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Original Research

Effect of Hydroalcoholic Extract of *Allium noeanum* Reut. ex Regel on Ethylene Glycol- Induced Kidney Stone in Male Wistar Rats

Mehrdad Goudarzi[1], Maryam Eskandari Mehrabadi[2], Zahra Salemi[2]*, Jamal Amri[1], Mitra Noori[3], Ali Rahbari[4], Amirhossein Alizadeh[2], Atefeh Soltani[5], Mitra Panahi[6], Farshad Eskandari Mehrabadi[7]

[1]*Traditional and Complementary Medicine Research Center, Arak University of Medical Sciences, Arak, Iran*
[2]*Department of Biochemistry, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran*
[3]*Department of Biology, Faculty of Sciences, Arak University, Arak, Iran*
[4]*Department of Pathology, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran*
[5]*Department of Medical Sciences, Islamic Azad University, Aligodarz, Iran*
[6]*Department of Biology, Alexander College, Vancouver, Canada*
[7]*Islamic Azad University of Ayatollah Amoli, Amol, Iran*

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Abstract

We want to evaluate the effect of *Allium noeanum* Reut. ex Regel (Bonsor) known (traditional medicine agent) in calcium oxalate stones in kidney. 36 male rats were divided into 6 groups. I: healthy model + water, II: negative model + 1% ethylene glycol in water, III: 750 mg/kg of total extract +1% of ethylene glycol in water(Prevention), IV: 250 mg/kg flavonoid extract +1% of ethylene glycol in water (Prevention), V: 1500 mg/kg of total extract from 15th day+ 1% of ethylene glycol in water (Treatment), VI: 500 mg/kg of flavonoid extract from 15th of the study + 1% of ethylene glycol in water (Treatment).24-hour urine and blood samples were collected in 30th day for analysis. Pathology of kidneys was checked. Serum urea, uric acid, creatinine and urine calcium and oxalate were significantly increased, urine citrate was decreased in group II Vs I. ($P < .05$). Extract administration significantly decreased serum creatinine, urea and uric acid. Urine calcium and oxalate significantly decreased in treated groups. Urine calcium levels were significantly decreased in treated rats, but urine citrate levels were increased Vs group II. ($P < .05$). No crystal accumulation and tubular cast were observed in prevention groups. Hydroalcholic extract of *Allium noeanum* was able to reduce urine oxalate.

Keywords: *Allium noeanum* extract; Urinary calculim; Ethylene glycol; Rat; Calcium oxalate

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*Corresponding Author: Zahra Salemi
Department of Biochemistry, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran
Email: 1999fe1999@gmail.com

Introduction

Kidney stone is a serious problem and leads to severe complications including urinary tract obstruction, hydronephrosis, urinary infections and hemorrhage [1]. This disease has several risk factors such as genetic history, age and sex, the weather conditions of the place of residence, water consumption and its salts and dietary habits [2]. The risk of kidney stones is about 10 to 15 percent in developed countries, but rising to 25-20 percent in the Middle East, with a higher incidence in male [3]. In 2005, the annual incidence of this disease in Iran was estimated at 147.2 for men and 129.6 for women per 100,000 people. Also, in this year, the relapse rate was 16% after one year, 32% after 5 years, 53% after 10 years [4].

Therapeutic treatments are widely used to remove the bigger kidney stones which do not spontaneously repel and cause severe complications including dissolution of the kidney stone through conventional drugs, surgical operation, extracorporeal shock wave lithotripsy, percutaneous lithotomy, transureteral lithotripsy [5,6]. These procedures are expensive, invasive, painful and has caused serious complications. Accordingly, researchers are looking for alternatives methods such as medicinal plants or phytotherapy [7,8].

Allium noeanum (Bonsor) belongs to the Amaryllidaceae family. The inhabitants of the central regions of Iran believe this plant as an effective agent for kidney stones; though, scientific confirmatory research has not yet been carried out. Therefore, this study aimed to evaluate the effect of hydro alcoholic extract of Bonsor (Total and Flavonoid) on ethylene glycol induced-kidney stones in rats.

