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ORIGINAL ARTICLE

Solubility of Calcium Oxalate and Calcium Phosphate Crystallization in the Presence of Crude Extract and Fractions from *Kelussia odoratissima* Mozaff

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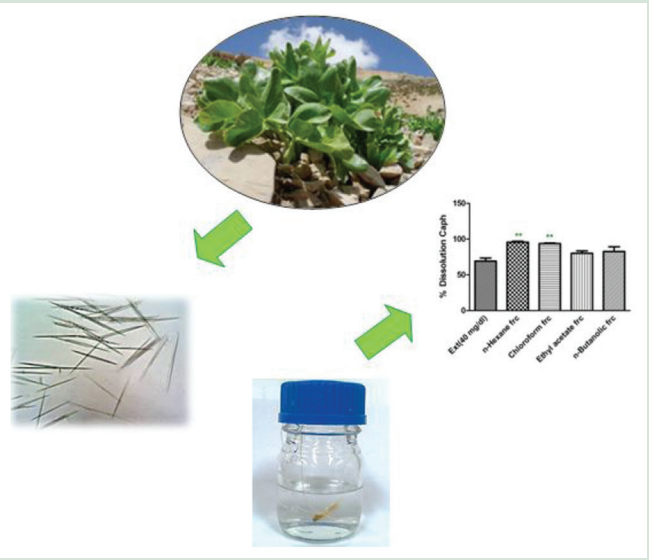
ABSTRACT

Background and Aim: Urinary tract stones are the third common urinary tract disease that may lead to renal failure. *Kelussia odoratissima* is traditionally used in the treatment of kidney stones in Chaharmahal va Bakhtiari region. This study was designed to investigate *in vitro* effect of crude extract and fractions of *K. odoratissima* on kidney stones (calcium oxalate and calcium phosphate). **Materials and Methods:** A total of 70% ethanolic extract of *K. odoratissima* was prepared by maceration method followed by liquid–liquid extraction with hexane, chloroform, ethyl acetate, and saturated *n*-butanol to get four fractions. Calcium oxalate and calcium phosphate were synthesized and then were treated with hydroalcoholic extract and fractions. Their effects on the dissolution of generated stone were assayed by calcium kit. The stones were synthesized and confirmed by fourier-transform infrared technique. **Results:** Results showed that total extract and its fractions had significant potency to dissolve calcium oxalate and calcium phosphate crystals. The results indicate the higher potency of fractions containing nonpolar compounds to dissolve calcium phosphate and calcium oxalate stones compared to the fractions containing polar compounds. *n*-butanolic fraction had the least effect and hexane fraction had the greatest effect on the calcium phosphate stones. Furthermore, the total extract has less dissolution ability, compared to the fractions. **Conclusion:** The obtained results of this study exhibited that the use of *K. odoratissima* extract and its fractions could help to dissolve urinary stones. Therefore, it can be effective in prevention and treatment of kidney stones on people who are prone to the formation of calcium oxalate and phosphate stones.

Key words: Calcium oxalate, calcium phosphate, extract, *Kelussia odoratissima*, kidney stone

SUMMARY

- Herbal medicines have been used throughout the world as a rich source of therapeutic agents for the prevention of different diseases. In Iranian traditional medicine, many plants like *Kelussia odoratissima* have been introduced to excretion and dissolution of kidney stones, or inhibition of formation. Results of this study showed that *K. odoratissima* extract and its fractions could dissolve urinary stones. These findings substantiate the traditional use of *K. odoratissima* in the treatment of urinary stones and kidney problems. The results of isolation and fractionation of the total extract of this plant support on the effective role of nonpolar and semipolar compounds of this plant in dissolving the studied kidney stones. Besides that, the phytochemical investigations on this plant comprise a good basis for finding potential molecules or compounds that serve as the leading contributors to dissolving kidney stones.



