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ORIGINAL PAPER

Dose-dependant preventive effect of a herbal compound on crystal formation in rat model

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Summary

Introduction: *To analyze the dose-dependent preventive effect of a plant-based herbal product on the new crystal formation in a rat model.*

Materials and methods: *A total of 42 rats were divided into 7 groups and zinc discs were placed into the bladder of rats to provide a nidus for the development of new crystal formation: Group 1: control, Group 2: 0.75 percent ethylene glycol (EG); Group 3: 0.75 percent EG plus 0.051 ml of the compound; Group 4: 0.75 percent EG plus 0.179 ml of the compound; Group 5: 0.75 percent EG plus 0.217 ml of the compound; Group 6: 0.75 percent EG plus 0.255 ml of the compound; Group 7 0.75 percent EG plus 0.332 of the compound). The analysis and comparison focused on the disc weights, changes in urinary oxalate and calcium levels, urinary pH, and the histopathologic evaluation of the inflammatory changes in the bladder after 14 days.*

Results: *According to the evaluation of discs placed in the bladders of the animals, animals receiving the herbal compound on a dose-dependent basis showed a limited increase in the disc weights values after 14 days, despite a considerable increase in animals receiving EG alone ($p = 0.001$). Further evaluation of the increase in disc weights on a dose-dependent basis in different subgroups (from Groups 3 to 7) demonstrated that the limitation of crystal deposition began to be more prominent as the dose of herbal compound increased. This effect was more evident particularly in comparisons between group 7 and others, according to LSD multiple comparison tests ($p = 0.001$).*

As anticipated, there has been no discernible change in the weight of the discs in the control group. Although urinary calcium levels in animals of Groups 2, 6, and 7 were significantly higher than the other groups, we were not able to demonstrate a close correlation between urinary oxalate levels and the increasing dose levels. Even though mean urine pH levels were statistically considerably higher in Group 3, there was no statistically significant correlation between the oxalate and calcium levels between all groups, and no association was seen with the administration of herbal agents. The transitional epithelium between the three groups of animals' bladder samples did not exhibit any appreciable difference according to pathological analysis.

Conclusions: *In this animal model, the treatment of the compound was successful in lowering the amount of crystal deposition surrounding the zinc discs, most noticeably at a dosage of 0.332 ml, three times per day.*

KEY WORDS: *Herbal compound; Rat model; Crystal formation.*

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INTRODUCTION

Urinary system stone disease, which is an endemic health issue, at least in some regions of the world, can irreparably harm the kidneys if not treated promptly and appropriately. The most significant feature of this disease is its recurrent nature, which is caused by insufficient metabolic evaluation and a lack of appropriate medical care, especially in individuals in high-risk groups (1, 2). While endourologists have made significant advancements in the minimally invasive therapy of calculi, there has been little progress made in the pharmacological management of urolithiasis. Allopurinol, potassium citrate, thiazide diuretics, and other medications have been utilized in medical therapy, with different degrees of claimed efficacy (3). The medical method has some efficacy in preventing recurrences, but there are other significant drawbacks, including some drug-related adverse effects and low patient compliance rates observed during extended therapy follow-up. Additionally, existing medical therapy techniques may fail due to the agents' insufficient impact on all fundamental relevant pathologic mechanisms at the kidney level given the very complicated etiology of urinary stone formation, which depends on multiple pathologic phases and/or mechanisms. In other words, the fundamental constraint of medical treatment is the impact through only one established pathogenetic process (after the formation of stones). Regarding this, despite the fact that certain models concentrate on the creation of novel medications that may have powerful effects on the various stages of stone formation (nucleation, supersaturation, and crystal growth) (4-7), such models constitute only a rough imitation of the events taking place in the organism (8).

Some herbal medications have been effectively used and assessed in terms of their short- or long-term efficacy as well as adverse effects to address the aforementioned challenges in the medical treatment and/or prevention of stone disease (6, 8-15). We used a herbal substance in this work that has been shown to have anti-apoptotic, anti-inflammatory, diuretic, nephroprotective, antioxidant, antibacterial, and spasmolytic activities. In some experimental tests, this substance has been proven to be effective at reducing the formation of stones (16).

