Hydroalcoholic Extract of Ziziphus Jujuba Leaf to Prevent Ethylene Glycol and Ammonium Chloride-Induced Kidney Stones in Male Rat: Is it Effective?

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Purpose: This study aimed to evaluate the effect of Ziziphus jujuba (Z. jujuba) leaf hydroalcoholic extract on the prevention/treatment of kidney stones.

Materials and Methods: Thirty-six male Wistar rats were randomly divided into six groups: control, Sham (kidney stone induction (KSI) by ethylene glycol 1% + ammonium chloride 0.25% through drinking water for 28 days), Prevention groups 1, 2 (KSI and Z. jujuba leaf (250 and 500 mg/kg, respectively) through gavage for 28 days), and Treatment groups 1, 2 (KSI and Z. jujuba leaf (250 and 500 mg/kg, respectively) from the 15th day). On the 29th day, the rats' 24-hour urine was assessed, the animals were weighed, and blood samples were taken. Finally, after nephrectomy and weighing the kidneys, tissue sections were prepared to examine the number of calcium oxalate crystals and tissue changes.

Results: The results indicated a significant increase in kidney weight and index, tissue changes, and the number of calcium oxalate crystals in the Sham group compared to the control; using Z. jujuba leaf considerably reduced them in experimental groups compared to the Sham. Body weight decreased in the Sham and experimental groups (except the prevention 2 group) compared to the control, while this observed reduction was lower in all experimental groups compared to the Sham. The mean urinary calcium, uric acid, creatinine, and serum creatinine in Sham and experimental groups (except the prevention 2 group) indicated a substantial increase compared to the control and decreased significantly in all experimental groups compared to the Sham.

Conclusion: Hydroalcoholic extract of Z. jujuba leaf is effective in the reduction of calcium oxalate crystals forming, and its most effective dose was 500mg/kg.

Keywords: calcium oxalate; Ziziphus jujuba; ethylene glycol; ammonium chloride; rat

INTRODUCTION

Kidney stone (urolithiasis, renal calculi, or nephrolithiasis) affects 5-15% of the world's population, with a recurrence rate of 50% and a high cost of treatment.[1] Despite the different types of crystals, the most prevalent type are calcium oxalate stones, located in the pelvis, ureter, bladder, and urethra.[2,3] The cause of this disease is not completely clear, but a diet rich in calcium, sodium, and protein,[1,4] as well as immobility in people with hypercalciuria type II and gastrointestinal diseases such as inflammatory bowel disease, involved in the developing of these stones.[5,6] Also, it is known that several gene polymorphisms are involved in the formation of kidney stones.[7]

Kidney stone symptoms include nausea, vomiting, hematuria, painful urination, urinary tract obstruction, hydronephrosis, and severe urinary tract bleeding, making surgery and stone breaking up an urgent need.[2,8] High costs and numerous side effects such as severe kidney tissue damage and general infection have caused patients to seek alternative treatments;[2,9] herbal products gained popularity today;[10,11] despite the benefits of these herbs, it is believed that more investigation is needed to evaluate the effects of herbs on kidney stones.[10]

In traditional medicine, Ziziphus genus is used in the treatment of kidney stones, but very few academic studies have been done so far. Z.jujuba is one of the thorny shrubs of the Rhamnaceae family.[11] Glycosides and alkaloids are distributed in all parts of plant. The saponin, ziziphin and the alkaloids Coclaurine, Isoboldine, Norisoboldine, Asimilobine, Iusiphine and Iusirine were isolated from Z. jujuba leaves.[12]

Moreover, Z. jujuba leaf extract is rich in linolenic, palmitic, oleic, and linoleic acids, and in β-Sitosterol, stigmasterol and flavonoid compounds (especially rutin and apigenin). Previous studies show that the fatty acids and flavonoids in the Z. jujuba leaves is responsible for their therapeutic and pharmaceutical effects.[12-14]

Since Z. jujuba is rich in antioxidants and based on previous studies[12,13], this study was conducted to evaluate the effect of oral consumption of hydroalcoholic extract of Z. jujuba leaf on the prevention/treatment of oxalate kidney stones caused by ethylene glycol and ammonium chloride.