Abbreviations Used: Caox: Calcium Oxalate, Caph: Calcium Phosphate, *K. odoratissima*: *Kelussia odoratissima* Mozaff

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INTRODUCTION

Urinary tract stones are among the most common and painful diseases that may occur for a person. History of kidney stone formation has coincided with the history of humanity. In fact, bladder and kidney stones have also been found even in Egyptian mummies.[1] If it is not treated, the rate of its recurrence is approximately 10% during a year, 33% within 5 years, and 10% during 10 years.[2] Epidemiological studies show that the average prevalence of kidney stones in men is between 5% and 7% and women is only between 3% and 6%.[3-5]

Calcium stones are composed of calcium oxalate and calcium phosphate that are caused due to increase in calcium, uric acid, urine oxalate, or decreased citrate. Noncalcium stones include uric acid, struvite, cysteine, and xanthine stones.[6,7] Calcium oxalate precipitate in acidic or neutral pH and calcium phosphate is formed in natural pH of urinary, that is, 5.5–6.[8,9]

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Pure calcium oxalate stones or mixed with calcium phosphate are more common and the reason of their formation is often unknown. Their pathogenesis is multifactorial and can be related to environmental factors such as air, occupation, country or the environment, various races, and chemical and physical factors which affect the formation of kidney stones.[6]

Herbal medicines have been known for thousands of years and greatly used throughout the world as a rich source of therapeutic agents for the prevention of different diseases. In Iranian traditional medicine, many plants have been introduced to excretion and dissolution of kidney stones, or inhibition of formation, although the mechanism of action of many of them is still unknown.[10]

Kelussia odoratissima Mozaff., which is traditionally used in the treatment of kidney stones in Chaharmahal va Bakhtiari region, is one of these plants. *K. odoratissima* from Umbelliferae family was introduced by Dr. Mozaffarian in 2003, with a scientific name of *K. odoratissima* Mozaff. and the old name of Amirkabiriaodoratissima Mozaffarian as a species of "Kelussiae."[11] It is called Kelussia in Persian.[12]

This plant is a wild plant which has a taproot and is a little thick with grass and hollow stems that grow to a height <1 m. Its growing period starts from mid-March until June 1st. This plant is native to certain areas of the country such as Chaharmahal va Bakhtiari, Isfahan, and Kohgiluyeh va Boyerahmad provinces. With regard to the literature data, there is no report of its existence in other regions until now.[11]

According to the research conducted on the pharmacological properties of this plant, analgesic and anti-inflammatory effects,[13] sedative effect,[14] fibrinolytic effect,[6] activity to decrease the secretion of acid and pepsin,[15] antihyperlipidemic effect,[16] inhibitory effect on contractions of uterus and ileum,[17,18] and memory boosting effect[19] have been reported. It contains total phenolic, flavonoid, and flavonol contents with the values of 96.2%, 56.1%, and 32.9%, respectively, and phthalides and fatty acids, that its cancer prevention[16] and liver protection effect,[20] have been attributed to. Furthermore, due to phenolic compounds and flavonoids, this plant has free radical scavenging and antioxidant effects.[21] In traditional medicine, *K. odoratissima* is used for treating some disorders such as rheumatism, colds, cough, hypertension, hyperlipidemia, diabetes, and stomachache.[16]

In 2010, Byahatti *et al.* investigated the effect of total extract and fractions containing phenolic compounds of leaves of *Bergenia ciliata* (Haw.) Sternb. on kidney stones *in vitro* and the results showed that isolated phenolic compounds could dissolve calcium phosphate (64.76%) more than the calcium oxalate stones (48.48%).[22]

Therefore, due to the necessity of obtaining a possible mechanism of *K. odoratissima* in treatment, this study aimed to investigate the *in vitro* effect of hydroalcoholic extract and fractions on calcium oxalate and calcium phosphate stones crystallization.

MATERIALS AND METHODS

Plant material

Aerial parts of *K. odoratissima* at an early stage of growing were collected from the central Zagros region in west of Iran on Zard-Kuh Mountain in March 2015 (32.3297° N, 50.1112° E; 2100 m above sea level). The sample identification was done by Dr. Hamzehali Shirmardi. The voucher specimen (No. 207) was deposited at the Herbarium of Shahrekord University of Medical Sciences (SKUMS). The aerial parts were then dried under standard conditions in the laboratory.

