

Platanus orientalis (Plane Tree) Extract Protects Against Hyperoxaluria Induced Kidney Damage

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ABSTRACT: The aim of this study is to determine whether *Platanus orientalis* (PO) which has anti-inflammatory, antioxidant and diuretic properties and used in the treatment of kidney stones as traditional folk medicine, will reduce or prevent the stone formation in the urinary system. To simulate the urolithiasis model 0.75% ethylene glycol (EG) and 1% ammonium chloride (AC) were applied to Sprague-Dawley rats. The rats were divided into four groups (n=8). The control group was given standard drinking water for 5 weeks. EG group received 0.75% EG in their drinking water containing 0.75% EG and 1% AC. PO extract (100 mg/kg) was given orally for 5 weeks to the preventive group and for last 2 weeks to the therapeutic group, respectively. At the end of experiment, 24-hour urine and kidney samples were obtained. In urine samples, calcium and citrate levels were decreased and oxalate level was increased in the EG group. In kidney samples myeloperoxidase, caspase-3, N-acetyl-β-glycosaminidase (NAG) activities, malondialdehyde (MDA), 8-hydroxy-2-deoxyguanosine (8-OHdG), tumor necrosis factor-α and interleukin-1β levels were increased while superoxide dismutase activity and glutathione levels were decreased in the EG group. These biochemical parameters returned to control levels in both PO treatment groups. Histological findings also correlate with these results. Our findings are suggested that PO treatments is effective in both preventive and therapeutic groups.

KEYWORDS: *Platanus orientalis*; kidney stone; urolithiasis; rat.

1. INTRODUCTION

Urinary system stone disease (urinary lithiasis) is one of the leading causes of chronic kidney failure. According to the latest data of the European Urology Association, its prevalence has increased to 8.8% and the recurrence rate is 20% within five years [1, 2]. Stone recurrence depends on multiple factors such as genetics, diet, or climatic changes [3].

While the most common urinary system stones are calcium oxalate and calcium phosphate stones, there are many stone types such as uric acid, cystine and struvite (magnesium ammonium phosphate) stones [4]. A wide variety of molecular mechanisms have been proposed that can cause stone formation. The supersaturation degree of the urine is the main thermodynamic force for initiation of stone formation [5]. On the other hand, it is thought that hyperoxaluria causes damage to renal epithelial cells, triggers the production of reactive oxygen molecules and oxidative stress which in turn contributes to crystal retention [6]. In cell culture and *in vivo* studies, it has been shown that the damage caused by oxalate crystals is accompanied by lipid peroxidation and subsequently the inflammation contributes to this damage with an increase in

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interleukin-1 β (IL-1 β) levels [7]. Besides, there are a lot of promoter or inhibitor factors that effect the mechanisms which cause stone formation [8]. While the low urine flow rate and volume, low urine pH, high levels of calcium, sodium, oxalate and uric acid in the urine are among the promoters of calcium oxalate stone formation, inorganic substances such as citrate, magnesium and organic substances such as nephrocalcin, urinary prothrombin fragment-1, osteopontin are among the inhibitors [6].

Urinary stones can either be fragmented with shockwave lithotripsy and excreted in urine and it is possible to disintegrate them by various open and endoscopic urological surgical methods [9]. On the other hand, medical treatments for urinary system stones are aimed to reduce the size of the stone, dissolve it completely and prevent recurrence. Antispasmodics such as hyoscine-N-butylbromide are used to facilitate the removal of stones from the ureter, as well as nifedipine and tamsulosin which have smooth muscle relaxant effects [10]. Potassium citrate is the most used medication for chemolysis and/or prevention of stone recurrence. For chemolysis of uric acid stones, alkalization of urine with sodium bicarbonate or potassium citrate can be performed while combined therapy with allopurinol an xanthine oxidase inhibitors can also be administered [11]. Nowadays, since approaches to the use of traditional folk plants for medicinal purposes have increased, studies are also increasing on this subject.

Different plant extracts such as *Camellia sinensis* [12], *Punica granatum* [13], *Petroselinum sativum* [14], *Daucus carota* [15], *Urtica dioica* [16], *Duranta erecta* [17] have been studied *in vivo* and shown to be remedial in the treatment of kidney stones. *Platanus orientalis* (PO) leaves are used for the treatment of burns, diabetes, diarrhea, inflamed wounds, kidney diseases, stomach diseases and rheumatism in folk medicine in Turkey [18]. PO is a traditional medicinal herb with anti-inflammatory [19], antinociceptive, antioxidant [20] and analgesic [21] properties which have been used in different parts of the world to expel kidney stones as well as for the treatment of gallstones, as diuretics, and for its analgesic and antipyretic properties. In the light of these studies, we investigated the effects of PO on urinary system stone disease.

2. RESULTS

2.1. *In vitro* anti-inflammatory and antioxidant effects and total phenolic/flavonoid contents of po ethanolic extract

PO extract with IC₅₀ value of 76.63 μ g/ml showed a high antioxidant activity against DPPH radical compared to the ascorbic acid (IC₅₀: 17.60 \pm 0.37 μ g/ml). In addition, PO extract with IC₅₀ value of 89.60 μ g/ml showed a significant antioxidant activity against ABTS radical compared to the trolox (IC₅₀: 17.22 μ g/ml).

