials and Methods

### Plant Material and Preparation of the Extract

The *Prunus mahaleb* L. plants were collected in Mashhad, northeast of Iran, in June 2017. The plant specimen was identified and authenticated by a member of our research team and a voucher specimen was deposited in the herbarium of Mazandaran University of Medical Sciences, Sari, Iran; voucher number E2-174141. Its seeds were grounded into powder and kept in a methanol concentration of 95% (v/v) for 10 hours in a Soxhlet extractor at 40 °C. The concentrate was obtained by freeze-drying the solution using a rotary evaporator, and subsequently, a red crystalline precipitate was collected. The yield of the extract was 10.05% (w/w). The product was placed in airtight containers and stored in a refrigerator for future use.

### Animals

Male BALB/c mice weighing between 15-20 g were obtained and housed under standard laboratory conditions (temperature 24±2 °C, under 12-hour light:dark cycle). Animal care and the experimental procedure were in accordance with the prescribed animal rights.<sup>17</sup> The study was approved by the Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (code: IR.MAZUMS.REC.95.2769).

### Ethylene Glycol-Induced Urolithiasis Model

Urolithiasis was induced by feeding the animals a mixture of drinking water with ethylene glycol (EG) and ammonium chloride (AC), with 99.5% and 99% purity, respectively. A total of 72 animals were randomly divided into six groups of twelve animals each.

Group 1: Received purified water (negative control)

Group 2: Received EG 0.75% (v/v) and AC 2% (w/v) in drinking water

Groups 3-5: Received the *Prunus mahaleb* L. extract at doses of 100, 300, 500 mg/kg body weight, respectively,<sup>18</sup> dissolved in distilled water and administered once daily by gavage.

Group 6: Received 888 mg/kg Sankol administered daily by gavage.<sup>19</sup>

To induce urolithiasis, groups 3-6 were also fed with EG 0.75% (v/v) and AC 2% (w/v) in drinking water throughout the 21 days of the experiment.<sup>20, 21</sup>

### Serum Analysis

The animals were sacrificed under thiopental anesthesia (60 mg/kg, intraperitoneally) and blood samples were directly collected from the heart. The serum was separated by centrifugation at 10,000 rpm for 10 minutes and analyzed for urea, uric acid, creatinine, calcium, and phosphorus levels using a Hitachi 917 auto-analyzer (Roche, Japan).<sup>22</sup>

### Kidney Histopathology and Homogenate Analyses

Upon collecting the blood, the kidneys were removed. The left kidneys of all mice were placed in containers with 10% formalin. After preparation of the paraffin sections, the samples were sliced into 5 mm sections using a rotary microtome and stained with hematoxylin and eosin (H&E) for histopathological examination. Under a light microscope (40× magnification), the CaOx microscopic crystals in the renal tubes were evaluated. The right kidneys of each group were individually used for homogeneous analysis. The kidneys were dried for 1 day at 60 °C and then individually packed in containers with 15 cc concentrated nitric acid and stored for 1 day at room temperature. The samples were then homogenized and centrifuged at 3,000 rpm for 10 minutes. Then, the supernatant was separated to determine the calcium phosphate and CaOx concentrations in the kidney homogenate using a Biotechnica BT-3000 automated analyzer device.<sup>12</sup>

### Acute Toxicity Test

To determine the maximum tolerable dose, assessment of the acute oral toxicity of *Prunus*

*mahaleb* L. extract was carried out according to a guideline from the Organization for Economic Co-operation and Development (OECD). The assessment included male mice.<sup>23, 24</sup> Three doses of 1000, 2000, and 5000 mg/kg; were administered as a single oral dose in six mice, which were followed for 14 days to monitor the mortality rate. At the end of this period, blood samples were taken and sent to a laboratory to evaluate changes in the liver enzymes.

#### Flavonoids and Total Phenolic Content Determination

The aluminum chloride colorimetric method<sup>25</sup> and Folin-Ciocalteu assay method were used to measure flavonoids and the total phenolic content, respectively.<sup>26</sup>

#### Statistical Analysis

The data were analyzed using GraphPad Prism Software (version 5.01). The results between the groups were analyzed using one-way ANOVA followed by the Tukey's test. Data were expressed as mean±SEM and P<0.05 was considered statistically significant.