Although calcium and oxalate may play a part in stone formation together, the most important risk factor for calcium oxalate stone disease has been revealed to be the presence of "*hyperoxaluria*". This condition affects 60-70% of people (17, 18). The most widely utilized agent to cause hyperoxaluria in animal models is *ethylene glycol* (EG), which has also been discovered to be the best agent for evaluating and analyzing an agent's efficacy when employed in such models (19). The formation of nidus for further crystal deposition and stone formation in animal models has been described using a variety of models, including the use of plastic discs, the insertion of suture material parts, the implantation of calcium and oxalate crystals, and the implantation of zinc discs into the bladders of the animals (17). Zinc disc implantation inside the bladder of hyperoxaluria-induced rats has been a widely used technique so far among these models. While the majority of animals' final stone composition measured on the zinc disc was calcium oxalate, as shown in several of these investigations, magnesium-ammonium-phosphate crystals (stones) were also shown in some other, more limited, experiments (17-19).

In the current work, we sought to assess the potential benefits of the herbal ingredient on the prevention of new urinary stone formation by restricting new crystal deposition on the "*zinc discs*" in a rat model.

Materials and methods

Herbal agent

We employed a plant-based herbal supplement composed by a stable mix that included rosmarinic acid, boldin, polysaccharides, the flavonoid quercetin, flavonglicozides, and essential fatty acids. Its nephroprotective, diuretic, anti-inflammatory, antioxidant, antimicrobial, and spasmolytic activities have been employed as the basis for *Resolutivo Regium*. In 250 ml bottles, the medication is offered in hydrolate form. The dose for adults is 7 ml administered three times per day. The herb *Sideritis angustifolia*, the leaves of *Melissa officinalis*, the flowers of *Opuntia ficus indica*, the leaves of *Peumus boldus*, the rhizomes of *Cynodon dactylon*, and the entire plant of *Spergularia rubra* are all included in the drug's composition. It also contains dried parts of the stem of *Enguisetum arvensis* and an aqueous distillate of those parts, as well as flowers.

Study design

The study protocol was accepted by the ethical committee of *Kafkas University Training and Research Hospital* (*June 28, 2022, Approval number: KAU-HAYDEK2022-120*). 42 male Sprague Dawley rats weighing between 300 and 350 grams were involved and divided into 7 groups. The lighting setup was set up to resemble the natural cycle of day and night. All rats in all groups received the appropriate cages, access to food and water without restriction, and normal (physiological) room temperature.

Small zinc discs were surgically inserted into the rats' bladders while they were under anesthesia in the first phase of the trial. Applications of ethylene glycol and herbal compounds were started daily on the second post-operative day. In an effort to determine the minimal dose necessary for the substance to effectively prevent the growth of stones, the drug has been administered in a dose-dependent manner. Regarding the treatment regimen in these subgroups, rats in Group 2 received water that had 0.75 percent EG added to it. For each rat in Group 3, 0.75 percent EG plus 0.051 ml of the compound was administered three times per day. Each rat in Group 4, received 0.75 percent EG plus 0.179 ml compound three times per day. Each rat in Group 5 received 0.75 percent EG plus 0.217 ml compound three times per day. For each rat in Group 6, 0.75 percent EG plus 0.255 ml compound was given three times daily.

Finally, 0.75 percent EG plus 0.332 compound ml was given in group 7. Treatment was continued for two weeks. Group 1 was the control group, which had the zinc disc, but no EG and no herbal compound.

Rats were sacrificed at the conclusion of the study, after 14 days of treatment with the aforementioned protocols, and urine samples from the harvested bladders were collected for the evaluation of urine pH as well as the urinary levels of calcium and oxalate. Harvested bladders were sent for histopathological analysis after the zinc discs were removed. Results of the urine test, histopathological findings, and zinc disc weight values were compared between each group.

Operative technique

Ketamine HCl (*Ketalar, Eczacibasi Inc., Istanbul, Turkey) and 10 mg/kg xylazine (Rompun, Bayer Turk Inc., Istanbul, Turkey*) were administered intramuscularly to induce anesthesia after a 6-hour fast. We assessed the impairment of the reflex arc reaction to compressing the claws in order to determine the efficacy of anesthesia. The skin was then cleaned with the appropriate antiseptic solutions after the incision site had been shaved with a blunt razor blade (*Poviodine, Istanbul-based Dioagnokim Inc.*) Sharp dissection was used to split the abdominal wall muscles after a 2 cm incision in the lower quadrants of the abdomen. After the urinary bladders were separated and exposed, a small incision was made to open the bladder lumen. Preparation zinc discs weighing 70 ± 2 mg were then placed inside the bladder lumen, and the existing incisions were sealed with 4/0 absorbable polyglactin (*Vicryl, Ethicon Inc., Somerville, NJ, USA*). After the bladder was returned to its original position, the skin and abdominal muscles were stitched together with 2/0 silk and 3/0 absorbable polyglactin (*Vicryl, Ethicaon Inc., Somerville, NJ, USA*). The rats were left for recovery after the closed incisions had been cleaned. After 14 days, the rats in each group had their bladders opened using the same surgical procedure as above, and urine samples were taken for