PO extract with IC₅₀ value of 57.91 μ g/ml was found to have a good anti-inflammatory activity against 5-lipoxygenase enzyme compared to indomethacin (IC₅₀: 22.39 \pm 0.26 μ g/ml).

Total phenolic and total flavonoid compound contents of PO extract was found as 141.40 mg/g equivalent to gallic acid and 35.21 mg/g equivalent to quercetin, respectively (Table 1).

Table 1. Antioxidant, anti-inflammatory activities and total compound contents of POE***

Assays	POE*	Ascorbic acid	Trolox	Indomethacin
DPPH radical scavenging activity (IC ₅₀ , μ g/ml)	76.63 \pm 0.55 ^b	17.60 \pm 0.37 ^a		
ABTS radical scavenging activity (IC ₅₀ , μ g/ml)	89.60 \pm 0.85 ^b		13.00 \pm 0.21 ^a	
Anti-lipoxygenase activity (IC ₅₀ , μ g/ml)	57.91 \pm 3.13 ^b			22.39 \pm 0.26 ^a
Total phenol content (mg GAE/g extract) **	141.40 \pm 3.34			
Total flavonoid content (mg QE/g extract) **	35.21 \pm 0.46			

*POE: *Platanus orientalis* ethanol extract

** The total phenolic and flavonoid contents were expressed as gallic acid equivalent (GAE) and quercetin equivalent (QE), respectively.

*** Each value in the table is represented as mean \pm S.D. (n=3). Different letter superscripts in the same line indicate significant differences (p<0.05).

2.2. Urinary calcium, oxalate, citrate, phosphate levels

Calcium and sitrate levels in urine were significantly decreased (p<0.05, p<0.001, respectively) while oxalate level in urine was remarkable increased (p<0.01) in the EG group (4.88 \pm 0.47 mg/dL, 2.94 \pm 0.28 mg/dL, 3.04 \pm 0.41 mg/dL, respectively) compared to control group (8.54 \pm 1.14 mg/dL, 6.36 \pm 0.54 mg/dL, 1.16 \pm 0.16

mg/dL, respectively). In the PO preventive group (8.20±0.51 mg/dL, 4.90±0.37 mg/dL, 1.57±0.25 mg/dL, respectively) calcium and citrate levels were slightly increased while oxalate level was decreased ($p < 0.05$) compared to EG group. In the PO therapeutic group (8.02±0.58 mg/dL, 4.36±0.57 mg/dL, 1.73±0.30 mg/dL, respectively) calcium level was not changed, significantly; citrate level was slightly decreased and oxalate level was slightly increased ($p < 0.05$) compared to the EG group. Phosphate levels in urine were not significantly changed among all groups (Figure 1).

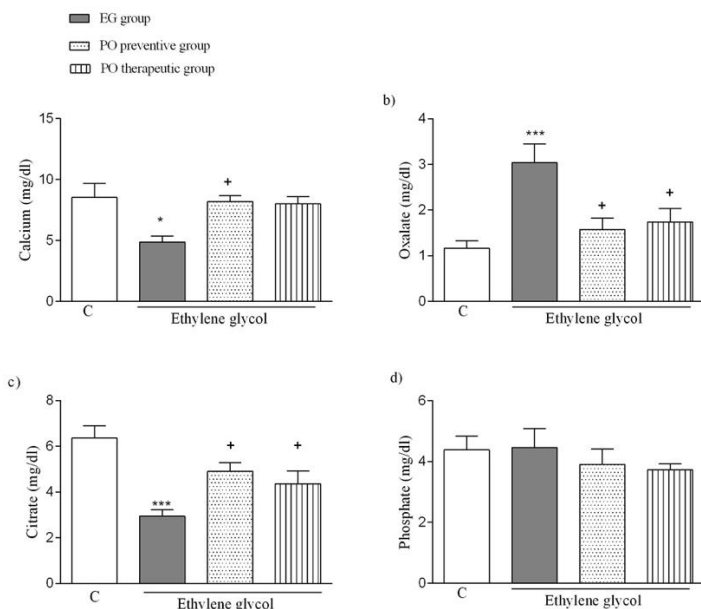


Figure 1. a) Calcium, b) Oxalate, and c) Citrate d) Phosphate levels in urine. * $p < 0.05$, *** $p < 0.001$ in comparison with control group; + $p < 0.05$ in comparison with EG group.

2.3. 8-OHdG levels

8-hydroxy-2-deoxyguanosine (8-OHdG) levels in the EG group and PO therapeutic group were found to be significantly higher ($P < 0.05-0.01$) compared to control group. Besides, in the PO preventive group it was significantly decreased ($P < 0.01$) compared to EG group (Figure 2).

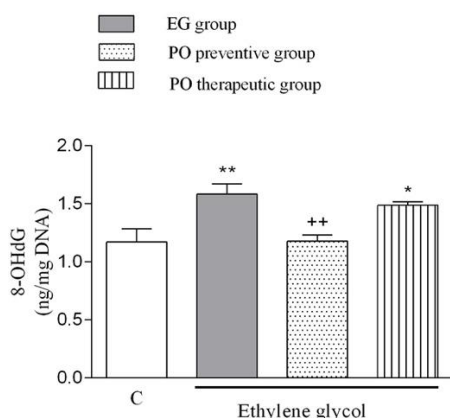


Figure 2. 8-OHdG levels in kidney tissues. * $p < 0.05$, ** $p < 0.01$ in comparison with control group; ++ $p < 0.01$ in comparison with EG group.