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Table 1. Results in the different groups.

Groups Parameters	Control	Sham	Prevention Group 1	Prevention Group 2	Treatment group 1	Treatment group 2
Left Kidney weight (g)	.86 ± .09	2.15 ± .19*	1.12 ± .07*+	1.08 ± .09*+	1.80 ± .14*+Ψ!	1.47 ± .16*+Ψ!#
Right Kidney weight (g)	.85 ± .08	2.13 ± .21*	1.10 ± .04*+	1.06 ± .13*+	1.68 ± .25*+Ψ!	1.40 ± .17 *+Ψ!#
Kidney weight (g)	1.70 ± .17	4.28 ± .37*	2.23 ± .09*+	2.15 ± .21*+	3.48 ± .39*+Ψ!	2.87 ± .25*+Ψ!#
Kidney Index (×10-3)	0.72 ± .08	2.27 ± .26*	1.03 ± .08*+	0.95 ± .09*+	1.69 ± .22*+Ψ!	1.38 ± .16*+Ψ!#
Weight of first Day (g)	210.83 ± 6.64	209.66 ± 5.60	208.33 ± 6.68	212.33 ± 6.12	210.83 ± 5.49	210.16 ± 5.91
Weight of Day 29 (g)	236.66 ± 9.04	188.83 ± 6.11*	216.66 ± 12.37*+	226.83 ± 4.70 +ΨP	206.66 ± 8.52*+!	209.16 ± 8.58*+!
Body Weight Change (g)	25.83 ± 7.67	-20.83 ± 6.82*	8.33 ± 8.18*+	14.50 ± 6.31+	-4.16 ± 7.41*+Ψ!	-1 ± 7.77*+Ψ!
UA urine (mg/24 h)	.65 ± .13	4.85 ± .90*	1.17 ± .10+	.73 ± .13+	3.18 ± .76*+Ψ!	2.47 ± .68*+Ψ!#
Ca urine (mg/24 h)	4.63 ± .48	7.73 ± .72*	5.7 ± .63*+	5.07 ± .77+	6.93 ± .70*+Ψ!	5.95 ± .38*+!#
Cr urine (mg/24 h)	39.20 ± 4.60	126.53 ± 22.30*+	61.13 ± 7.34*+	44.28 ± 9.70+ Ψ	106.17 ± 9.43*+ Ψ!	92.62 ± 7.16*+Ψ!
Volume of Urine (ml/24 h)	8.93 ± .99	18.27 ± 1.98*+	21.83 ± 3.09*+	22.37 ± 2.24*+	22.70 ± 2.40*+	23.36 ± 1.89*+
Cr serum (mg/dl)	.62 ± .20	2.95 ± .27*	.87 ± .12*+	.72 ± .08+	1.12 ± .16*+ Ψ!	.92 ± .07*+#
Calcium Oxalate Crystal count (n)	.00 ± .03	28.50 ± 10.17*	19.50 ± 6.53*+	11.83 ± 5.91*+Ψ	21.33 ± 5.27*+	11.33 ± 6.15*+Ψ
Tubulointerstitial changes	.00 ± .00	2.96 ± .75*	.93 ± .40*+	.66 ± .47*+ Ψ	1.24 ± .45*+Ψ!	.85 ± .44*+!#

*Significant different with control group ($P \leq .05$)
+ Significant different with sham group ($P \leq .05$)
Ψ Significant different with prevention group 1 ($P \leq .05$)
! Significant different with prevention group 2 ($P \leq .05$)
Significant different with treatment group 1 ($P \leq .05$)
Abbreviations: mg, milligram; kg, Kilogram; mL, milliliter; dL, Deciliter; h, hour; UA, Uric Acid; Ca, Calcium; Cr, Creatinine; n, number.
**All data represent Mean ± SD. According to Duncan test, means that have at least one letter in common in each row, have no significant difference. $P < .05$ is considered statistically significant.