## Results

#### Acute Toxicity Test

The value of LD<sub>50</sub> was more than 500 mg/

kg body weight. Mice mortality or morbidity during the 14-day test period was not observed. The results of the biochemical studies showed no changes in the liver enzymes except for an increased serum ALT level in one sample (table 1). Hence, the therapeutic doses (1000, 2000, and 5000 mg/kg body weight) used in the present study were considered safe.

#### Serum Analysis

Administration of EG 0.75% (v/v) and AC 2% (w/v) induced renal stone and caused impairment of the renal functions. The serum urea, uric acid, and creatinine levels were significantly elevated (P<0.001, P<0.01, P<0.05, respectively). In addition, compared to the control group, the serum phosphorus and calcium levels were remarkably increased in the calculi-induced animals. Nevertheless, treatment with *Prunus mahaleb* L. seed extract grossly reduced the elevated serum levels of urea, uric acid, creatinine, calcium, and phosphorus in the animals receiving EG+AC (table 2).

#### Kidney Homogenate Analysis

In comparison with the control group, the levels of oxalate, phosphate, and calcium were significantly (P<0.001, P<0.01, and P<0.01, respectively) increased in the groups receiving EG+AC. Whereas, treatment with

**Table 1:** The effects of *Prunus mahaleb* L. seed extract and Sankol on serum parameters in ethylene glycol- and ammonium chloride-induced urolithiasis (mg/dl). Values are expressed as mean±SEM (n=12 animals per group)

Groups	Control (purified water)	EG/AC	P value <sup>a</sup>	EG/AC+ Extract 100	P value <sup>b</sup>	EG/AC+ Extract 300	P value <sup>b</sup>	EG/AC+ Extract 500	P value <sup>b</sup>	EG/AC+ Sankol	P value <sup>b</sup>
Urea	24.50±1.10	53.50±1.32	0.0007	51.75±2.49	0.0426	35.50±1.55	0.0415	29.25±0.62	0.00013	27.50±0.69	0.001
Uric acid	2.20±0.19	6.85±0.20	0.0017	5.55±0.44	0.0019	3.10±0.17	0.00017	2.85±0.18	0.00018	2.50±0.12	0.0052
Creatinine	0.69±0.01	1.21±0.04	0.0104	1.13±0.04	0.005	0.87±0.01	0.00009	0.70±0.01	0.0048	0.71±0.03	0.0355
Calcium	8.30±0.03	10.93±0.12	0.002	10.07±0.07	0.0014	9.73±0.07	0.0018	9.08±0.06	0.0062	9.06±0.03	0.0024
Phosphate	2.95±0.02	5.70±0.05	0.0001	5.22±0.03	0.0007	4.86±0.05	0.0009	3.68±0.08	0.0009	3.62±0.05	0.00009

Values are expressed as Mean±SEM (n=12 animals/group). <sup>a</sup>Comparison between control group and EG/AC group; <sup>b</sup>Comparison between All Groups and EG/AC group; P value less than 0.05 were considered statistically significant; EG: Ethylene glycol; AC: Ammonium chloride

**Table 2:** The effect of *Prunus Mahaleb* L. seed extract and Sankol on kidney homogenate parameters in mice with induced urolithiasis (mg/g). Data are expressed as mean±SEM (n=12 animals per group)