2.4. Caspase-3 activity

Renal tissue caspase-3 activity was significantly increased ($p < 0.001$) in EG group compared to control group. When the treatment groups were examined, caspase-3 activity in PO preventive group was significantly decreased ($p < 0.001$) compared to EG group. Caspase-3 activity in PO therapeutic group was also decreased ($p < 0.01$) compared to EG group (Figure 3).

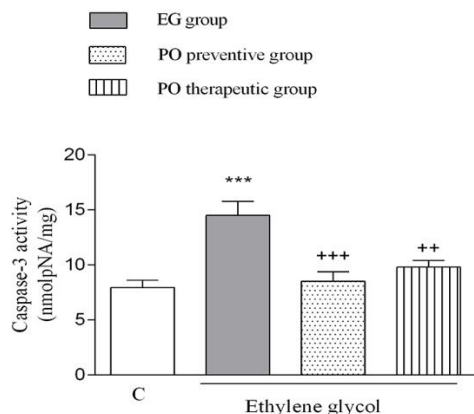


Figure 3. Caspase-3 activity in kidney tissues. *** $p < 0.001$ in comparison with control group; ++ $p < 0.01$, +++ $p < 0.001$ in comparison with EG group.

2.5. MPO, SOD activities and MDA, GSH levels

The activity of MPO was significantly increased ($p < 0.01$) in EG group and slightly increased ($p < 0.005$) in PO therapeutic group when they compared to control group. Besides these, MPO level of the PO preventive group was found to be significantly decreased ($p < 0.01$) compared to EG group.

Our results show that, there was slightly increase ($p < 0.05$) in the MDA levels in the EG group compared to control and there were slightly decrease ($p < 0.05$) in the PO preventive group and PO therapeutic group compared to EG group.

When the levels of GSH were examined, remarkable decrease in the EG group was observed compared to control group and a slight decrease was observed in the PO therapeutic group compared to control group. There was no significant change observed in the PO therapeutic group compared to EG group. Contrarily, a slight increase was observed in the PO preventive group compared to EG group.

Similar to GSH levels, SOD activities were also significantly decreased ($p < 0.01$) in EG group compared to control group. While the SOD activities were not remarkable increased in the PO therapeutic group, in the PO preventive group they were slightly increased ($p < 0.05$) compared to EG group (Figure 4).

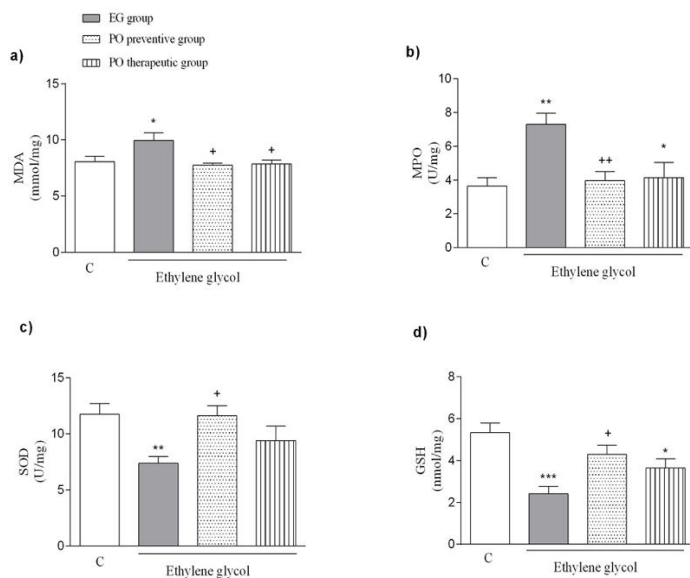


Figure 4. The levels of a) MDA, b) MPO, c) SOD, d) GSH in kidney tissues. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison with control group; + $p < 0.05$, ++ $p < 0.01$, in comparison with EG group.

2.6. NAG activity

N-acetyl- β -glucosaminidase (NAG) activity in the EG group was significantly increased when compared to control group ($p < 0.01$). In the treatment groups, NAG activities in the PO preventive group were found to be significantly decreased ($p < 0.01$) and in the PO therapeutic group were found to be slightly decreased ($p < 0.05$) compared to EG group (Figure 5).

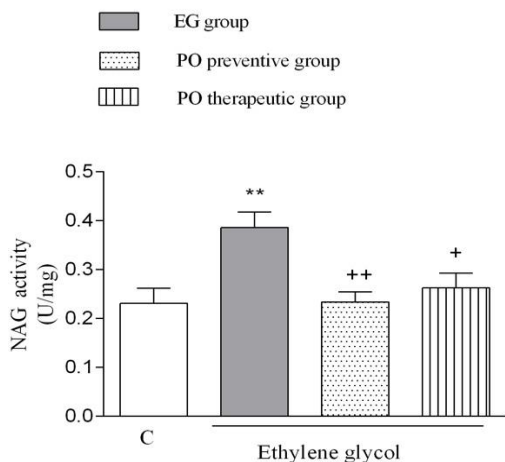


Figure 5. NAG activity in kidney tissues. ** $p < 0.01$ in comparison with control group; + $p < 0.05$, ++ $p < 0.01$, in comparison with EG group.