MATERIALS AND METHODS

Experimental animals and study design

This experimental laboratory research has been registered in the ethics committee of Jahrom University of Medical Sciences-Iran(IR.JUMS.REC.1397.110). Only one of the authors was aware of the group allocation at different stages of the experiment during the experiment. In this study, 36 healthy adult male Wistar rats (weight: 190-210 grams, 8-10 weeks) within their cages under 12:12 light: dark cycles with 24 ± 1°C room temperature, 50-55% humidity, and ad libitum access to food and water.

According to previous research, the animals were randomly divided into six groups (each group containing six rats): Control group (without receiving any treatment), Sham group (kidney stones induction (KSI) by ethylene glycol 1% + ammonium chloride 0.25% through drinking water for 28 days), Prevention groups 1 and 2 (KSI and hydroalcoholic extract of Z. jujuba leaf concentrations of 250 and 500 mg/kg (1 mL) respectively through gavage all these 28 days), and the Treatment groups 1 and 2 (KSI and Z. jujube leaf xetract with concentrations of 250 and 500 mg/kg respectively through gavage (1 mL) from the day 15 for two weeks). All treatments were performed daily at 8-10 am.

Preparation of Z. jujuba leaf extraction

To prepare Z. jujuba, leaves were collected from Jahrom city trees, registered with Herbarium code 1151 in the Islamic Azad University of Jahrom. The leaves were dried and powdered; 100 grams of the powder were poured into a one-liter beaker with 40cc ethanol (96%). Then, the beaker was positioned on the shaker for 24 hours. After filtering the solution, ethanol (75%) was added to the remained waste and shaken for more than 12 hours. The filtered solution was concentrated by rotavapor at 50°C and at 90-rpm speed, up to 1/3 of the primitive volume. Finally, the filtered solution was positioned in an incubator (50° C). After a few days, the powder was ready to be prepared at different concentrations.[15]

Evaluation of Biochemical parameters

On the 28th day, the rats were placed in metabolic cages for 24 hours to assess urine volume, uric acid, calcium, and creatinine. Then, the animals were weighed and anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg), and blood samples were taken from the hearts.

Analysis of urine and blood samples

Following assessing urine and blood, samples were centrifuged (1,500×g for 10 minutes), and the supernatants were stored at −20 °C. Urine (uric acid, calcium, and creatinine) and serum (creatinine) parameters were evaluated using the Pars-Azmoon Kits.

Kidney weight and Histopathological analysis

The next step was nephrectomy and washed kidneys with cold 0.9% NaCl to calculate kidney index (the ratio of both kidneys weight to body weight), histological examination, and counting the number of calcium oxalate crystals. The kidneys were weighed and placed in formalin (10%). For tissue staining, after performing the usual steps of tissue passage, 5 microns of paraffin sections were serially prepared. Five sections from each kidney were randomly selected, stained with H&E, and studied under a light microscope (Olympus BX31, Tokyo, Japan). From each section, ten fields were randomly chosen with a magnification of ×100 and ×400. Eventually, the average number of calcium oxalate crystals was counted, and tubulointerstitial changes such as tubular cell necrosis, atrophy, dilation, interstitial inflammation, hyaline cast, and tubular atrophy were evaluated using a semi-quantitative method:(16) 0 = none, 1 = trace (< 10%), 2 = mild (10-25%), 3 = moderate (26-50%) and 4 = marked (> 50%).

Statistical analysis

Statistical analysis was performed using SPSS version 21 software. After assessing the normality of the data by Kolmogorov-Smirnov test, we used one-way analysis of variance (one-way ANOVA) followed by Tukey's test and Duncan's test to analyze them.