Extraction and fractionation

Aerial parts of the plant were chopped, then macerated with 70% ethanol and shaked for 48 h. The extract was filtered and concentrated by rotary evaporator and finally dried in a freeze dryer. The crude extract was sequentially fractionated with hexane, chloroform, ethyl acetate, and saturated *n*-butanol through liquid–liquid extraction. Hexane, chloroform, ethyl acetate, and *n*-butanol fractions were concentrated by rotary evaporator and then were dried in freeze dryer.[23]

In vitro calcium oxalate and calcium phosphate synthesis

An equimolar solution of calcium chloride dihydrate in distilled water and sodium oxalate in 10 ml of 2N H_2SO_4 was allowed to react in sufficient quantity of distilled water in a beaker. The resulting precipitate was calcium oxalate. Calcium phosphate was prepared by combining equal volumes of equimolar solutions of calcium chloride dihydrate and disodium hydrogen phosphate (Na_2SO_4) with 10 cc of 2 N sulfuric acid. Both precipitates were neutralized by ammonia solution and washed with distilled water and dried at 60°C for 4 h.[24] Furthermore, synthesized stones were confirmed through fourier-transform infrared (FT-IR) technique.

Semipermeable membrane preparation

Dialysis tubing cellulose membrane (D9652 SIGMA) [Figure 1] was placed in distilled water for 3–4 h and then in 0.3% sodium sulfide solution at 80°C for 1 min. Then, the membrane was washed with distilled water at 60°C for 2 min and dipped in 0.2% sulfuric acid solution, thoroughly washed with distilled water and stored in the refrigerator in Tris buffer at pH = 7–7.4.[25]

Estimation of calcium oxalate and calcium phosphate dissolution

The test was carried out by Byahatti *et al.* method with some modifications.[22] One milligram calcium oxalate or calcium phosphate with different concentrations of extract was dipped in the semipermeable membrane [Figure 1] in a flask containing 60 cc of 0.1 M Tris buffer. One group as negative control contained only 1 mg of calcium oxalate or calcium phosphate. Then, flasks were incubated at 37°C for 7 h. The calcium content of total extract and fractions was also determined without the presence of stones and these values were considered in final calculations.

Semipermeable membrane contents of each group were transferred to test tubes. Then, 2 cc of 1 N sulfuric acid was added, and concentration of calcium was measured after the treatment with the hydroalcoholic

Figure 1: Semipermeable membrane contains calcium oxalate and extracts in Tris buffer

extract of *K. odoratissima* and its fractions using calcium kit by Pars azmoon Co. and BT-3000 device. The dissolution percentages of calcium oxalate or calcium phosphate were calculated by using the formula, Percent dissolution = (calcium content of stone– (calcium content of stone in the presence of extract or fractions– calcium content extract or fractions))/calcium content of stone × 100. Experiments were conducted in triplicate and expressed as mean ± standard deviation.

Statistical analysis

The effect of crude extract and fractions on the dissolution of synthesized stone was assayed by one-way ANOVA and Tukey's *post hoc* test in GraphPad Prism 5 software. The level of statistical significance was considered $P < 0.05$.

RESULTS

The hydroalcoholic extract was prepared from aerial parts of *K. odoratissima* by maceration method and then hexane, chloroform, ethyl acetate, and *n*-butanolic fractions were obtained by liquid–liquid extraction. The stones were synthesized *in vitro* and confirmed by FT-IR technique [Figures 2 and 3].

In FT-IR spectrum [Figure 2], the band at 3432 cm^{-1} is related to OH group of coordinated water molecules. The band at 1154 cm^{-1} is related to the stretching frequency of phenolic C-O group connected to calcium ion. An absorbance at 674 cm^{-1} characterizes the stretching vibration of C-C, which in general calcium oxalate.[22]

In FT-IR spectrum [Figure 3], bands at 3405 cm^{-1} and 1114 cm^{-1} are related to the stretching vibration of OH group of coordinated water molecules and stretching vibration of PO_4^{3-} group, which verify calcium phosphate stone.[23]

Dissolution of calcium phosphate crystals by crude extract

One-way ANOVA analysis showed that different concentrations of total extract had no significant difference in the dissolution of calcium phosphate stone ($P \geq 0.05$) [Figure 4]. Concentrations of 20, 40, and 60 mg/dl of total extracts had the potency to dissolve calcium phosphate crystals with the values of 79.00 ± 4.60, 72.09 ± 12.89, and 80.89 ± 5.50, respectively.