2.7. TNF- α and IL-1 β levels

The amount of TNF- α and IL-1 β significantly increased in the EG group compared to control group ($p < 0.01$). In the PO preventive group both TNF- α and IL-1 β levels were decreased compared to EG group ($p < 0.01$). Besides, TNF- α and IL-1 β levels in PO therapeutic group were remarkable decreased when compared to EG group ($p < 0.01$, $p < 0.05$, respectively) (Figure 6).

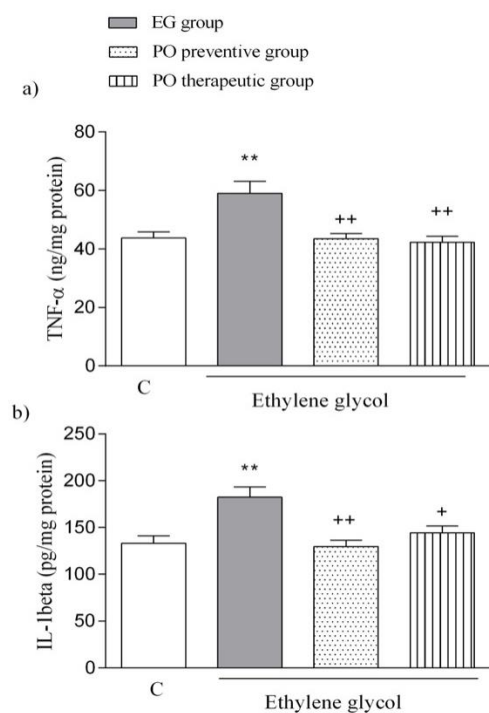


Figure 6. TNF- α ve IL-1 β levels in kidney tissues. ** $p < 0.01$ in comparison with control group; + $p < 0.05$, ++ $p < 0.01$, in comparison with EG group.

2.8. Histopathological analyses

While smooth structures were observed in both cortex and medulla in the control group (Figure 7a), enlargement of both cortical and medullary tubules, shedding of tubule cells and suppuration in the lumen were observed in the EG group (Figure 7b). In the treatment groups, although the degeneration regressed in the PO preventative group, it continued (Figure 7c), while in the PO therapeutic group tubule enlargement regressed both in the cortex and in the medulla (Figure 7d).

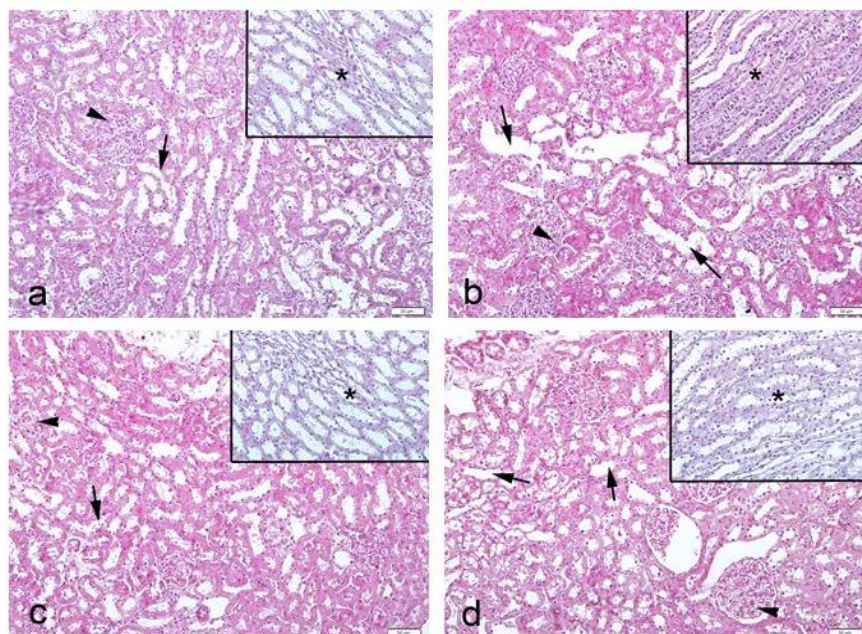


Figure 7. Representative light micrographs of renal tissues in experimental groups. a) control group showed regular tubules (arrow) and glomeruli (arrowhead), medulla (*). b) EG group showed suppuration tubules (arrow), glomeruli (arrowhead), degenerated tubules medulla (*) due to severely enlarged and intraluminal cell shedding. c) PO preventative group showed tubule enlargements (arrow), glomeruli (arrowhead), decreased degeneration medulla (*). d) PO therapeutic group started to improve and decreased intratubular spills (arrow), glomeruli (arrowhead), improved tubule structure in the medulla (*). All thumbnails are at the same magnification and belong to kidney medullary region.

3. DISCUSSION

Kidney stone disease is an important health problem as it increases risk of chronic kidney disease, end-stage renal failure, cardiovascular disease, diabetes, and hypertension [2, 5, 31, 32]. In order to prevent this burden due to urolithiasis, it is necessary to examine the alternative approaches that can be effective in preventing stone formation or excreting the already formed stones.