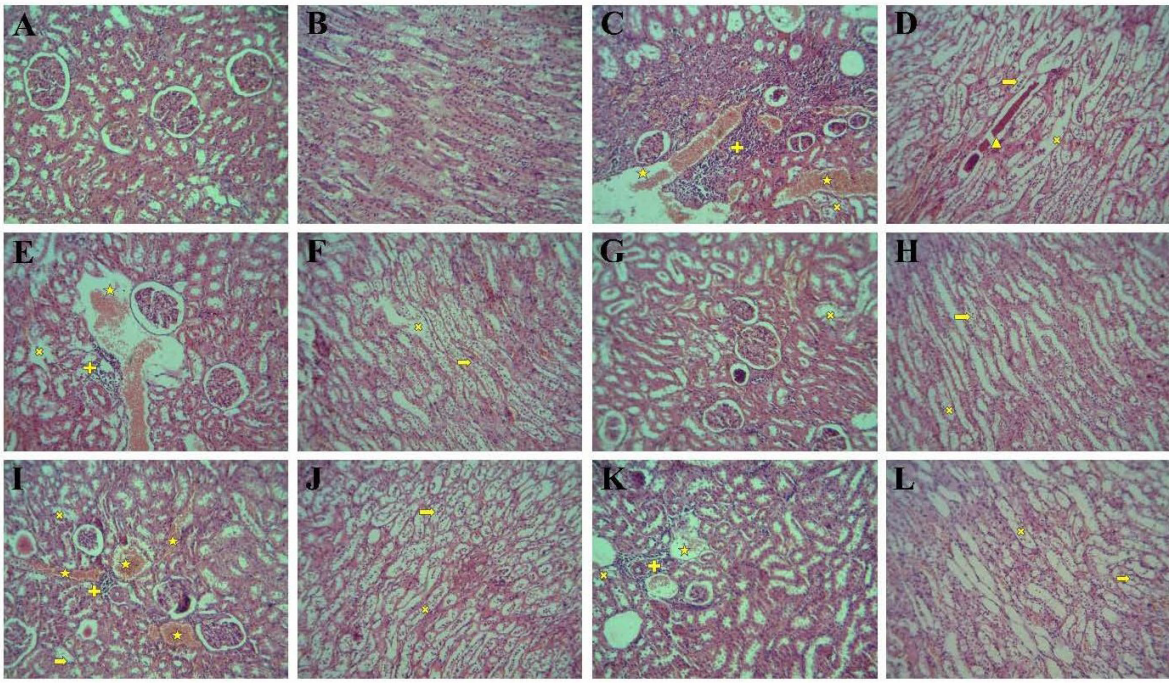

Figure 1. Histological changes of kidney in the studied groups. **A)** Control group/Cortex; No tissue changes were observed and the kidney tissue was completely normal. **B)** Control group/Medulla; No tissue changes were observed and the kidney tissue was completely normal. **C)** Sham group/Cortex; Star: Hyperemia - Plus sign: Infiltration of inflammatory cells. **D)** Sham group/Medulla; Cross: Tubular destruction - Arrow: Pyknoses nucleus - Triangle: Kidney stones. **E)** Prevention group 1/Cortex; Star: Hyperemia - Plus sign: Infiltration of inflammatory cells - Cross: Tubular destruction. **F)** Prevention group 1/Medulla; Cross: Tubular destruction - Arrow: Pyknoses nucleus. **G)** Prevention group 2/Cortex; Cross: Tubular destruction. **H)** Prevention group 2/Medulla; Cross: Tubular destruction - Arrow: Pyknoses nucleus. **I)** Treatment group 1/Cortex; Star: Hyperemia - Plus sign: Infiltration of inflammatory cells - Cross: Tubular destruction - Arrow: Pyknoses nucleus. **J)** Treatment group 1/Medulla; Cross: Tubular destruction - Arrow: Pyknoses nucleus. **K)** Treatment group 2/Cortex; Star: Hyperemia - Plus sign; Infiltration of inflammatory cells - Cross: Tubular destruction - Arrow: Pyknoses nucleus. **L)** Treatment group 2/Medulla; Star: Hyperemia - Arrow: Pyknoses nucleus (Type of staining: Hematoxylin and Eosin; Magnification ×100).

