The Effects of Aqueous Extract of Eryngium Campestre on Ethylene Glycol-Induced Calcium Oxalate Kidney Stone in Rats

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Purpose: This study aimed to evaluate the anti-inflammatory effect of *E. campestre* using the aqueous extracts, obtained from the aerial parts, on Ethylene Glycol (EG)-induced calcium oxalate kidney stone in rats.

Materials and Methods: 64 male Wistar rats were randomly divided into 8 groups. Group I was considered as negative control and received normal saline for 30 days, group II as kidney stone control received EG for 30 days, groups III to VI as prophylactic treatment received EG plus 100, 200 or 400 mg/kg extracts for 30 days and groups VI to VIII received EG as therapy from day one and 100, 200 or 400 mg/kg extract from the 15th day. On the 30thday from the start of induction, rats were euthanized. Blood was collected and the kidneys were immediately excised. Slides from each one's kidneys were prepared and stained with Hematoxylin & Eosin method. Also levels of interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) were determined in rat's serum by competitive ELISA kit.

Results: E. campestre reduced IL-1β and IL-6 levels, showing a significant reduction for both cytokines in all prophylactic groups, especially at the dose of 400 mg/kg (*P*-value < .001). Moreover, IL-1β (p = .011) reduced significantly in the therapy groups in 400 mg/kg dose. Crystal count reduction was seen in all prophylactic and therapy groups in comparison with group II.

Conclusion: These results suggest that the E. campestre extract has potent suppressive effect on pro-inflammatory cytokine production in rat. Also, E. campestre decreases crystal deposition in the kidney of the hyperoxaluric rat.

Keywords: cytokines; *E. campestre*; inflammation; kidney stone

INTRODUCTION

Kidney stone is among the oldest and the most common diseases known to human and the third most prevalent disorder of the urinary tract that affects 10-15% of the total population around the world[(1)]. The prevalence of kidney stones in Iran was 5.7% in 2005, with slight increase prevalence in men (6.1%) than women (5.3%)[(2-4)]. There are several risk factors related to the formation of urinary calculi, such as gender, genetics, dietary habits and climate. Also, metabolic acidosis, hypertension and urinary tract infections are associated with urinary tract stone. Calculi usually form when urine becomes supersaturated with particular calcium salts such as calcium oxalate[(5)]. Based on chemical composition, the most common types of kidney stones are calcium, magnesium ammonium phosphate, uric acid, and cysteine. In this regard, calcium-containing stones constitute about 75% of all the urinary calculi and can exist as either calcium oxalate or calcium phosphate (Apatite), or a mixture of both[(6,7)]. Calcium oxalate (CaOx) stones are responsible for a large proportion of calculi in primary and secondary forms of hyperoxaluria[(8,9)].

Current therapy approaches for kidney stone are mainly supportive like drinking plenty of water and the use of anti-inflammatory drugs. Although,stone pieces may discharge naturally, but bigger pieces should be removed by surgery. Also, ultrasound shock waves are used to break the larger stones into tiny pieces. However, this has been associated with a number of undesirable side-effects, such as tubular necrosis, hemorrhage and kidney fibrosis[(10)]. Herbal medicine recently attracted research attention to avoid the adverse effects of current anti-urolithiasis therapies[(11-14)].

The genus Eryngium, belonging to the subfamily Saniculoidea of Apiaceae, consist of approximately 317 species around the world and are well-known to possess acetylenes, flavonoids, coumarins and triterpensaponins[(15)].Eryngiumcampestre L. (Apiaceae) (field eryngo) is scattered in Spain, France, Germany, Balkan Peninsula and some countries in Africa and Asia including Iran[(16)]. Also, it has been used in European herbal medicine as an infusion to treat kidney and urinary tract inflammations.[(17)]

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Table1: Effect of *E. campestre* on CaOx crystal deposits in urolithiasis induced rat.