Materials and Methods

Preparation of extract and fractions

Aerial parts of *Allium noeanum* was collected from Shazand in Markazi Province, Iran (CMG1 = Mehrdad Godarzi Collection Number in Arak University Herbarium, not listed in herbarium index). Samples were authenticated by taxonomist Dr. Mitra Noori based on available references [9,10]. The voucher specimen was deposited at the Department of Biology, Faculty of Science, Arak University, Iran (CMG1, 07 June 2016). Aerial parts were dried in the shade for a week, powdered, and 300 g of the leaf powder was extracted with 70 % ethanol and the extract was vacuum concentrated to dryness in a rotary evaporator. After dividing the extract into two parts, first extract labeled as total extract and the second was used for flavonoids isolation. The flavonoids in the second extract were isolated and detected using two-dimensional paper and thin-layer chromatography according to reported methods (Markham 1982). Main active ingredients were measured in both extracts. Both, total and flavonoid extracts were kept in dark vials and stored in cool conditions until further use. Two-dimensional paper chromatography (2-D PC): Two hundred milligrams of the leaf powder was boiled for 2 min in 5 ml of 70 % EtOH, then cooled and left to extract for 24 h. The extract was then filtered, evaporated to dryness by rotary evaporation at 40° and dissolved in 2 ml of 80 % MeOH for performing 2-D PC.

About 2 µl of the extract was applied and rutin (quercetin 3-O-rutinoside) was used as a standard, the paper was developed in mixture of n-butanol:acetic acid:water(4:1:5) (BAW; first direction). HOAc 15 % was used for the second direction. The dried chromatogram was studied using UV at 366 nm and Rf values were calculated for both dimensions. After acid hydrolysis, co-chromatography was done by applying hydrolyzed flavonoid extract and standards on thin-layer cellulose chromatogram. TLC plate was run in a mixed solvent, viewed under UV at 245 and 366 nm and the Rf -values and color was recorded for each spot in comparison with standards. Results showed aerial part of the species contains 4 flavonoid sulphates, aglycones and flavones C and C-/O glycosides were not find in the species. Apigenin, narigenin, Rutin and vitexin were found in crude extract. But in special flavonoids extract after asetolysis, Isorhamnetin, Kaempferol, Luteolin, Morin, Myercetin, Quercetin and Rhamnethin were identified in the species aerial part. In our previous article, we provided details of the results [11].

Experimental protocol

Animals

In this experimental study, 36 Wistar rats weighing 180-220 g were taken and transferred to the Animal Laboratory of Arak University of Medical Sciences. Animals kept in clean cages under conditions of light (12 h light-dark cycles), temperature (22 ± 2 °C), relative humidity (55 ± 5%) and had free access to food and drinking water. All research and laboratory animal care processes were conducted according to the Guide for the Care and Use of Laboratory Animals (8th edition; National Academies Press; 2011) and approved by Review Board and Ethics Committee of Arak University of Medical Sciences (Ethics number: IR.ARAKMU.REC.1395.238)

EG-induced urolithiasis and animals study design

In this experimental study, 36 male Wister rats were randomly divided into 6 groups. Urolithiasis was induced by 1% ethylene glycol in the experimental animals [12,13]. Finally, the groups were designed as follows:

Group I (HM): This group was selected healthy model group (HM: healthy model) which received water and normal food for 30 days.

Group II (NM): This group served as the negative model group (NM: negative model) that received drinking water containing 1% ethylene glycol for 30 days.

Group III (P750) and IV (P250): These groups were considered as preventive groups that received drinking water containing 1% ethylene glycol plus 750 mg/kg total extract (P750: prevention by 750 mg/kg total extract) (group III) and 250 mg/kg flavonoid extract of *Allium noeanum* (P250: prevention by 250 mg/kg flavonoid extract) (group IV) from day 1st to 30th.

Group V(T1500) and VI(T500): These groups were considered as curative groups that received drinking water containing 1% ethylene glycol for thirty days and from the 14th day until the end of the study these groups received 1500 mg/kg total extract (T1500: treatment by 1500 mg/kg of total extract) (group V) and 500

mg/kg flavonoid extract of *Allium noeanum* (T500 treatment by 500 mg/kg of flavonoid extract) (group VI).

Measurements

Urine analysis

In the last day, the 24-hour urine was collected separately in metabolic cage for evaluating calcium, oxalate, citrate and and pH. Samples were centrifuged for 5 min at 2500 rpm. Pars Azmun kits and spectrophotometer (JENWAY 6505, Europe Union) were used to measure these parameters.