RESULTS

Examination of left, right, and both kidneys weight and kidney index indicated weight rise in Sham, treatment, and prevention groups in comparison with the control one. Using Z. jujuba leaf extract considerably decreased weight scores compared to the Sham, which was significantly higher in the prevention groups. However, among the treatment groups, the higher dose showed better effects (**Table 1**).

Evaluating the first-days body weight in all groups indicated no difference, while after 29 days, the weight alteration in the Sham and experimental groups was significant. It should be noticed that using Z. jujuba leaf extract increased animals' weight in comparison with the Sham group ($P \leq 0.05$); in prevention groups, this increase was substantial (**Table 1**).

Assessing body weight and its changes in different groups indicated decrease in Sham and experimental groups (exception prevention group 1) compared to the control group. There was a significant increase in the experimental groups' body weight and its changes compared to the Sham; the prevention groups' weight change was even higher than the treatment groups) $P \leq 0.05$((**Table 1**).

Assessing 24-hour urine uric acid changes in different groups demonstrated a substantial increase in the Sham and the treatment groups compared to the control. In contrast, the prevention groups did not indicate any significant differences compared to the control group. A reduction in urine uric acid was shown between treatment group 2 and the other treatment group) $P \leq 0.05$(. 24-hour urine calcium and creatinine changes in different groups indicated an increase in Sham and experimental groups) $P \leq .05$(except the prevention group 2, compared to the control. The experimental groups showed a significantly higher growth compared to the Sham group) $P \leq 0.05$(. Urine 24-hour volume in different groups indicated a substantial increase in the Sham and experimental groups compared to the control. In addition, a substantial increase was observed in the Z. jujuba groups in comparison with the Sham) $P \leq 0.05$(. Experimental groups did not show any significant difference (**Table 1**). Assessing serum showed that only the prevention group2 brought serum creatinine to the control) $P \leq 0.05$(. Treating animals with a lower dose of Z. jujuba showed the highest serum creatinine level among the experimental groups (**Table 1**).

Calculation of stone numbers in different groups indicated a substantial increase in the Sham and experimental groups compared to the control, but the Z. jujuba groups had lower stones than with the Sham group. Higher doses in both prevention and treatment groups showed less stone count) $P \leq 0.05$(. Evaluating tissue changes in different groups indicated increase in the Sham and experimental groups compared to the control. This reduction was significant in the experimental groups compared to the Sham. Higher doses in both prevention and treatment groups were considerably more effective) $P \leq 0.05$(. The prevention group had the least changes (**Table 1**).

Results of histopathological changes

In this study, the normal glomerular structure, renal tubule and regular interstitial area in the medulla and cortex of the control group were observed. We observed

that the tubular damage with hyperemia, infiltration of inflammatory cells, tubular destruction, pyknoses nucleus and kidney stones (calcium oxalate crystals) are revealed in the EG-treated (sham) group. In the treatment and preventive groups tubular damage, hyperemia, infiltration of inflammatory cells, tubular destruction, pyknoses nucleus and calcium oxalate crystals were reduced (**Figure 1**).

DISCUSSION

Numerous individuals around the world endure from issues related to urinary tract stones. Calcium stones are the foremost common and account for about 75% of the entire stones. These days, numerous researchers have based their investigations on therapeutic plants.[17,18] What this study showed is the beneficial role of Z. jujuba in kidney stone even by affecting the formation of calcium oxalate crystals, while the higher dose is associated with better effects. Our results showed an increase in urine volume, which probably indicates the diuretic role of the extract. It is widely known that diuretic products can reduce the amount of calcium released into the urine and help prevent kidney stones. On this basis, Z. jujuba leaf significantly increased the 24-hour urine calcium changes in such a way that the prevention group (500 mg/kg Z. jujuba leaf) reduced calcium levels to the control. Comparison among groups demonstrated that the most effective dose of Z. jujuba is 500 mg/kg in prevention ($P = .003$, $P = .001$, $P = .047$, $P = .015$) and treatment ($P = .005$, $P = .001$, $P = .035$, $P = .011$) group compare with control, sham, prevention group 1, and treatment group 1. Z.spina-christi is another species of Ziziphus genus. The benefits of Z.spina-christi and Z. jujuba extracts (seed, leaf, and so on) on renal function were shown previously.[13,19-22]