both microbiological and biochemical analysis. The crystal-covered and coated zinc discs were removed for weighting and stone analysis. The rats' bladder walls were removed and sent to pathology. The urine samples were stored and transported at 4°C while the harvested bladders were fixed in 4% neutral formaldehyde, embedded in paraffin blocks, and cut into 4-6 µm sections, and stained with hematoxylin and eosin. After that, the rats were sacrificed.

Histopathological evaluation

Prior to paraffin embedding, 10% formalin was used for the fixation of the bladder tissues. After the procedure, blocks were cut 5-6 µm slices and stained with hematoxylin-eosin. A pathologist expert with animal models analyzed the slices. All samples were examined under the light-microscopy and vascular congestion, level of edema (none, mild, moderate, and severe), level of inflammation, the thickness of the epithelium (in millimeters), changes in the epithelium (dysplasia, calcification, fibrosis, mitosis), and epithelial cell layers were recorded.

Laboratory analysis

Urine calcium levels were assessed using the photometric o-Cresolphthalein complex method (*Cobas c501 analyzer-Roche Diagnostics, Germany*). Urine oxalate levels were measured using a rat ELISA kit (*SunRed, China; Catalogue No: 201-11-5547*). Urine pH was assessed using strips for urinalysis (*Dirui, China; Catalogue No: 231011501001*).

Statistical analysis

The normality of the distribution of continuous variables was tested by the Shapiro-Wilk test. One-Way ANOVA and LSD multiple comparison tests were applied to investigate the difference between groups in terms of numerical variables and Kruskal Wallis tests were used to compare non-normal data across groups. Statistical analysis was performed with SPSS for Windows version 24.0 and a p-value < 0.05 was accepted as statistically significant.

Results

Evaluation of our data obtained in seven groups of animals revealed the following findings:

Table 1.
Evaluation of laboratory findings.

Parameters Groups	Oxalate (ng/L) Mean ± SD	pH Mean ± SD	Calcium (mg/dl) Mean ± SD	Zinc disc weights (mg) Mean ± SD
1 (n = 6) Control group **	1111.63 ± 223.41	8.47 ± 0.1	2.57 ± 0.26	97.83 ± 2.93
2 (n = 6)	1533.73 ± 137.15	8.33 ± 0.2	4.17 ± 0.4	161.17 ± 11.86
3 (n = 6)	1401.22 ± 197.88	8.65 ± 0.19	2.72 ± 0.29	124.83 ± 10.3
4 (n = 6)	1293.33 ± 81.96	8.5 ± 0.33	3.08 ± 0.21	107 ± 1.9
5 (n = 6)	1381.73 ± 261.7	8.17 ± 0.49	1.68 ± 0.21	101 ± 2
6 (n = 6)	1348.02 ± 104.73	8.22 ± 0.17	4.88 ± 0.53	101 ± 4.6
7 (n = 6)	1419.82 ± 208.79	8.1 ± 0.37	4.23 ± 0.42	79.17 ± 2.4
P value	0.018 *	0.026 *	0.001 *	0.001 *

* *p: Significant at 0.05 level.*
** *Group 1 (Control) is the one that had the zinc disc, but no EG (Ethylene Glycole) and no herbal compound.*

Effects of the medication on crystal deposition

Evaluation of the weights of the zinc disks placed in the bladders of animals receiving EG in addition to normal diet and water in group 2 revealed a significant increase at the end of 14 days when compared with the baseline (Group 1) values. (p = 0.001, LSD multiple comparisons) (Table 1). On the contrary, however, very little increase in the weight of the disks in the control group (Group 1) animals has been noted as expected with mean values of 97.83 ± 2.93 during this evaluation, compared to 70 mg. of the free zinc disc weight (Table 1).

Evaluation of the weight of the disks in animals receiving herbal compounds during the study period (in addition to EG administration) demonstrated a reduced (not significant) increase in these values after a 14-day follow-up (p = 0.001, Table 1). Evaluation of the increase in disc weights on a dose-dependent basis in different subgroups (from Groups 3 to 7) demonstrated that the limitation of crystal deposition began to be more prominent as the dose of herbal compound increased. This effect was more evident particularly in comparisons between group 7 and others, according to LSD multiple comparison tests (p = 0.001) (Table 1).