	Control groups		Prophylactic groups			therapy groups		
Parameter	NC	KSC	P100	P200	P400	T100	T200	T400
CaOxcrystals	0.7 ± 0.6*	33.5 ± 9.1	17 ± 8.5*	5.8 ± 2.6*	5.31± 1.5*	19 ± 5.1*	12 ± 6.9*	9.9 ± 4.2*

* The mean differences in comparison with KSC group is significant at the < 0.01 level.

This study aimed to evaluate the anti-inflammatory effect of E.campestre, using the aqueous extracts obtained from the aerial parts, on Ethylene Glycol-Induced CaOx kidney stone in rats.

MATERIALS AND METHODS

Preparation of extract

E.campestre was collected from the northern parts of Iran and its herbarium code was determined at the School of Pharmacy and Pharmaceutical Sciences, Mazandaran University of Medical Sciences (Code: 1442). Aerial parts were separated, dried in shade and powdered to a fine grade using a grinder. Thirty grams of powder was dissolved in distilled water in a volume of 600 ml and stirred by a stirrer for 24 hours in the room temperature. The resulting mixture passed through sterilized gauze and subsequently purified with Whatman paper. Finally, the extract was placed in an incubator at 40 °C to completely dry. Three grams of dry extract (10 percent) obtained from every 30 grams of dried plant powder. The extract was kept in a refrigerator and the doses of the extract were prepared in distilled water before oral administration to rats.

Experimental protocol

This experimental study was conducted in 2017 in 30 days in Birjand University of Medical Sciences. This study received an ethics code (code: Ir.bums. REC.1396.76) from the University Ethics Committee. A total of 64 male Wistar rats weighing 200–250 g were divided randomly into8 groups (each group contained 8 rats). Rats were housed under controlled standard conditions at a temperature of 25 ± 2 °C and 12-hour darkness and lightning cycles with free access to standard food and drinking water. Ethylene glycol (EG) was used to induce hyperoxaluria in rats[18]. The followings are the details of the experimental procedure, adopted for the study:

Group I or negative control (NC): the standard diet and drinking water; they received 1 ml of normal saline by oral gavage once daily.

Group II or kidney stone control (KSC): standard diet and 0.1 % of EG in drinking water during the study period; they received 1 ml of normal saline by oral gavage once daily.

Group III (prophylactic 100): standard diet and 0.1 % of EG in drinking water during the study period; they received 100mg/kg of extract by oral gavage once daily for 30 days in the final volume of 1 ml.

Group IV (prophylactic 200): standard diet and 0.1 % of EG in drinking water during the study period; they received 200mg/kg of extract by oral gavage once daily for 30 days in the final volume of 1 ml.

Group V (prophylactic 400): standard diet and 0.1 % of EG in drinking water during the study period they received 400mg/kg of extract by oral gavage once daily for 30 days in the final volume of 1 ml.

Group VI (therapy 100): standard diet and 0.1 % of EG in drinking water during the study period, and from the 15th day until the end of the study they received the extract at a dose of 100mg/kg daily in the final volume of 1 ml.

Group VII (therapy 200): standard diet and 0.1 % of EG in drinking water during the study period, and from the 15thday until the end of the study they received the extract at a dose of 200mg/kg daily in the final volume of 1 ml.

Group VIII (therapy 400): standard diet and 0.1 % of EG in drinking water during the study period, and from the 15thday until the end of the study they received the extract at a dose of 400mg/kg daily in the final volume of 1 ml.

