Original Article

# Effect of Cucumis melo L. seeds extract on renal mRNA levels of Interleukin-10 and Cyclooxygenase-2 in calcium oxalate urolithiasis rats

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## Abstract

**Introduction:**While cyclooxygenase-2 induces inflammatory responses, interleukin-10 is involved in terminating inflammatory responses of macrophages in kidney stones. Cucumis melo has a diuretic and anti-oxidant effect and is, therefore, effective in the disposal of urine sediments. This study aimed to examine the effect of hydro-ethanolic extract of C. melo seed on mRNA levels of interleukin-10 and cyclooxygenase-2 coding genes in kidneys of urolithiasis male rats.

**Materials and Methods:** 36 male rats were randomly divided into normal control, urolithiasis control, positive control, and three treatment groups with different C. melo seed extract treatment. Calcium oxalate stones induction was performed by oral administration of ammonium chloride (3 days) and ethylene glycol (38 days). Potassium citrate and hydro-ethanolic extract of C. melo L. seed were co-administered orally with ethylene glycol for 38 days. After the treatment period, the animals were anesthetized and their left kidneys were removed for evaluation of mRNA levels of the IL-10 and COX-2 genes by quantitative real-time PCR.

**Results**: The results showed that daily oral administration of potassium citrate and ethanolic extract (150, 300, and 600 mg/kg body weight) significantly reduced IL-10 gene transcription in experimental rats compared to the control group. The concentration of 150 mg/kg bodyweight of extract had a stronger effect than potassium citrate. Also, daily oral treatment of potassium citrate and extract (150, 300, and 600 mg/kg body weight) significantly reduced cox-2 gene expression in experimental rats compared to the control group. C. melo seed hydro-ethanolic extract can decrease the inflammation induced by renal stones by reducing the mRNA level of Cox-2 coding genes. Moreover, mRNA level of IL-10 was decreased due to the effect of C. melo seed extraction in termination of inflammation.

**Conclusions**: Hydro-ethanolic extract of C. melo seed can significantly decrease the inflammation in urolithiasis which is a major cause of kidney stones formation and growth.

**Keywords:** Kidney stone, Urolithiasis, Interleukin-10, Cyclooxygenase-2, Cucumis melo L.

## 1. Introduction

Urolithiasis, also known as kidney stones, is the oldest and the third most common urinary tract disease. According to available reports, 10 to 12 percent of people in industrialized countries (10% of men and

3% of women) develop kidney stones during their lifetime [1–3]. Kidney stones are a multifactorial disease, associated with several factors including genetics, diet, and low activity. Calcium-containing stones (75-90 %) , ammonium-magnesium phosphate (15-10 %), uric acid (3-10 %) and cystine stones (0.5-1 %) are the most common kidney stones [4]. The overall risk of developing kidney stones varies in different parts of the world. The risk of developing kidney stones in adults seems to be higher in the western regions of the world (5-9 % in Europe, 12 % in Canada, 13-15 % in the United States) than in the eastern regions (1-5 %) [5]. Stones can cause a variety of symptoms, including pain, blockage, infection, and bleeding as the stones move through the urinary tract. Treatment and management of kidney stones using complex surgical techniques is costly and does not prevent the regeneration of kidney stones [6–8].

Decreased antioxidant capacity or long-term supersaturated urine leads to oxidative stress and kidney stones formation [9,10]. The release of reactive oxygen species (ROS) into the intercellular space destroys renal tubular cells [11]. Renal tubular cell damage is an important risk factor for the onset of renal stone formation. Exposure of these cells to calcium oxalate crystals induces oxidative stress, increase in lipid peroxidation, production of free radicals, and release of arachidonic acid by phospholipase A [12]. Under oxidative stress, these events are amplified and kidney stone production is intensified.

Arachidonic acid is a substrate of cyclooxygenase-2 (COX-2). Cyclooxygenase-2 catalyzes the committed step reaction in the biosynthesis pathway of prostaglandins [13]. Prostaglandins play a key role in the development of inflammatory responses. They are significantly increased in the tissues involved in inflammation and are effective in the development of inflammation [14]. The mRNA, protein levels and COX-2 activity increase when renal tubular cell damage occurs [15].

Interleukin-10 is a cytokine with multiple effects (pleiotropic) in regulating immunity and inflammation [16]. IL-10 is mainly produced by activated macrophages and also is an inhibitor of activated macrophages and dendritic cells. These functions represent their role in the regulation of innate and cellular immune responses [17].