Serum analysis

All animals were anesthetized with ketamine (75 mg/kg b.w) and xylazine (10 mg/kg b.w). Blood samples were collected by cardiac puncture and centrifuged at 3000 rpm for 5 minutes, serum was quickly separated. Serum samples aliquot were stored at -80 C. The serum levels of urea, creatinine, uric acid and calcium were measured in all groups by Pars Azmun kits and spectrophotometer (JENWAY 6505, Europe Union).

Histopathology study

Both kidneys were removed and fixed in 10% formalin. After dehydrated and embedding in paraffin, slides with thickness of 3 μm were prepared. Then, 3 slides were selected from each kidney and stained with Hematoxylin and Eosin. The number of calcium oxalate crystals was counted in 10 microscopic fields (×40 magnification) of each slide under a light microscope.

Statistical analysis

All the data were expressed as means ± standard error mean (SEM) of two replicates for six rats in each group. First, we checked the normality of the data. One-way ANOVA followed by Bonferroni's posttest were used to compare the data. The P-value < 0.5 was considered statistically significant All statistical analyses were performed with GraphPad Prism software (Version 6.00).

Results

Urine parameters

Calcium levels

As shown in Table 1, the mean of calcium level in the group NM was significantly higher than the group HM ($P < 0.01$). In groups P750 and P250, both total extract ($P < 0.01$) and flavonoid ($P < 0.001$) have been able to reduce the amount of calcium vs. the group NM. Notably, the results have been shown that flavonoid extract was significantly more effective than total extract for reducing urine calcium ($P < .05$). In groups T1500 and T500, total extract and flavonoids ($P < 0.001$) significantly decreased urinary calcium levels. But there was no significant difference between them ($P > .05$). However, in these groups the results showed that total extract was better than flavonoid.

Oxalate levels

According to Table 1, urine oxalate was significantly increased in group NM vs. group HM.

Urine oxalate was significantly reduced in both P250 and P750 compared with NM (P <

0.001)., although there was not any significant difference between them (P > 0.05) (P250 was numerically more effective than P750). It also was significantly reduced in both T1500 (P < 0.01) and T500(P < 0.001) than NM. Although there was not any significant difference between them (P > 0.05) (T500 was numerically more effective than T1500).

Citrate levels

The finding demonstrated that urine citrate was significantly decreased in group NM vs. group HM. The urine citrate levels of the groups P750, P250, T1500 and T500 increased significantly as compared to the group NM (P < .05). It is worth to note, the effect of total extract on increasing citrate in prevention and treatment groups was higher than flavonoid.

Urine pH

Urine pH (P < .05) increased significantly in group NM, compared with group HM urine pH

Table 1. Effect of hydroalcholic extract (flavonoids and total extracts) of *Allium noeanum* on urine parameters in the studied groups[¥]

Urine parameters [#]	I (HM)	II (NM)	III (P750)	IV(P250)	V(T1500)	VI(T500)
Calcium (mg/24 h)	5 ±0.16	11.2 ±0.32[a]**	8.9±0.11[b]**	6.5±0.27[b]***	6.6±0.23 [b]***	6.3±0.32 [b]***
Oxalate (mg/24 h)	4.7±0.66	14±0.48[a]***	8.1±0.33[b]***	7.2±0.11[b]***	9±0.11[b]**	5±0.25[b]***
Citrate (mg/24 h)	0. 4±0.09	0.2±0.44[a]*	0.4±0.17[b]*	0.42±0.17[b]*	0.8±0.15[b]*	0.62±0.15[b]*
Urine pH[#]	6.1±0.119	7.1±0.108[a]*	6.9±0.131	6.9±0.108	6.8±0.085	6.7±0.086

[¥]These results were evaluated at the end of the 30th days in all groups

[#] Data are expressed as mean ± SEM for each group (n=6).