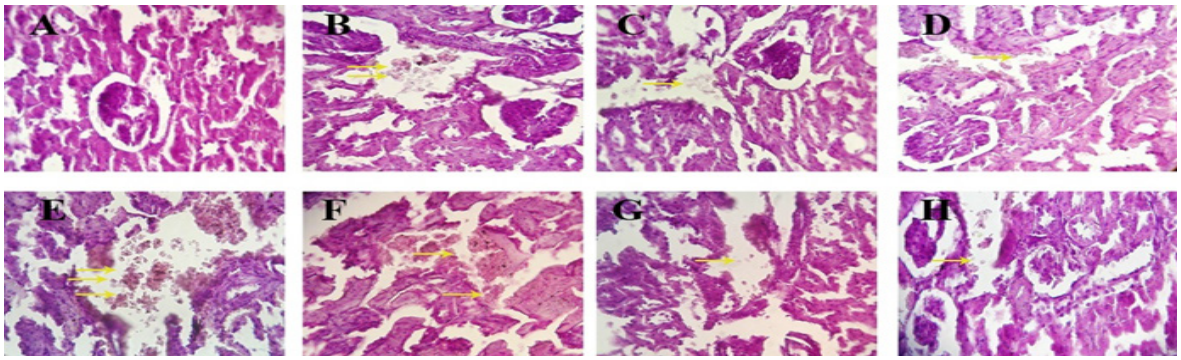

Figure 1: Photomicrographs of H&E stained paraffin sections of rat kidney tissues. A. There were no deposits of crystals in the normal control group. E. KSC group showed significantly increased levels of crystal deposits. B, C, D. Prophylactic group (doses 100,200and 400 mg/kg, respectively) had significantly decreased crystals in comparison to KSC group. F, G, H. Interventiongroup (doses 100,200and400 mg/kg, respectively) also had a significant decrease of crystals in comparison with KSC group. Original magnification x40.Abbreviations: KSC, Kidney Stone Control.

Table2: Effect of the aqueous extract of E. campestre on serum levels of IL-1 and IL-6 in different groups in comparison to KSC

groups	IL1 Mean	Standard Deviation	IL6 Mean	Standard Deviation	IL1(*P*-value compare to KSC)	IL6 (*P*-value compare to KSC)
KSC	1012.26	182.48	130.80	19.80		
T100	821.90	142.15	101.24	38.55	0.206	0.142
T200	803.38	182.09	94.27	20.31	0.123	0.030
T400	734.07	135.58	102.12	16.68	0.011	0.168
P100	772.68	112.09	89.75	20.75	0.046	0.009
P200	745.80	133.34	81.06	24.29	0.017	0.001
P400	654.82	191.08	71.71	12.26	$P < 0.001$	$P < 0.001$
NC	592.62	98.62	56.80	8.50	$P < 0.001$	$P < 0.001$

Abbreviations: T, Therapy group; P, Prophylactic group; NC, Normal Control; KSC, Kidney Stone Control.

Histopathologic examination and Cytokine Measurement

On the 30th day from the start of EG induction, the animals were anesthetized and euthanized. Blood was collected and the kidneys were immediately excised and washed in ice-cold saline and placed in 10% formalin; they were then embedded in paraffin and on each slide, 3 sections of 5μm were stained by Hematoxylin & Eosin. The CaOx crystal deposition, induced by EG in tissue kidney, was studied by the pathologist, using the light microscopy with a magnification of ×40 in 10 microscopic fields.

Serum was separated by centrifugation at 3000×g for 15 minutes. Levels of IL-1β and IL-6 were determined in all groups by rat competitive ELISA kit (EASTBIOPHARM, China, lot number: E20170620008) according to the manufacturers' instructions (eastbiopharm.com Inc.).

Statistical analysis

All data was analyzed using SPSS version 18.0 (New York: McGraw-Hill) and are expressed as mean ± SD. One-way ANOVA (LSD post hoc test) was used to compare the mean values of the cytokines. A *P*-value < 0.05 was considered statistically significant for the differences.

RESULTS

Histopathologic examination

The histopathologic status of calcium deposits in rats' kidneys paraffin sections was analyzed in the field view of ×40 magnification (**Figure 1**). Almost no CaOx deposits were found in group I. **Table 1** provides information about the average distribution of CaOx deposits in kidneys of rats in different groups. The mean numbers of CaOx deposits in the kidney specimens of both prophylactic and therapy groups were significantly lower than that in the kidney stone control group ($P < .001$). Rats who received 200 or 400 mg/kg extract had shown more decline in crystal deposits (average number of crystal deposits = 8.25) compared to those who received 100 mg/kg extracts daily with average number of crystal deposit being 18 in prophylactic and therapy groups. In total, crystal deposits in prophylactic groups were lower (mean crystals = 9.3) compared to therapy groups (mean crystals = 13.63).