Medicinal plants have been used for thousands of years in various countries to prevent the spread and recurrence of kidney stones [18]. Melon is a plant of the Cucurbitaceae family with the scientific name Cucumis melo var. Inodorus [19]. Melon seeds are commonly used in the treatment of kidney stones, ulcers of the urinary tract and stomach, chronic fever, inflammation of the liver and kidneys, and general weakness of the body. Various biological activities of melon have been reported, including antioxidants, analgesics, anti-inflammatory, and anti-microbial [20].

In the present study, kidney stones were developed by oral treatment of ethylene glycol in model rats. Then, the effect of hydro-ethanolic extract of Cucumis melo seed on mRNA level of IL-10 and COX-2 encoding genes in kidney tissue of model rats was determined by RT-qPCR method and compared with the control rat group.

## 2.Materials and Methods:

Preparation of Cucumis melo seed extract

Cucumiss melo L. was purchased from Varamin (Tehran province, IRAN). The seeds were separated and dried in the dark at 24 °C and then completely pulverized using a mechanical mill. To prepare the hydro-ethanolic extract, 300 g of the seeds powder was mixed with 2.5 L of 80 % ethanol and kept in a closed container in a

dark place for 72 hours at 24 °C. The resulting mixture was filtered using a paper filter (Whatman paper). The resultant solution was concentrated using a rotary apparatus and placed at 50 °C for complete drying. The obtained extract was kept in a closed container at 4 °C until the next use.

Animals

In this study, 36 male Wistar rats (180 ± 20 g) were used. Rats were obtained from the Pasteur Institute (Karaj, IRAN) and kept in the research laboratory for one week to adapt to the conditions. Throughout the experiment, rats were kept in standard cages under standard conditions (24 ± 2 °C, 12-hour light and dark cycle). Also, during the experiment, the rats had free access to standard food and water. All experimental steps were performed after receiving the required permission from the National Ethics Committee in Biomedical Research (IR.IAU.VARAMIN.REC.1398.012).

Experiment design

The rats were randomly divided into 6 groups and every 3 rats were kept in separate cages. The treatment of each group during the 38 days of the study was as follows: Healthy control group maintained under standard conditions. The urolithiasis control group received ammonium chloride (1% W/W in drinking water) and ethylene glycol (0.75% V/V in drinking water) for 3 days for induction of kidney stones. The positive control group received citrate-potassium (2.5 g/kg body weight in drinking water) in addition to ammonium chloride and ethylene glycol. The three experimental groups received Cucumis melo seed extract (150, 300, and 600 mg/kg body weight) in drinking water in addition to ethylene glycol. At the end of the treatment period, the rats were anesthetized with chloroform and the left kidney of rats was removed to study gene expression. These samples were immediately stored at -70 °C.(table 1)

**Real-time quantitative PCR**

RiboExTM total RNA extraction kit (GeneAll) was used for extraction of total RNA from the kidney tissues. DNAase Max® kit (Qiagen) was used to remove the remaining DNA. cDNA synthesis was performed using HyperscriptTM RT Mastermix (GeneAll). Total RNA (400 ng) was mixed with DNase and RNase-free water to a final volume of 10 µl and was incubated at 65 °C for 5 min. The master mix (10 µl) was added to the reaction mixture and thermal steps were performed in a thermocycler (Bio-Rad). RT-qPCR was performed using a QuantiNova SYBR® Green PCR Kit (Qiagen) in a Rotor-Gene Q (Qiagen) real-time PCR cycler. The thermal and time program for RT-qPCR is provided in table 2. GAPDH gene was used as the internal control. The sequence of primers and product length are shown in Table 3. The REST software was used to analyze RT-qPCR results. RT-qPCR was performed in three replicates for each sample and the average Ct was used for fold change calculations. The changes in the expression of the target genes compared to the normal control group ($\Delta\Delta ct = \Delta ct$ target group - $\Delta ct$ normal) and relative expression of each gene were calculated according to the $2^{-\Delta\Delta ct}$ equation. Thus, the raw expression levels and normal control for genes are not shown in graphs.

Statistical analysis

The SPSS software (ver. 20) was unitilized for statistical analysis of RT-qPCR results.

A one-way ANOVA test was used to determine the significance of differences between groups, the significance level, and SD (Standard Deviation) of the data. Graphs were drawn using Excel software. P values of less than .05 were regarded as statistically significant.

## 3.Results

mRNA level of cyclooxygenase-2 gene

The results of the present study showed that daily oral administration of potassium citrate (PC) at a concentration of 2.5 g/kg body weight significantly decreased mRNA level of COX-2 compared with the urolithiasis group (34.7%). Further, hydroethanolic extract of Cucumis melo seed extract at concentrations of 150, 300, and 600 mg/kg bodyweight decreased by 93.7%, 30% and 53.1%, respectively ($p < .001$). Concentrations of 150 and 600 mg/kg bodyweight of the extract have a stronger effect in the reduction of mRNA level of COX-2 than potassium citrate ($p < .001$) (Figure 1).