Current treatments methods applied to patients with urolithiasis are planned according to size and location of the stone. Although stones larger than 6 mm can be removed surgically, excretion of stones can be facilitated by drugs such as alpha blockers and calcium channel blockers for smaller stones. Lifestyle changes such as increasing fluid intake, decreasing intake of carbonated drinks and drug supplements such as thiazid diuretics, allopurinol, and potassium citrate are used to prevent recurrence [10, 33]

The EG model is frequently used in studies to investigate the pathophysiology of urinary system stone disease as well as new drug alternatives to reduce stone formation or treatment against stone induced kidney damage. Accordingly, we studied with EG which increased oxalate excretion and decreased calcium and citrate excretion to induce hyperoxaluria which is necessary for stone formation. Ethylene glycol is broken down to four organic acids including glycolaldehyde, glycolic acid, glyoxylic acid, and oxalic acid *in vivo*. While glycolic acid causes severe acidosis, oxalate precipitates as calcium oxalate in the urinary system [34].

As expected in supersaturation mechanism, while EG administration increased oxalate levels in the urine of rats, it was observed that urine calcium and citrate levels decreased. In addition, these levels were found to return to control levels in rats treated with the PO extract in our study. Supersaturation, the first step in stone formation, depends on the increase in the oxalate concentration in the urine. Hyperoxaluria,

hyperuricosuria, hypercalciuria, hypocitraturia and hypomagnesuria are seen in the formation of CaOx stones, which are the most common renal stones [15].

Studies have shown that some traditional herbal extracts are used in the treatment of urinary stone diseases and suggested that these extracts could be used as an alternative, considering the side effects of current treatment methods [35]. PO is a traditional herb used in dermatologic, ophthalmologic, and rheumatic diseases, as an antinociceptive in toothache and in diseases such as dysentery and asthma [36]. At the same time, as shown in *in vitro* studies, PO has played an important role in the prevention and treatment of urolithiasis with its antioxidant and anti-inflammatory activity. Ahmed et al. reported that polyphenols can effectively inhibit the formation of calcium oxalate urinary stones in relation to their antioxidant, anti-inflammatory, diuretic, and angiotensin converting enzyme (ACE) inhibitory effects. The current study revealed that PO had a high phenolic and flavonoid content [37]. In addition, El-Alfya et al. suggested that PO ethanolic extract contained 5,7,4'-trihydroxy-3,6-dimethoxyflavone-3'-O- β -D-xylopyranoside, kaempferol, quercetin, platanoside, tiliroside, afzelin, quercitrin, hyperoside, nicotiflorin, rutin, p-coumaric acid, and cryptochlorogenic acid [38]. Therefore, the phenolic compound content of PO may have contributed significantly to the prevention of stone formation. Based on these findings, the effects of PO on urinary system stone disease were investigated in this study to evaluate its therapeutic or preventive properties.

Intracellular reactive oxygen species (ROS) are primarily produced by NADPH oxidase and mitochondria [39, 40]. ROS activate the transcription factors such as nuclear factor- κ B, activation protein-1 through regulate the signal molecules such as protein kinase C, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase. The activation of these molecules allows the genes involved in the production of crystallization modulators such as osteopontin, bikunin and α -1-microglobulin [41].

Hyperoxaluria and CaOx crystals have been shown to increase the formation of ROS by causing inflammation in the kidney and cause tissue damage [42-44]. ROS are highly reactive and chemically unstable molecules and can bind to macromolecules (proteins, lipids, carbohydrates), disrupt their structure, and eventually cause tissue damage [45]. Studies in experimental models and clinical findings have shown the role of ROS in the formation of CaOx kidney stones. Although ROS generation can be controlled under normal conditions, pathologically excessive formation, causes tissue damage. Exposure to increased oxalate and CaOx / calcium phosphate (CaP) crystals increases the damage through excessive ROS formation, and this in turn disrupts kidney functions, increases the accumulation of these crystals and a vicious circle develops. As these mechanisms are taken into consideration; studies are widely carried out with the use of antioxidants to prevent and ameliorate this condition.

There are various endogen antioxidants in human body that scavenge ROS such as SOD, GSH, catalase, glutathione peroxidase, α -tocopherol, ubiquinone [36, 40]. Additionally, some antioxidant compounds can be taken with food [46]. Superoxide dismutase is an important antioxidant enzyme that catalyzes the conversion of superoxide radical to hydrogen peroxide and oxygen molecules. Moreover, GSH, a key antioxidant molecule which is present in all cell types, protects the cell against oxidative damage through the thiol groups in its structure. Recent studies have shown that SOD activities and GSH levels are reduced in rats with urolithiasis. Yousefi Ghale-Salimi et al. have reported that taraxasterol treatment increases SOD activity in rats with kidney stone [47]. Similarly, Wang et al. have shown that decreased SOD activities in rats with urolithiasis increased with α -lipoic acid [48] and *Endothelium Corneum Gigeria Galli* extract [49] treatment, respectively. Anundi et al. showed that decreased GSH levels triggers lipid peroxidation then causes cell lysis [50]. In accordance with these studies, our study also showed that SOD activities and GSH levels were decreased in rats due to EG, while increased with PO extract when given as preventive treatment for SOD activity and both treatments for GSH.