Effects of herbal compound application on urine parameters

The mean urinary oxalate levels within Groups 2, 3 to 7 were similar (p = 0.018, Table 1) with no statistically significant difference between the groups from 2 to 7. Only in Group 1, the oxalate levels were lower than in the others. The difference was particularly prominent between Groups 1 and 2 (p = 0.001).

Urinary calcium levels in animals of Groups 2, 6, and 7 were significantly higher than the other groups as shown in Table 1. We were not able to demonstrate a close correlation between urinary oxalate levels and the increasing dose levels.

Comparative evaluation of the mean urinary pH levels (Table 1) showed that although it was significantly higher in Group 3 animals (p = 0.026) during the 14-day evaluation, this finding has been accepted to be an isolated finding with no attributed correlation with herbal agent administration.

Crystals accumulated on the zinc disks were analyzed at the end of the trial and data revealed them to be composed of calcium oxalate in 17 and magnesium ammonium phosphate in 25 rats.

Effects of the compound on bladder inflammation

Following the two weeks of the trial period, the bladders were harvested and evaluated with respect to the possible histopathologic alterations. Despite a mild degree increase in vascular congestion and edema formation in the animals of Groups 4, 5, and 7, pathological evaluation findings revealed no significant difference regarding the presence as well as the extent of edema formation, vascular

congestion, and inflammatory cell infiltration between the groups. Additionally, no significant alteration was found to note with respect to the pathological changes in transitional epithelium like calcification, mitosis, fibrosis, dysplasia, or a number of epithelial cell layers.

Discussion

Prevention of new stone formation particularly in the risk group cases is the most important aim of the medical management for urolithiasis. Despite minimally invasive treatment of urinary calculi with endoscopic/ureteroscopic treatment alternatives has gained more importance with their safe and practical characteristics, highly limited advancements have been achieved in the prevention of urinary stones, Regarding the agents used with this aim, currently, potassium citrate, allopurinol, thiazide diuretics, and tiopronin are the most commonly applied ones depending on urinary pH and the chemical composition of the stone(s) treated. However, in addition to the ongoing controversies regarding the efficiency and optimal treatment duration of agents, certain side effects resulting in the discontinuation of the drug administration constituted another important limitation in decreasing the patient compliance rates, particularly during long-term follow-up. Based on these facts, physicians began to consider phytotherapeutic agents, in other words, herbal compounds as a valuable option for the effective medical management of urinary stones.

Regarding the underlying pathogenetic mechanisms of calcium oxalate stone formation, accumulated information has clearly demonstrated that hyperoxaluria is one of the most important and crucial factors in this cascade. For that reason, EG is the most commonly used agent to induce hyperoxaluria status and form calcium oxalate crystals in animal models (17). However, some drawbacks have been stated for the use of this model regarding its detrimental effects like metabolic acidosis, cellular injury, and necrosis in tubular epithelial cells which will compromise interpretation of the real effects of either high oxalate levels or the crystals formed as a result of its application (20). In an attempt to reduce the urinary excretion of stone-forming risk factors as well as to inhibit the accumulation of stone crystals, certain herbal medications have been used in animal studies with a certain level of efficacy. With this aim, some experimental studies have pointed out that such herbal compounds could serve as an encouraging, efficient, and also safe alternative due to the limited toxic side effects observed with their application (21). This also emphasizes that phytotherapy can be used as a complementary or direct approach to decrease the established side effects of the commonly used treatment alternatives. Literature-derived data show that these herbal compounds may exhibit anti-oxidant, diuretic, vasodilator, spasmolytic, nephroprotective, antibacterial, and anti-inflammatory effects (22-24). To augment such valuable effects some extra active ingredients like essential oils, flavonoids, saponins, xanthine derivatives, and glycosides are also added to these structural units (16). A variety of herbal agents including *Rubus idaeus* (25), *Phyllanthus niruri* (11), *Herniaria hirsute* (22), *Alisma Orientalis* (23), and *Costus spiralis Roscoe* (24) have been applied with their proven effects of antiurolithiatic activity. Although the precise underlying mechanisms causing these preventive effects have yet to be identified, some researchers have shown that these substances have an impact on the levels of oxalate, calcium, and malondialdehyde in the urine of animals who have stones (25). *Rubus Idaeus* (European raspberry) on this aspect was found to prevent renal tubular damage by limiting the formation of hyperoxaluria and also the accumulation of calcium oxalate crystals with reduced malondialdehyde excretion in urine. A tropical plant named *Phyllanthus niruri* is known to limit the development of calcium crystals without changing the urinary magnesium or citrate levels. *Herniara hirsute*, a flowering plant, is probably acting by dissolving the residual crystals deposited in the kidney (22). This plant extract decreases CaOx crystal binding to the tubular epithelium, without making any important difference in the urinary pH, volume, or chemistry. (26). *Alisma Orientalis* is known to inhibit stone formation steps like crystal formation, aggregation, and growth (23). The findings of these studies suggest well that these compounds can be efficiently used to inhibit urinary stone development and stone episodes even if the exact pathophysiology is not fully known.