[a]Compared with group I

[b]Compared with group II

*Significance level was considered at P < .05

**Significance level was considered at P < 0.01

***Significance level was considered at P < 0.001

Serum parameters

Uric acid, urea and creatinine levels

The serum parameters at the end of the study is shown in table 2. Uric acid ($P < 0.01$), urea ($P < 0.001$) and creatinine ($P < 0.01$) was significantly increased in group NM compared with group HM. The concentration of uric acid ($P < .05$), urea ($P < 0.01$) and creatinine ($P < .05$) in groups p750 and P250 were significantly decreased. These results indicated that the prevention with total extract was better than flavonoid, although it was not significant. In group T1500 and T500, the uric acid level ($P < 0.01$), urea ($P < 0.01$) and creatinine ($P < .05$) dramatically decreased vs. NM and the flavonoid effect was better than total extract, but the difference was not significant.

Table 2. Effect of hydroalcholic extract (flavonoids and total extracts) of Allium noeanum on serum parameters in the studied groups[¥]

HC, NC, P750, P250, T1500 and T500.

Serum parameters	I (HM)	II (NM)	III (P750)	IV(P250)	V(T1500)	VI(T500)
Uric acid (mg/dl)	3.5 ±0.44	5.8 ±0.23[a]**	4±0.14[b]*	4±0.36[b]*	3±0.23 [b]**	2.7±0.32 [b]**
Urea (mg/dl)	37±0.56	60±0.32[a]***	40±0.31[b]**	42±0.21[b]**	49±0.11[b]**	45±0.25[b]**
Creatinine (mg/dl)	0. 5±0.15	0.8±0.18[a]**	0.6±0.16[b]*	0.62±0.01[b]*	0.6±0.19[b]*	0.6±0.12[b]*

[¥]These results were evaluated at the end of the 30th days in all groups

[#] Data are expressed as mean ± SEM for each group (n=6).

[a]Compared with group I

[b]Compared with group II

*Significance level was considered at $P < .05$

**Significance level was considered at $P < 0.01$

***Significance level was considered at $P < 0.001$

Histopathological result

Pathologic evaluations of kidney tissue sections revealed no evidence of oxalate deposits and did not inflammation in group HM. But, in the group NM, which has not been treated, oxalate deposits and inflammation, collecting ducts, tubular atrophy, dilation, and tubular cell necrosis were detected in kidney tissue. In prevention (group P750 and P250) and treatment (group T1500 and T500) groups, the oxalate deposits and tissue damage were decreased compared with group NM in both of flavonoid and total extract of *Allium noeanum* (No statistical analysis was performed).

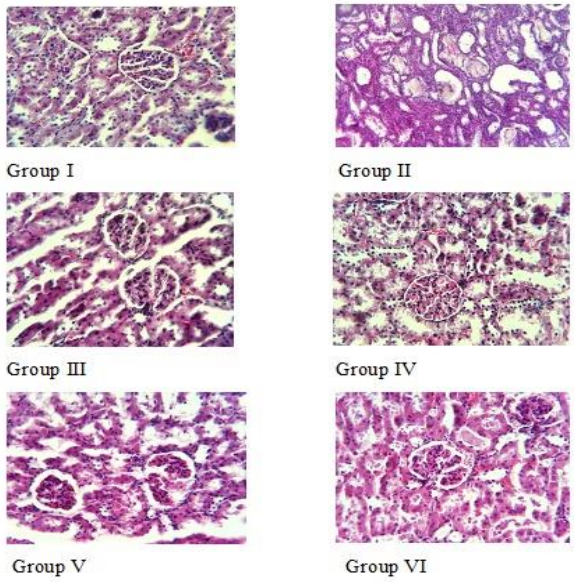

Figure 1. Group I(HM) showing normal tubules and collecting ducts; Group II(NM) showing calcium oxalate crystals and secondary renal tubular dilatation; Group III(p750), IV(P250) (prevention groups) and Group V(T1500) and VI(T500) (curative groups) presenting normal urinary tubules without calcium oxalate crystals. Magnification of x400, optical microscopy and staining of hematoxylin-eosin were used for all sections.

Discussion

Medicinal herbs are widely used in different parts of the world and considered as a good alternative to chemical drugs due to their lower side effects and naturalness. The utilizing of medicinal plants to repel or dissolve kidney stones has been commonplace since ancient times and is increasing due to the convenience and low cost.

The effect of hydroalcholic extract of *Allium noeanum* has not been studied so far. In traditional medicine, it is recommended to dissolve or eliminate renal stone. Therefore, the present study is the first report on the effect of *Allium noeanum* on calcium oxalate stone in experimental model of urolithiasis.