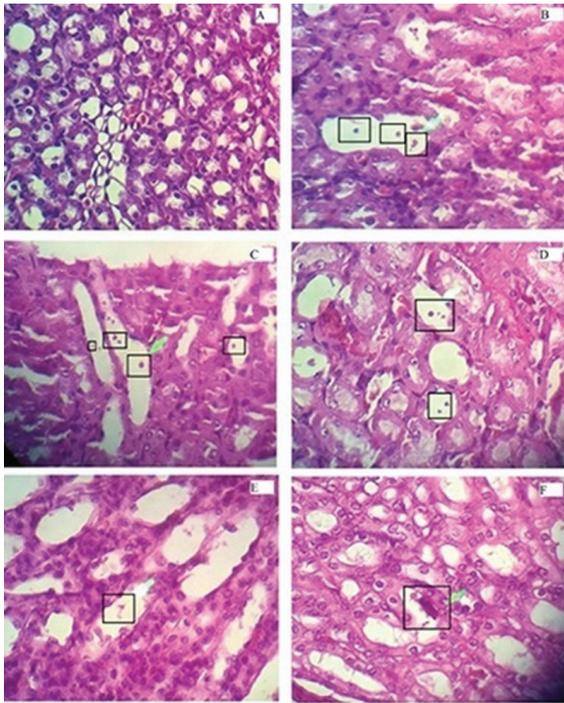
Groups	Control (purified water)	EG/AC	P value <sup>a</sup>	EG/AC+ Extract 100	P value <sup>b</sup>	EG/AC+ Extract 300	P value <sup>b</sup>	EG/AC+ Extract 500	P value <sup>b</sup>	EG/AC+ Sankol	P value <sup>b</sup>
Calcium	0.24±0.01	0.72±0.02	0.0035	0.67±0.01	0.0071	0.59±0.01	0.0065	0.35±0.01	0.0099	0.28±0.01	0.0039
Phosphorus	2.13±0.09	3.82±0.06	0.0049	3.72±0.07	0.0063	3.20±0.01	0.0018	2.54±0.02	0.0008	2.43±0.04	0.0006
Oxalate	1.36±0.03	6.38±0.11	0.0006	5.86±0.01	0.0098	4.92±0.07	0.0024	2.28±0.08	0.00009	1.69±0.05	0.00095

Values are expressed as Mean±SEM (n=12 animals/group). <sup>a</sup>Comparison between control group and EG/AC group; <sup>b</sup>Comparison between All Groups and EG/AC group; P value less than 0.05 were considered statistically significant; EG: Ethylene glycol; AC: Ammonium chloride

**Table 3:** The effect of *Prunus. Mahaleb* L. seed extract and Sankol on kidney homogenate parameters in mice with induced-urolithiasis (mg/g). Data are expressed as mean±SEM (n=12 animals per group)

Groups	Calcium	Phosphorus	Oxalate
Control (purified water)	0.24±0.01	2.13±0.09	1.36±0.03
EG/AC	0.72±0.02 <sup>ab</sup>	3.82±0.06 <sup>ab</sup>	6.38±0.11 <sup>va</sup>
EG/AC+Extract 100	0.67±0.01 <sup>b</sup>	3.72±0.07 <sup>b</sup>	5.86±0.01 <sup>b</sup>
EG/AC+Extract 300	0.59±0.01 <sup>b</sup>	3.20±0.01 <sup>tb</sup>	4.92±0.07 <sup>b</sup>
EG/AC+Extract 500	0.35±0.01 <sup>ab</sup>	2.54±0.02 <sup>vb</sup>	2.28±0.08 <sup>vb</sup>
EG/AC+Sankol	0.28±0.01 <sup>ab</sup>	2.43±0.04 <sup>vb</sup>	1.69±0.05 <sup>vb</sup>

<sup>†</sup>P<0.05; <sup>††</sup>P<0.01; <sup>†††</sup>P<0.001; <sup>a</sup>Comparison with group 1; <sup>b</sup>Comparison with group 2



**Figure 1:** Microscopic images (H&E stain, 40×) of the kidney tissues. The presence of kidney stone crystals is indicated by black squares. Figures A-F correspond to groups 1-6, respectively.

*Prunus mahaleb* L. seed extract reduced the crystalline components in the renal tissue dose-dependently (table 3).

#### Histopathology

In comparison with the normal architecture of kidney tissue in the control group (figure 1A), histopathological reports showed crystal deposits in different parts of the kidney in the EG+AC group (figure 1B). Simultaneous treatment with *Prunus mahaleb* L. seed extract dose-dependently reduced deposition and damage resulting from the EG+AC treatment and prevented crystalline-induced renal tissue injuries (figure 1C-1E).