Dissolution of calcium oxalate crystals by crude extract

One-way ANOVA analysis showed that different concentrations of total extract had no significant difference in the dissolution of calcium oxalate stone ($P \geq 0.05$) [Figure 5]. In brief, results indicate that the total extract

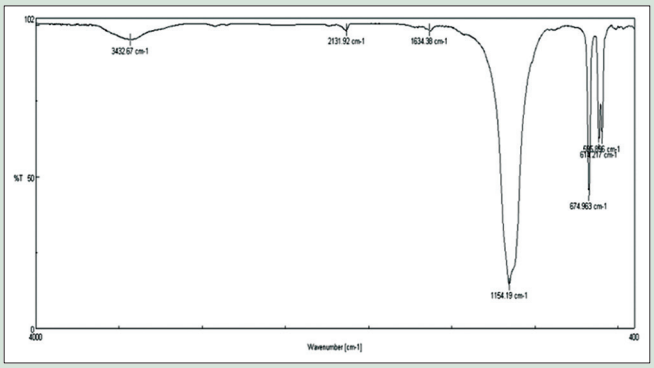

Figure 2: Fourier-transform infrared spectrum of calcium oxalate stone

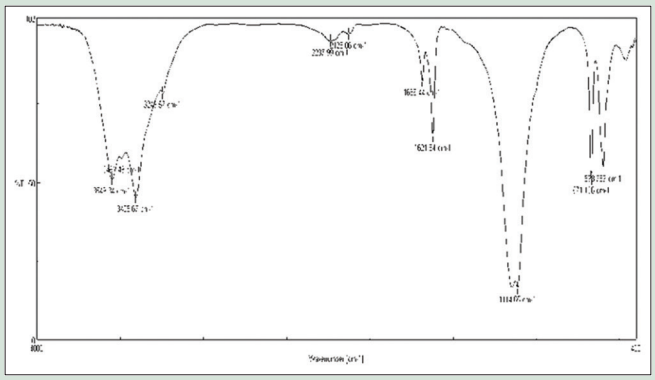

Figure 3: Fourier-transform infrared spectrum of calcium phosphate stone

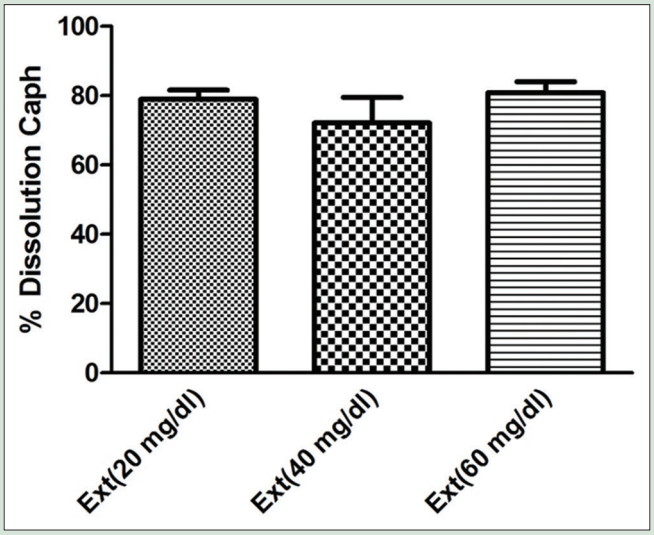

Figure 4: The dissolution of calcium phosphate crystals by *Kelussia odoratissima* extract

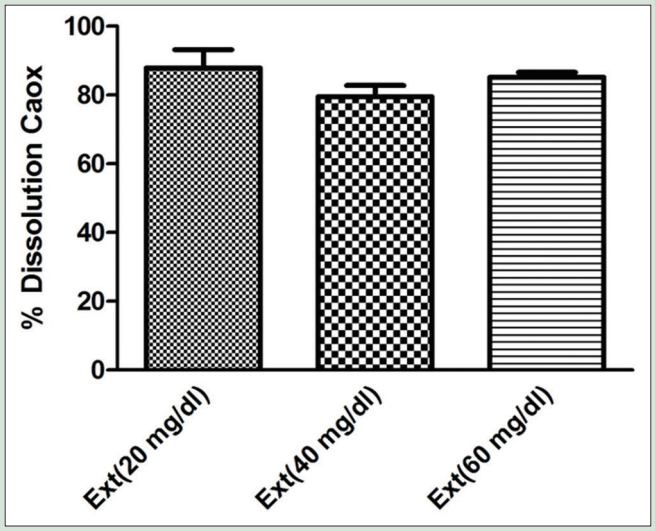

Figure 5: The dissolution of calcium oxalate stones by *Kelussia odoratissima* extract

of *K. odoratissima* had high potency to dissolve both calcium oxalate and calcium phosphate crystals *in vitro*. Dissolution percentage of calcium oxalate crystals by above-mentioned concentrations of crude extract of *K. odoratissima* in concentrations of 20, 40, and 60 mg/dl was calculated as 87.89 ± 9.24, 79.54 ± 5.58, and 85.21 ± 2.38, respectively.