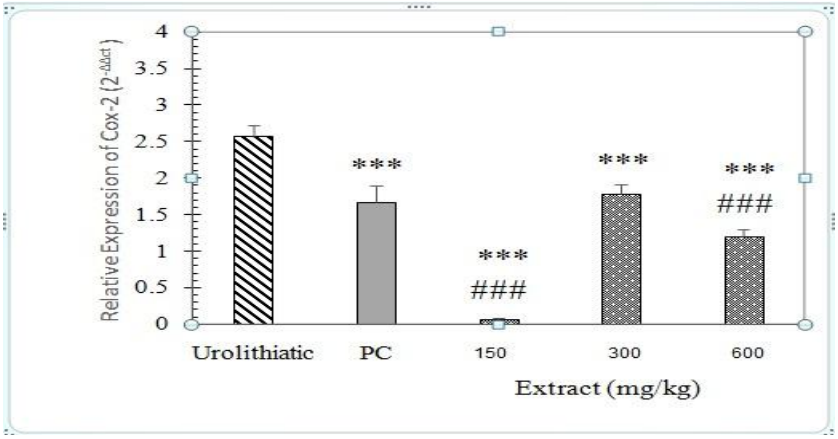


**Figure-1**. Effect of daily administration of potassium citrate (PC) and hydroethanolic extract of Cucumis melo seed extract at concentrations of 150, 300 and 600 mg/kg bodyweight for 38 days on the mRNA level of COX-2 in the kidney of urolithiasis rats. Each column shows Mean ± SD. Significant differences from the urolithiasis group ($p < .001$) and potassium citrate group ($p < .001$) have been indicated with *** and ###, respectively. potassium citrate; PC.

mRNA level of Interleukin-10 gene

The IL-10 mRNA levels in the kidney tissues of rats are shown in Figure 2. Daily oral administration of potassium citrate (PC) at a concentration of 2.5 g/kg bodyweight significantly decreased mRNA level of interleukin-10 (IL-10) compared with the urolithiasis control group (95.6%, $p < .001$). Concentrations of 150, 300 and 600 mg/kg bodyweight of melon seed hydroethanolic extract went down by 96%, 96% and 96.4%, respectively.

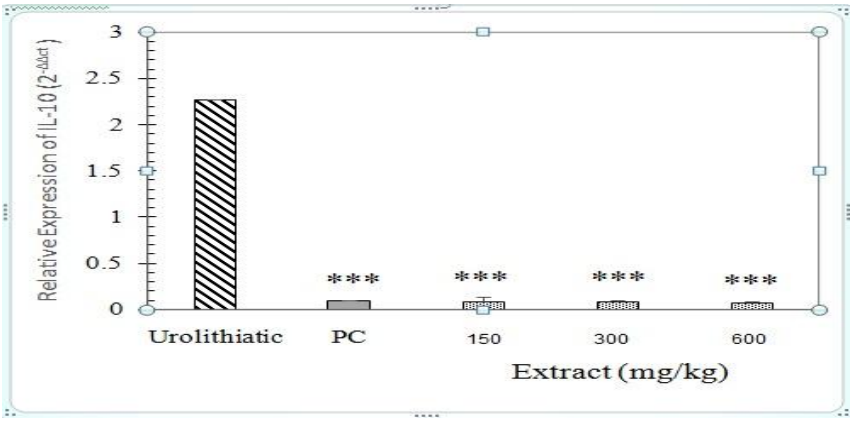

**Figure-2**- The effect of daily administration of potassium citrate (PC) at a concentration of 2.5 g/kg bodyweight and hydroethanolic extract of Cucumis melo seed at concentrations of 150, 300 and 600 mg/kg bodyweight for 38 days on the mRNA level of interleukin- 10 (IL-10). Each column shows Mean ± SD. Significant differences from the urolithiasis group ($p < 0.001$) have been indicated with ***. potassium citrate; PC.

## 4.Discussion

Urolithiasis is the third most common urinary tract disease. According to the reports, urolithiasis affects 10 to 12% of people in industrialized countries [1,3,21]. The complex and costly surgical procedures do not prevent the recurrence of kidney stones [6,7]. Due to the little effectiveness of diuretics such as potassium citrate and the disadvantages of surgical methods, the use of medicinal plants or natural products has recently been considered. Numerous medicinal plants are known to have diuretic, anticonvulsant, antioxidant properties and inhibit crystalization, primary nucleus formation and crystal accumulation. These medicinal plants are used in the treatment of kidney stones [18,22].