ROS cause lipid peroxidation of membranes and tissue damage in the kidney [51]. Lipid peroxidation is a chain reaction that begins with the removal of a hydrogen atom from unsaturated fatty acids of membrane phospholipids, resulting in the formation of a large number of reactive compounds [7]. Malondialdehyde (MDA) is one of the end products of this chain reaction and well-known sign of oxidative stress [52]. In this study, increased MDA levels showed that oxalate crystals cause oxidative stress, and this result is consistent with studies in the literature [53]. On the other hand, decreased MDA levels with PO extract demonstrated that PO have antioxidant properties.

NAG activity which is an indicator of oxidative stress mediated renal tubular damage, increased in rats with kidney stone induced by EG and significantly decreased in both treatments' groups. NAG is a lysosomal enzyme found in the kidney, especially in the proximal tubule cells. Evidence on the literature as urinary NAG level is directly proportional to the urinary oxalate level and can be increased by nephrotoxic medication, diabetic nephropathy, and acute kidney injury [54, 55].

8-OHdG is highly sensitive and the most used oxidative DNA damage marker. Guanine is a base that the most susceptible to oxidation among DNA bases. 8-OHdG is formed as one of the DNA repair products when guanine is exposed to attacks of ROS such as hydroxyl radical and superoxide anyone [56]. In addition, increased 8-OHdG levels were found in rats with experimental kidney stones [57]. Sener et al. showed that in rats with EG induced kidney stones 8-OHdG levels were increased due to oxidative damage and decreased with the administration of melatonin, an antioxidant agent [58]. In correlation with these data, we found that 8-OHdG level increased in rat given EG, and these levels decreased to control levels in treatment groups in this study. 8-OHdG induces apoptosis by activating various caspases and causing an increase in the expression of p53 [59]. Caspase-3 is a death protease that catalyzes the specific cleavage of many cellular proteins and can be activated by intrinsic and extrinsic pathways [60]. It has been shown that in various studies that caspase-3 activities increase in rats with EG-induced kidney stone and decrease to control levels with various treating agents [58, 61]. Concordantly, in this study we have shown that caspase-3 activity increased in rats with urolithiasis and significantly decreased with PO extract treatments.

Inflammation in urolithiasis cases usually occurs through cells such as leucocytes, monocytes, macrophages that gather around crystal deposits in the renal papillae and in some cases, inflammation can reach the renal cortex [40]. In previous studies the MPO activities have been shown to be increased in nephrolithiasis as an indicator of inflammation caused by neutrophil infiltration [58, 62]. Additionally, increased MPO activities were reported in various kidney diseases such as pyelonephritis, glomerulonephritis [62]. Myeloperoxidase is a peroxidative enzyme that is released from activated neutrophils and cause the HOCL production that damages macromolecules [63]. In our study MPO activity was increased in rats with kidney stone and returned to control levels in treatment groups. TNF- α , a proinflammatory cytokine, stimulates related receptors to trigger tubular epithelial necroptosis in nephrocalcinosis based on calcium oxalate accumulation through the increase in the expression of tubular adhesion molecules [64]. Another proinflammatory cytokine, IL-1 β is secreted for inflammation and tissue remodeling in various crystallopathies including nephrocalcinosis [65]. It was shown in an experimental study that both TNF- α and IL-1 β levels were increased in rats with kidney stones formed with EG [53]. In our study we found that TNF- α and IL-1 β levels were increased in rats with urolithiasis and returned back to control levels with PO preventive and therapeutic treatments. Based on these data, we concluded that PO is beneficial in urolithiasis through its anti-inflammatory effect.

4. CONCLUSION

In this study, we demonstrated that if the PO ethanolic extract, when used before stones were formed, may have a protective effect against urinary system stone disease. Also, if urinary system stones were formed, PO ethanolic extract can be beneficial by reducing the damage caused by stones in the kidney. Based on our findings, we suggested that PO is beneficial as adjunct to current treatment approaches in the treatment of the urinary system stone disease. Also, this study confirms traditional use of PO against kidney stones.

5. MATERIALS AND METHODS

5.1. Plant material and preparation of extract

Leaf parts of the PO were collected in October 2019 from Istanbul, Turkey and identified by Dr. İsmail Şenkardeş, a lecturer botanist in Marmara University Faculty of Pharmacy, Department of Pharmaceutical Botany. A few leaf samples of the dried plant were recorded in the Herbarium of the Marmara University Faculty of Pharmacy (Mare No: 22084).

The leaves of PO, dried in a sunless and ventilated room, were pulverized, weighed 90 g and extracted with 96% ethanol in the Soxhlet apparatus. The solvent was continued to be consumed until it became colorless, and the solvent of the extract was evaporated to achieve dryness in a rotary evaporator (Rotapvapor® R-300) to obtain ethanolic extract. The yield of extract was found to be 19.16 \pm 0.96%. Extract was stored at 4°C during the analyses.

5.2. Quantification method

The total phenolic compound of the extract was made using Folin-Ciocalteu solution by adapting it to the microplate according to the method proposed by Gao et al. [22]. The total flavonoid compound of the extract was adapted to the microplate according to the method proposed by Zhang et al. [23, 24].