Dissolution of calcium phosphate crystals by fractions

Considering the nonsignificant effect of different concentrations of total extract in the dissolution of both stones, the concentration 40 mg/dl was used in the next steps assay.

As shown in Figure 6, the concentration of 40 mg/dl of hexane, chloroform, ethyl acetate, and *n*-butanolic fractions had the potency to dissolve calcium phosphate crystals with the values of 95.59 ± 2.20, 93.53 ± 1.44, 80.22 ± 5.56, and 82.52 ± 11.61, respectively.

One-way ANOVA analysis showed that there are significant differences between mentioned groups in the dissolution of calcium phosphate crystals ($P < 0.001$). As shown in Figure 6, Tukey's *post hoc* test analysis showed that the significant difference was between crude extract with *n*-hexane fraction and chloroform fraction ($P < 0.01$). Dissolution potency of calcium phosphate stone by mentioned fractions was decreased in the following order: *n*-hexane fraction ≥ chloroform fraction > *n*-butanol fraction ≥ ethyl acetate fraction ≥ crude extract.

Dissolution of calcium oxalate crystals by fractions

The dissolution potency of calcium oxalate crystals by *n*-hexane, chloroform, ethyl acetate, and *n*-butanolic fractions was 92.46 ± 5.98, 93.59 ± 2.80, 69.97 ± 5.06, and 66.82 ± 2.36, respectively. One-way ANOVA analysis showed that there are significant differences between different fractions in the dissolution of calcium oxalate crystals ($P < 0.001$). As shown in Figure 7, Tukey's *post hoc* test analysis demonstrated that the differences were significant between crude extract with *n*-hexane fraction, chloroform fraction, and *n*-butanol fraction ($P < 0.05$) and between *n*-hexane fraction with ethyl acetate fraction and *n*-butanol fraction ($P < 0.001$). Furthermore, a significant difference was between chloroform fraction with ethyl acetate fraction and *n*-butanol fraction ($P < 0.001$). Dissolution potency of calcium oxalate crystals by mentioned fractions was decreased in the following order: Chloroform fraction ≥ *n*-hexane fraction > crude extract ≥ ethyl acetate fraction ≥ *n*-butanol fraction.

DISCUSSION

Urinary stones usually occur in concentrated urine. The presence of oxalate and phosphate, calcium in urine causes the formation of crystals and eventually urinary stones.[9] Diet modification, nutritional supplements, and some drugs are usually recommended for the prevention of kidney stone formation, depending on the type of kidney stone and urinary properties.[3] At present, for most of the urinary stones, supportive treatments such as drinking plenty of water and prescription of analgesics are used. Narcotics (morphine or pethidine) and other analgesics such as NSAIDs (NonSteroidal Anti-Inflammatory Drugs) can be used for pain relief. Anyway, the consumption of mentioned drugs, especially opioids, is associated with some side effects. Smaller stones are spontaneously excreted in 99% of cases;[26] for larger stones which were not spontaneously excreted and caused the severe complications, aggressive therapies, and surgery are done. In addition to the high cost imposed to the patient, surgery and breaking the stones have several side effects including urinary tract infection, which may cause severe injury to kidney tissue and also general body infections. Considering abovementioned problems, consumption of herbal products has been given special attention.[4] In the belief of ethnobotany, it has been noted that *Keluss* juice is extremely useful for destruction and fragmentation of kidney and bladder stones. In this study, the effect of *K. odoratissima* Mozaff. extract on the dissolution of urinary stones was investigated, and the results showed that total extract and its fractions had the significant potency to dissolve kidney stones (calcium oxalate and calcium phosphate). The results indicate the higher potency of fractions containing nonpolar compounds to dissolve calcium phosphate and calcium oxalate stones compared to the fractions containing polar compounds. *n*-butanolic fraction had the least effect, and hexane fraction had the greatest effect on the calcium phosphate stones. Furthermore, the total extract has less dissolution ability, compared to the fractions. In fact, the synergistic effect of the total extract compounds will be rejected in