#### Total Phenol and Flavonoid Contents of the Extract

The flavonoid content was calculated using the standard curve equation ( $y=0.0006x+0.0276$ ,  $r^2=0.9992$ ) and expressed

as quercetin equivalents. The total flavonoids content in the extract was  $27.68\pm 0.03$  mg/g quercetin equivalent. In addition, the phenol content was measured using the Folin-Ciocalteu assay method with the standard curve equation ( $y=0.0061x+0.0682$ ,  $r^2=0.9992$ ) and expressed as gallic acid equivalents. The total phenol content in the extract was  $69.50\pm 0.1$  mg/g gallic acid equivalent.

#### Discussion

The results of the acute toxicity test and serum analysis confirmed the effectiveness of *Prunus mahaleb* L. in treating kidney stones. Our findings were in line with previous studies on the effectiveness of *Prunus mahaleb* L. plant for the treatment of respiratory tract infections<sup>4-6</sup> as well as in the context of traditional medicine.<sup>7</sup>

Previous studies have described the process of kidney stone formation and associated symptoms.<sup>8, 27</sup> It was shown that CaOx stones are the most common type of kidney stone, while other types of stone are caused by calcium phosphate, uric acid, struvite, cysteine, and rarely by xanthine.<sup>14</sup> Treating rats with EG led to the formation of oxalate deposits in the kidney and its appendages. Oxalate derivatives showed urinary over-excretion or occasional crystals in the kidney,<sup>28</sup> and the AC compound produced metabolic acidosis. The combination of EG and AC was shown to cause high levels of CaOx crystals in the kidney, with recommended doses of 0.75% (v/v) and 2% (w/v), respectively.<sup>29</sup> Rats treated with EG and AC showed increased excretion of oxalate crystals in the urine. Increased calcium and phosphorus release provide suitable conditions for the formation and deposition of calcium phosphate crystals.<sup>30</sup> The presence of stones in the urinary tract reduces urinary flow, glomerular filtration rate, and excretion of nitrogenous substances, which in turn leads to an increase in the creatinine, urea, and uric acid levels in blood.<sup>31</sup>

The results of the histopathological test showed inflammation and sedimentation in the EG+AC group, while there were slight changes in

the Sankol and extract groups in comparison with the control group. The groups treated with *Prunus mahaleb* L. showed a reduction in the size and the number of CaOx deposits in the kidney sections. This finding indicated undamaged kidney structure in the groups receiving the extract similar to those receiving the standard medicine.

Serological results showed increased serum creatinine, urea, and uric acid in the EG+AC group, indicating kidney damage. Given the fact that these groups were on the same diet, those with lower urinary calcium excretion, i.e. higher serum calcium levels, had less chance of developing urolithiasis. We also found that the group receiving *Prunus mahaleb* L. extract in 500 mg/kg dose responded better to the treatment. Lower levels of creatinine and urea in this group indicated less damage to the kidneys. Such a dose-dependent effect was further confirmed by a better response to the treatment in the group receiving a dose of 300 mg/kg compared to 100 mg/kg group. The effectiveness of the extract in preventing the formation of a kidney stone is attributed to the antioxidant properties of the plant, which is also due to the high phenol and flavonoids content. Moreover, a reduced amount of urea in urine excretion can be effective. Finally, it is recommended to adjust the causative agents and inhibitors of the extract to substantiate the findings of the present study.

## Conclusion

The results showed that the use of *Prunus mahaleb* L. extract effectively prevented the formation of kidney stones. Since this plant is widely used in Iranian herbal medicine, clinical trials are recommended to confirm the findings of the present study.

## Acknowledgment

We would like to express our gratitude to Akbari family for their financial contribution. Laboratory assistance by Ms. Amjadi at the Cancer Research Center (Imam Khomeini Hospital, Sari, Iran) is very much appreciated.

**Conflict of Interest:** None declared.

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