Serum analysis

Based on the serum levels of IL-1β and IL-6 presented in **table 2**, the values of normal control group were significantly lower than their values in EG administered KSC group ($P < .001$). The IL-1β and IL-6 serum levels of rats received 400 mg/kg extracts in prophylactic groups showed a significant decrease in comparison with the KSC group ($P < .001$). Furthermore, the reduction of IL-1β and IL-6 levels remained significant at lower doses of extracts (100 & 200 mg/kg) in the prophylactic groups. While the reduction of IL-1 in treatment groups was significant in 400 mg/kg dose ($p = .01$), it remained unchanged for IL-6 in these groups. From the values, it was evident that both doses (200 and 400 mg/kg) of the extract are effective in reducing the serum levels of inflammatory cytokines in prophylactic groups (**Figure & Table 2**). The results clearly demonstrated that the high dose of 400 mg/kg extract is more

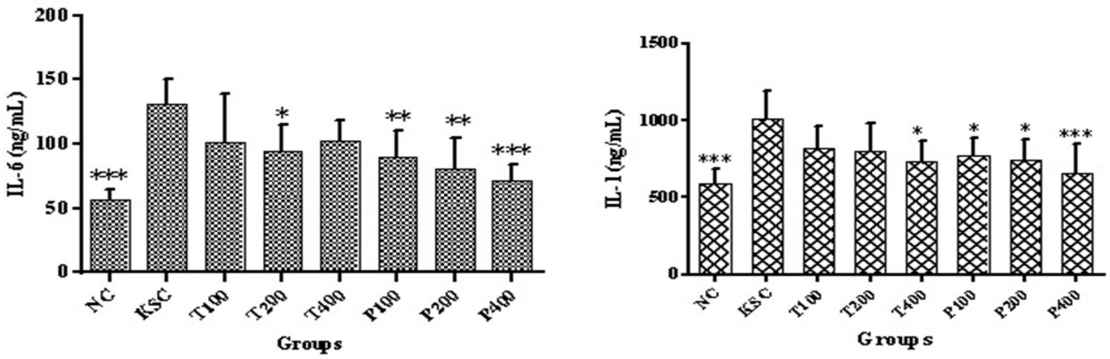

Figure 2. Effect of the aqueous extract of E. campestre on serum levels of IL-1 and IL-6 in different groups in comparison to kidney stone control (KSC) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < .001$). Abbreviations: T, Therapy group; P, Prophylactic group; NC, Normal Control; KSC, Kidney Stone Control.

effective in reducing the serum levels of IL-1β and IL-6 inflammatory cytokines.