Reducing the formation of kidney stones can be explained by a decrease in urinary calcium output within 24 hours in rats. Saponins prevent kidney stone formation in animal studies by stimulating kidney ATPases. Therefore, this steroid forms an intracellular reservoir of Na+ -K- -ATPase, which increases ATPase activity in the renal tubule, and changes in sodium pumps of the cortex may lead to a change in urine composition.[17,18] The renin-angiotensin-aldosterone system (RAAS) controls salt and water balance, blood volume, and blood pressure.[23] This system plays a fundamental physiological role in maintaining the body's homeostasis.[24] Mohebbati et al. found that Z.jujuba extract, another member of the Ziziphus family with similar components, reduced the function of the RAAS and this may be due to the effect of the ingredients of Z.jujuba on aldosterone compounds.[25] Therefore, based on the similarity of these two plants (Z.jujuba and Z.spina-christi), this hypothesis can be expressed that the role of Z.jujuba as a diuretic is another confirmation of its effect on RAAS system and renal diseases.

In the kidney stone groups, tissue damage and the deposition of calcium oxalate crystals were increased significantly, in addition to the reduction of the Bowman's capsule diameter and renal glomerulus.[26] In an interesting way, tissue sampling showed no calcium oxalate crystal, tissue damage, or a significant change in the diameter of different parts of the nephron in groups receiving Z. jujuba extract. In the Z. jujuba groups (both treatment and prevention), the rate of tissue damage and deposition of calcium oxalate crystals decreased, and the best was the highest dose. The effects of Z. jujuba can be attributed to its antioxidant properties. Experimental studies have shown that antioxidant-rich substances reduce the risk of calcium oxalate stones developing.[27,28] Oxidants and free radicals can create a suitable environment for the growth of calcium oxalate crystals in kidney cells. At low concentrations of free radicals, crystals develop in calcium-rich areas, while at higher concentrations, crystals form in areas with a single layer of superficial damaged cells.[29-31] Elevated oxalate secretion may also induce oxidative stress and an increase in free radicals of renal epithelial cells.[32] These conditions create an interaction between the crystals and renal tubular cells, which leads to the formation and growth of calcium oxalate crystals.[32,33] As the study conducted by Almeer et al., Z.spina-christi has beneficial effects against mercury-induced renal toxicity and reversed kidney alterations to near normal values. The authors believe that these effects resulted from Z.spina-christis' chelation and antioxidant, which minimize the pathological changes induced by mercury.[20] Or in another study by abdel-wahhab and colleagues, in animals treated with Zizyphus spina-christi L. extract alone or plus aflatoxin (AF), to induce oxidative stress, a significant improvement in all biochemical parameters and histological picture of the liver, kidney, and the testis was detected. This study proved that Zizyphus extract has a powerful protective role against aflatoxicosis due to its antioxidative properties.[19] In addition, studies agreed that herbs with flavonoids and antioxidants could reduce creatinine levels and control tissue damage in the kidney.[34,35]

According to the results of the present study and other studies, Z. jujuba extract is effective in the reduction of calcium oxalate crystals forming, which can be due to the phenolic compounds, flavonoids, fatty acids, antioxidants, and especially the diuretic properties of this plant. However, there is a need for further studies on their antioxidants role.

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SUMMARY

The results described in this manuscript showed that:

- Z. jujuba extract could decrease serum BUN, uric acid and creatinine levels
- Z. jujuba extract has an effect on the prevention and treatment of kidney stones, which can be due to phenolic compounds, flavonoids, fatty acids, and antioxidants and diuretic properties of this plan. These Results will be very informative for your journals audience to focus on herbal benefits in protecting kidney from various damage and harmful metabolites.

CONFLICT OF INTEREST

The authors have no conflicts of interest relevant to this article.

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