Overall, our results demonstrated that total hydroalcoholic extract in the prevention and treatment group decreased urinary calcium oxalate and also serum uric acid, urea and creatinine levels in comparison with NM. Urinary citrate level in treatment and prevention groups increased compared to NM. On the other hand, these extracts reduced the tissue damage in the kidney of treated animals compared NM. Oxalate is an accelerating factor in Calcium oxalate stone formation. Thus, reducing the risk of stone formation in this investigation can be explained by its reducing effect on the urinary oxalate level. Citrate is regarded as an effective inhibitor of Calcium oxalate stone formation. Elevated levels of urinary citrate in treatment groups may indicate that this extract increased citrate levels to reduce the incidence of oxalate kidney stones and thus, the kidneys excreted more citrate than NM [14].

To the best of our knowledge, this is the first report regarding the effect of total hydroalcoholic extract on prevention and treatment of kidney stone. Lastly, several other herbal extracts were also demonstrated to have an inhibitory effect on urinary stone formation. For example, Saremi et al. [15] proposed an aqueous extract of *Malva neglecta* Wallr to prevent the formation of calcium oxalate stones in kidney rats. Azaryan et al. [4] also showed that the aqueous extract of *Cerasus avium* has a good effect on the excretion of renal oxalate stones. Li et al. (2015), the aqueous extract of *Fructus aurantii* inhibits the formation of calcium oxalate stones and has anti-renal effects [16]. Vyas et al [17] and Hiremath et al [18] respectively showed that the hydroalcoholic extract of *Pergularia* daemia and Vernonia cinerea Less. are effective

in the prevention and treatment of renal stone. Our analysis showed that the major component of *Allium noeanum* extract is phenolic compounds, including flavonoids; therefore, we suggest that flavonoids might be responsible for the anti-kidney stone effect of the *Allium noeanum*. We examined the effect of flavonoid isolated from the *Allium noeanum* on kidney stone induced by ethylene glycol. The results showed that flavonoid extract had significant effects and decreased the urinary calcium, oxalate and citrate, as well as serum uric acid, urea and creatinine in both curative and prevention groups compared to NM. The present study indicates that following the use of ethylene glycol, urinary citrate concentrations in the group NM significantly decreased, compared to the group HM. These findings are consistent with previous studies which describe that increased citrate excretion is involved in calcium oxalate formation [19]. In line with our results, Perez et al. [20] reported that the administration of isoflavonoids isolated from *Eyshinardtia polistachya* reduced the size of kidney stones in mice. Also, Larobi and colleagues [21] found that a flavonoid-rich plant extract disperses Calcium oxalate particles in the urine and causes them to disappear. Studies have shown that Ethylene glycol can induce inflammation and Calcium oxalate precipitation and it causes oxidative damage through production of reactive oxygen species, such as superoxide and hydrogen peroxide [22]. It was supposed that the main factor of oxalate stones pathogenesis is the reactive oxygen species (ROS), which is generated due to crystals of calcium oxalate interaction with renal tubular epithelial cells. Under normal conditions, production of ROS is under control, regulating crystallization modulator production. If ROS overproduction or antioxidant factors decrease occur, they cause oxidative stress, inflammation and injury. Calcium oxalate provokes ROS-mediated inflammatory responses. Through generation of ROS, lipid peroxidation take place that mediate oxalate-induced membrane injury [23,24].

Researchers in several studies have proven that flavonoid extracts can inhibit the formation of kidney stones due to anti-inflammatory and anti-oxidant properties [25-27]. We guess that extract of *Allium noeanum* (Bonsor) also has anti-inflammatory and antioxidant properties, which can prevent the formation of kidney stones. Therefore, it is suggested that in future studies the anti-inflammatory and antioxidant properties of this extract should be studied. We had some limitations in our study such as small sample size, lack of available data about *Allium noeanum* and the constituents of its extract. The last is because no studies have been conducted on this plant and its active compounds so far. Further investigations are recommended to reach a conclusive result.

Conclusion

In conclusion, our results showed that total hydroalcoholic and flavonoids extracts of *Allium noeanum* have beneficial effects on the treatment and prevention the formation kidney stones in animals.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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