5.3. *In vitro* anti-inflammatory activity of *Platanus orientalis*

The anti-inflammatory activity described by Phosrithong et al. was accomplished with some modifications. The method adapted to 96-well microplate format [25]. 20 μ l ethanol and 20 μ l borate buffer were added onto 10 μ l extract/standard at different concentrations then 25 μ l type-V soya lipoxygenase solution (20.000 U/ml) in the buffer was added. After the mixture was incubated for 5 minutes at 25°C, 100 μ l of 0.6 mM linoleic acid solution was added and the change in absorbance at 234 nm was recorded for 6 minutes. Indomethacin was used as reference standard. A dose-response curve was drawn to determine the IC₅₀ values [24, 25].

5.4. *In vitro* antioxidant activity of *Platanus orientalis*

5.4.1. DPPH radical scavenging activity

The antioxidant activities of the extract were examined according to the method of Zou et al [26]. Briefly, the stock solution prepared in dimethyl sulfoxide at a concentration of 5 mg/ml from the extract was diluted 1:8 and solutions in various concentrations were prepared. 10 μ l of each solution were transferred to microplate wells. 190 μ l of 0.1 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution was added on them. After keeping the mixtures in the dark for 30 minutes, their absorbance was measured against dimethyl sulfoxide to be used blindly at 517 nm. Ascorbic acid (0.25-0.016 μ g/ml) solution was used as standard. A dose-response curve was drawn to determine the IC₅₀ values.

5.4.2. ABTS radical scavenging activity

For the production of ABTS⁺ radical cation, 7 mM ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) was mixed with 2.45 mM potassium persulfate and the reaction took place at room temperature in the dark for 16 hours. ABTS⁺ solution was diluted with 96% ethanol solvent at 734 nm for an absorbance value of 0.700 \pm 0.050. The stock solution prepared in dimethyl sulfoxide at a concentration of 5 mg/ml from the extract was diluted 1:8 and solutions in various concentrations were prepared. 10 μ l of each solution were transferred to microplate wells. 190 μ l ABTS⁺ solution was added on them. After keeping the mixtures in the dark for 30 minutes, their absorbance was measured against trolox to be used blindly at 734 nm and results were expressed as IC₅₀ values [26].

5.5. Animals and experimental design

Adult 32 Sprague-Dawley rats (200-300g) were obtained from Marmara University Experimental Animal Application and Research Center (DEHAMER). The rats were housed in controlled room (humidity 50%; temperature 25 \pm 2°C; and 12:12 h dark/light cycle) with free access to water and a commercial laboratory complete food. All the experimental protocols were approved by Marmara University Animal Experiments Local Ethics Committee (MUHDEK) (59.2019.mar).

The rats were divided into four groups, each containing eight rats. The control group was given drinking water for five weeks and during the last two weeks of this period saline was administered by oral gavage. Ethylene glycol group received 0.75% EG in their drinking water for five weeks; in the first five days they fed with drinking water containing 0.75% EG and 1% AC [27]. During this period, saline was administered daily for last two weeks. Preventive group received 0.75% EG in their drinking water and PO extract at 100mg/kg via oral gavage daily for five weeks; in the first five days they fed with drinking water containing 0.75% EG and 1% AC. Therapeutic group received 0.75% EG in their drinking water for five weeks; in the first five days they fed with drinking water containing 0.75% EG and 1% AC. During this period, PO extract at 100mg/kg via oral gavage daily was administered daily for last two weeks (Figure 8). After the experimental period, 24-hour urine was collected and then all rats were sacrificed to obtain kidney tissue samples. After the rats were sacrificed, kidneys were removed from each animal and washed with cold 0.9% NaCl and stored at -80°C until further use.