The diuretic effects of melon as well as inhibition of primary stone nucleus formation and facilitating its excretion have been proven. These effects seem to play an important role in the inhibition of kidney stone formation and recurrence [23]. Numerous reports have been published on the role of various medicinal plants in the expression of genes involved in inflammation, but so far, no study has been conducted to investigate the effect of Cucumis melo seed extract on the mRNA levels of IL-10 and Cox-2 genes.

The results of this study show that the presence of potassium citrate as a drug used in the treatment of kidney stones significantly reduces the mRNA levels of Cox-2 gene compared with the untreated group. Similar results were obtained in the Cucumis melo seed extract. The concentration of 150 mg/kg bodyweight had a stronger effect than potassium citrate. The mRNA level of the COX-2 gene was significantly lower than potassium citrate. Several cytokines and inflammatory mediators of various inflammatory cells can induce the production of COX-2. COX-2 induced under conditions of chronic and acute inflammation causes the synthesis of prostanoids. Prostanoids are involved in the inflammatory process in various diseases, including kidney stones [24]. COX-2 dependent prostaglandins play an important role in kidney function by participating in functions such as inflammation, maintaining sodium and water homeostasis, controlling renin release, vasodilation, and inhibition of vasoconstriction. Besides, prostaglandins facilitate the water excretion process by reducing the sensitivity of the tubules to the anti-diuretic hormone [13].

Compared to the urolithiasis group, the mRNA level of the IL-10 gene in the potassium citrate group was significantly decreased. However, in the presence of all three concentrations of hydro-ethanolic extract of melon seed, the mRNA level of the IL-10 gene was not only lower than the untreated group but even significantly lower than the potassium citrate treatment group. IL-10 is a cytokine with multiple (pleiotropic) effects in regulating immunity and inflammation. The biological effects of interleukin-10 are due to its ability to inhibit many functions of activated macrophages. Macrophages secrete cytokines and produce stimuli to respond to microbial infection that increase T cell activation and cellular immunity. IL-10 terminates these responses by acting on activated macrophages and returns the system to the rest state after the

microbial infection has been eradicated [24].

The presence of stones activates monocytes and causes inflammatory responses in kidney tissue [25]. The transcriptional level of TNF-α, IL-1β, IL-8, and IL-10 genes in monocytes is significantly increased in the presence of calcium oxalate crystals. Therefore, hydro-ethanolic extract of Cucumis melo seed and the potassium citrate reduces the supersaturation of calcium oxalate, the expression of IL-10 and COX-2 genes, and inflammation at the site of stone formation. Due to the high antioxidant capacity of Cucumis melo seeds, this extract inhibits oxidative stress through ROS, which is considered one of the factors contributing to stone production. Consequently, stone production is stopped and following the diuretic effect of Cucumis melo seed extract, the formed primary nuclei are also discarded.

## 5.Conclusion

In conclusion, one of the therapeutic effects of potassium citrate in the treatment of kidney stones can be the inhibition of inflammation results from the presence of primary stone nuclei in the kidney and thus reducing the transcription of IL-10 and COX-2 genes. Hydro-ethanolic extract of Cucumis melo seed extract also decreases the transcription of IL-10 and COX-2 genes and inflammatory responses to kidney stones. Similar to the potassium citrate, hydro-ethanolic extract of melon seed decreases transcription of IL-10 and Cox-2 genes and, in turn, reduces kidney stones-related inflammatory responses.

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## Conflict of interest

The authors declare no conflict of interest.

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| Groups | Ammonium chloride | Ethylene glycol | Citrate-potassium (PC) | Cucumis melo seed extract |
|---|---|---|---|---|
| Normal control | - | - | - | - |
| Urolithiasis control | + | + | - | - |
| PC treatment | + | + | + | - |
| Extract treatment | + | + | - | + |

**Table 1**. Groups and treatments received by each group of rats.

| Steps | Cycles | Duration (s) | Temperature (°C) |
|---|---|---|---|
| Initial denaturation | 1 | | 95 |
| Denaturation | 40 | 30 | 95 |
| Annealing | 40 | 60 | 55-60 |
| Extention | 40 | 15 | 72 |
| Hold | 1 | 30 | 95 |

**Table 2**. Thermal and time program for RT-qPCR.

| Gene | | Sequence (5'→3') | PCR product (bp) |
|---|---|---|---|
| IL-10 | Forward | AGCTGCGACGCTGTCATC | 155 |
| | Reverse | TGTCACGTAGGCTTCTATGC | |
| COX-2 | Forward | TCCTCCTTGAACACGGACTT | 185 |
| | Reverse | AGGTTTCAGGGAGAAGCGTT | |
| GAPDH | Forward | TGCCAGCCTCGTCTCATAG | 197 |
| | Reverse | ACTGTGCCGTTGAACTTGC | |

**Table 3**. Sequence and PCR product size of genes.