DISCUSSION

Nephrolithiasis incidence and prevalence is reported to be increasing annually around the world, along with a decrease in the age of onset, probably because of changes in lifestyle, diet, and climate[19].In the present study, EG was used to produce hyperoxaluria in male Wistar rats. As the urinary system of male rats resembles that of humans, kidney stone formation in EG fed rats is frequently used to mimic the Urinary calculi formation in human[20]. Preceding studies showed that 14 days administration of EG, increases oxalate concentration in urine which leads to the formation of renal calculi[21].
The Pathological process of stone formation involves crystal nucleation, growth, aggregation and their retention in the kidneys. Although the triggers and the exact mechanisms of crystal formation are not well understood, crystal deposition has been linked with intra-renal inflammation[22,23].From previous histopathological findings, EG causes tissue damage in the renal tubular epithelium and tubules dilatation. Enhancement of renal tubular epithelial cell damage leads to emptying the contents of the cell, attracting inflammatory cells, and thereby inflammatory response in the renal tissue[24]. Inflammation leads to the development of collagen deposit and epithelium transformation which is conducive to biomineralization processes in the tubular basement membrane[24]. The pharmacological effects of Eryngium species correlated with the presence of high triterpenoid saponin content[25], flavonoids[26,27], phenolic acids[28] coumarin derivatives[29], acetylenes[30,31], Rosmarinusacid, and chlorogenic acid, known as antioxidants[32,33].Exclusively, phytochemical analysis revealed significant concentrations of sterols and anti-inflammatory effects[34]. E. campestre contained high amounts of sitosterol and stigmasterol, while cholesterol was detected in small amounts[31]. Phytosterols (sitosterol, stigmasterol, and campesterol) are responsible for antioxidant effects in some diseases[35]. *E. campestre* extracts showed very significant inhibition of the bone marrow acute phase response by reducing the phagocytic leukocytes count and by lowering neutrophils and monocytes infiltrates[34]. In spite of the extensive usage of Eryngium species in the treatment of inflammatory disorders around the world, the number of scientific investigations evaluating the anti-inflammatory or anti-nociceptive activity of Eryngium is limited. In this study, microscopic examination of kidney sections obtained from EG induced urolithic rats showed crystal deposition in the kidneys of lithogenic groups along with inflammation, which might be attributed to oxalate. Based on the findings, the administration of extract in a prophylactic regimen (100, 200 & 400 mg/kg) reduced CaOx crystal deposition in the kidney (**Table1**). In the therapy groups (group VI to VIII), the extract at the dose of 200 mg/kg also reduced the count of crystal deposition significantly (**Table1**). Crystal nucleation induced by super saturation of urine with CaOx and crystal growth caused by oxidative stress, resulted from renal epithelial exposure to CaOx crystals[36]. The antioxidant or anti-inflammatory potential of E. campestre extract showed a protective effect against crystal deposition in the kidney of rats that received the extracts. E. campestre extracts reduced the level of IL-1β and IL-6 cytokines significantly in all prophylactic groups($P < 0.001$) (Figure & Table2). Moreover, E. campestre extract possessed the inhibitory effects on IL-1β at the high dose of 400 mg/kg in the treatment groups compared with the negative control group ($P < 0.01$). However, all the prophylactic groups (group III to V) exhibited a marked decrease in IL-1β and IL-6 levels at a high dose (400 mg/kg) of extract in comparison to treatment groups. Therefore, the results revealed the significant anti-inflammatory activity of E. campestre extracts in EG-induced inflammation in rats with a prophylactic regimen. Considering the activation of the inflammasome by a wide variety of triggers during the inflammatory conditions, the extracts may interfere with the inflammasome-dependent IL-1β signaling pathway, which warrants further studies[37]. Simona Conea et.al. showed that the anti-inflammatory activities of the extract were supported by inhibition of phagocytes and nitro-oxidative stress reduction. The effect is likely related to a synergic activity of the detected sterols, triterpenoid saponins, and polyphenolic compounds[37]. Overall, the results obtained in the present study are consistent with the anti-inflammatory effects of the eryngium reported in the previous studies[34,38]. Since inflammatory cytokines have significantly decreased in the treatment group compared with the KSC group, it is suggested to measure anti-inflammatory cytokines such as IL-10 and IL-4 in future studies. Also, additional tests such as flow cytometry and western blotting can be further examined to assess the effects of this extract on inflammation and EG- induced calcium oxalate kidney stones.

CONCLUSIONS

E. campestre canlower the level of proinflammatory cytokines such as IL-1β and IL-6 in rats, demonstrating a potent bioactive potential to suppress the inflammatory response. Also, E. campestre decreased crystal deposition in the kidney of the hyperoxaluric rat. This study suggests E. campestre extract as an anti-urolithiasis agent for prophylactic treatment of CaOx kidney stone inflammation.

CONFLICT(S) OF INTEREST

The authors declare no conflict of interest.

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