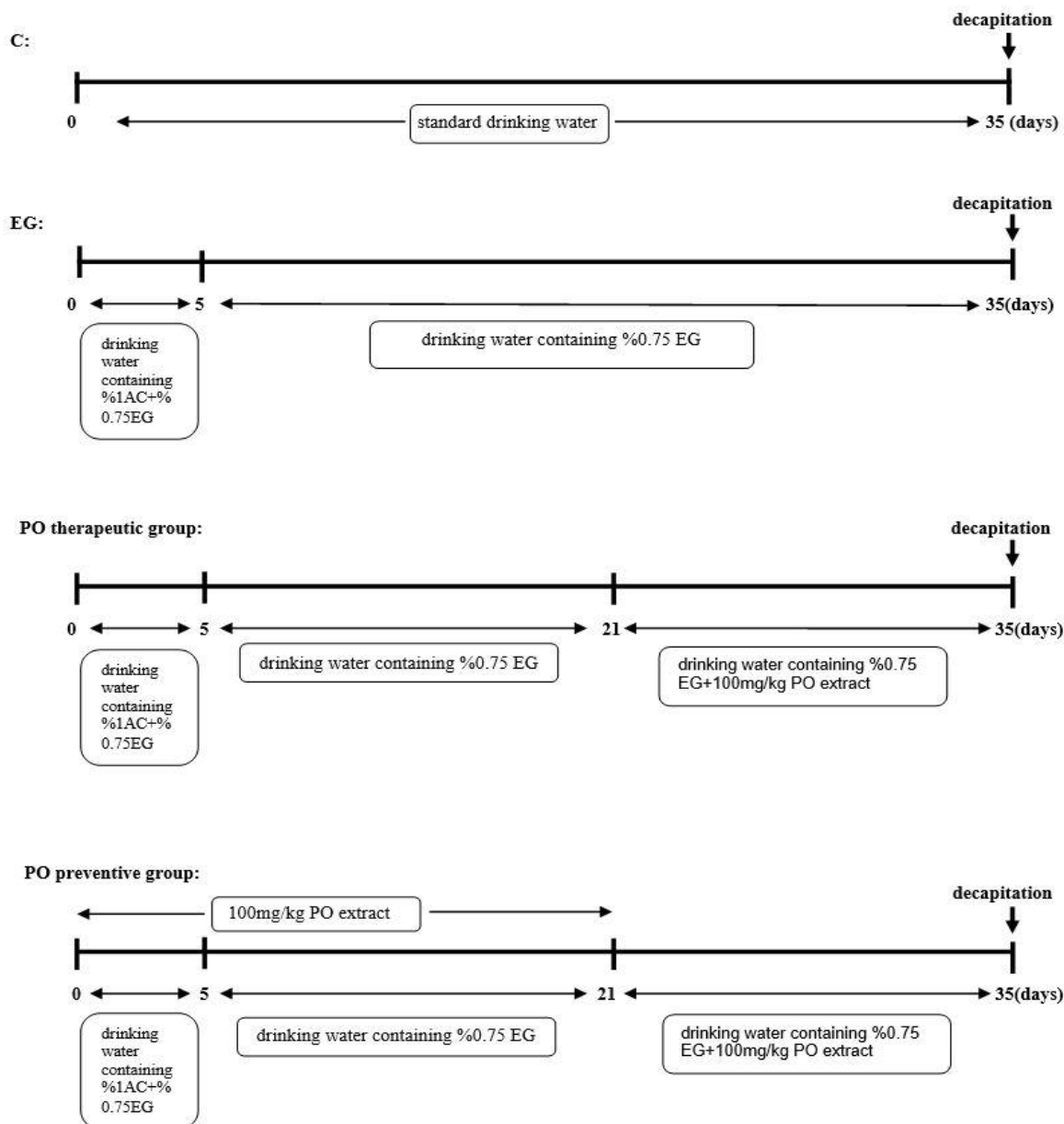


Figure 8. Schematic diagram of experimental design.

5.6. Biochemical analysis

5.6.1. Analysis of urine samples

At the end of the experimental period all the animals were housed in individual cages and a 24-hour urine samples were collected before sacrifice. Urinary calcium, oxalate, citrate, and phosphate levels were measured with commercial kits from Colorimetric Abbott BNSRL, Italy.

5.6.2. Analysis of kidney samples

Tissue samples were homogenized and were centrifuged at 1000 x g for 10 minutes at 4°C and supernatants were separated. Tissue levels of TNF- α were analyzed by ELISA kit (Sunred, 201-11-0765) with sensitivity of rat TNF- α was 5.127 ng/L. Tissue levels of IL-1 β were analyzed by ELISA kit (FineTest, ER1094) with sensitivity of rat IL-1 β was 18.75 pg/ml. Tissue levels of caspase-3 protein were analyzed by ELISA kit (Sunred, 201-11-5114) with sensitivity of human Caspase-3 was 0.0033 ng/ml. Tissue levels 8-OH-dG were analyzed by ELISA kit (FineTest, ER1487) with sensitivity of 8-OH-dG was 0.938 ng/ml.

NAG enzyme activity was measured by spectrophotometric method using commercial kit (Sigma Cs0780). According to the kit protocol, NAG enzyme activity is the formation of p-nitrophenol in the reaction that takes place by hydrolyzing the NP-GlcNAc substrate in an acidic environment (pH=4.7) at 37°C. Tissue samples (10µl) were used after homogenization with cold buffer and reaction was measured the conversion of p-nitrophenol to the yellow-colored p-nitrophenylate ion at a wavelength of 405 nm.

The MDA levels of homogenized tissue samples were assayed thiobarbituric acid reactive substances [26]. The results are expressed as nmol/g tissue. Glutathione measurements were performed using metaphosphoric acid (Na₂HPO₄) for protein precipitation following the Ellman procedure [28]. Glutathione concentrations were expressed in µmol GSH/mg protein. The MPO activity was measured in tissues as previously described [29]. Myeloperoxidase results were expressed as U/mg protein. Superoxide dismutase activity in tissue samples was measured as previously described [30]. Superoxide dismutase results were expressed as U/mg protein.

5.7. Histopathological analysis

The kidney tissues were fixed in 10% formalin solution. Then they were embedded in paraffin, cut into 4-µm-thick sections, stained for histopathology with hematoxylin and eosin (H&E). All stained sections were evaluated using light microscope (Olympus BX51, Tokyo, Japan).

5.8. Statistical analysis

All data were analyzed using GraphPad Prism 6.0® software (GraphPad Software, Inc., San Diego, CA). Results were expressed as mean±standard error of mean (S.E.M). Data of groups was compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of p<0.05 were regarded as significant.

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