In one of these models, a herbal agent was applied to prevent the ethylene glycol-dependant apoptosis and calcium oxalate crystal accumulation in tubular cells of the kidney and it was found to be enough effective in this aspect (16). However, the administration of the compound in this study did not alter the urinary calcium and oxalate levels indicating that the inhibitory effect on stone development is independent of the urinary concentrations of these ions. In other words, obtained results suggested that factors other than calcium and oxalate may also play a role in the pathogenesis of urolithiasis.

On the other hand, in addition to the levels of urinary stone-forming risk factors (calcium, oxalate, uric acid), urinary pH levels are also very important in stone formation. Some researchers suggested that urinary pH levels in animal models can be changed between the range of 5.0 to 9.0 depending on dietary alterations (27). Related to this issue, measured pH levels in this study varied between 7.5 and 9.0 with slightly increased levels reported in Group 3 (Table 1). Additionally, no significant change was found to note with respect to the pH changes in other groups.

In this study, we used a phytotherapeutic compound that was produced from fractions of a few different plant extracts. Of these ingredients, extracts of *Opuntia ficus indica* (28), *Rosmarinus officinales* (29), and *Cynodon dactylon* (13) have been found to exhibit potent inhibition of urinary stone growth. We evaluated its potential inhibitory effects on crystal formation in the rat model in a dose-dependant-based manner and our results revealed that although animals receiving EG showed a significant increase in crystal formation around the zinc disc placed, no or limited change was noted in animals receiving the compound. In other words application of this agent seemed to be protective enough against new crystal formation. The herbal compound application was more effective at a dosage of 0.332 ml, 3 times a day in reducing the extent of crystal deposition in this animal model.

Although the exact mechanism of this litholytic effect is not clear, the excretion of oxalate and calcium seemed to have no role because no significant correlation was assessed. We believe that the potent antioxidant and anti-inflammatory effects of this herbal medication, shown well in other studies, may be responsible for the limitation of crystal formation. Lastly, pathological evaluation of the bladder tissue specimens revealed no significant difference regarding the presence of edema formation, vascular congestion, inflammatory cell infiltration, and pathological changes in transitional epithelium between the groups.

In light of the published data so far in the literature and our current findings as well, we may claim that the above-mentioned effects of the herbal medication could play a role also at the kidney level to limit the formation of crystals in the tubules.

Our study has certain limitations. First of all, the main disadvantage is gender singularity as the study was performed on male animals. Regarding this issue, although relatively smaller-sized stones were formed in female rats than in the male ones in a study with a zinc disc model (19), the efficiency of the herbal treatments were found to be similar in both genders (24). On the other hand, spot urine samples were collected for analysis at the time of sacrification, instead of using a metabolic cage and collection of all excreted urine. However, human clinical studies have clearly shown that spot urine also may be useful enough for metabolic assessment (30). Last but not least, after stone formation, a herbal compound application is used to prevent stone growth but not chemical dissolution.

In conclusion, our current findings demonstrated evident crystal deposition on the surface of the zinc discs, due to the hyperoxaluria induced by ethylene glycol. The herbal compound administration was effective in reducing the extent of crystal deposition around the zinc discs and this effect was found to be the most prominent at a dosage of 0.332 ml, 3 times a day. Although the probable mechanism of this litholytic effect is not clear, it was shown not to be related to the excretion of oxalate and calcium. The potent antioxidant, as well as anti-inflammatory effects of this herbal medication shown in other studies, may be responsible for the limitation of crystal formation. However, we believe that further studies are needed to outline the possible effects of the herbal compound on the limitation of new stone formation in humans.

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