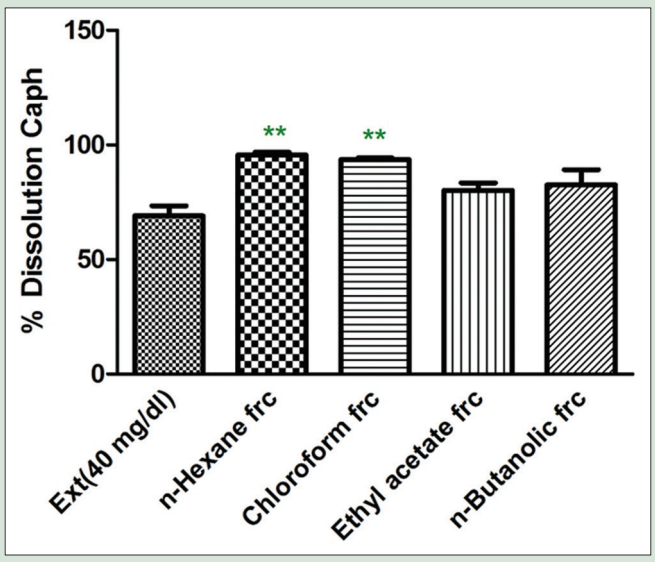

Figure 6: The dissolution of calcium phosphate stones by fractions; **: significant difference Ext (40 mg/dl) versus other groups ($P < 0.01$)

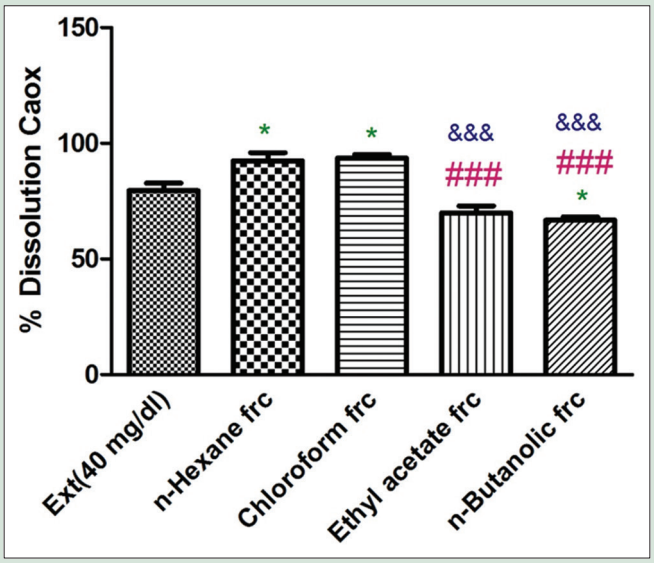

Figure 7: The dissolution of calcium oxalate crystals by fractions; *: Significant difference Ext (40 mg/dl) versus other groups ($P < 0.05$); ###: Significant difference n-hexane fraction versus other groups ($P < 0.001$); &&&: Significant difference Chloroform fraction versus other groups ($P < 0.001$)

this study. It also seems that the combination of nonpolar and semipolar compounds had more potency for dissolution of calcium phosphate and calcium oxalate crystals *in vitro*. In case of calcium oxalate stones, the dissolution ability of *n*-butanolic fraction compared to total extract was reduced. Thus, the nonpolar and semipolar compounds present may be playing a contributing role in the anti-crystallization action. Results have stated that these fractions contained a substance that promoted the nucleation of crystals.

The study on the effect of *K. odoratissima* Mozaff. *in vivo* and clinical trials in the future can confirm the using of this plant as a supplement to the dissolution of kidney stones. In addition, phytochemical studies of this plant can help to find probable molecules or lead compounds for strong busting of urinary stones.

Studies of Byahatti *et al.* in 2010 showed that phenolic compounds of ethanol extract from *Bergenia ciliata* had suitable ability to dissolve calcium oxalate and calcium phosphate stones *in vitro* (67.74% and 36.95%, respectively) compared to Cystone that commonly used (67.74% and 48.48%),[22] where the ability of *K. odoratissima* extract and its fractions is more prominent than the results of mentioned study. Furthermore, compared to the results of Cystone, the ability of by *K. odoratissima* Mozaff. in the dissolution of stones has been more.

In a study conducted by Frąckowiak *et al.* in 2010, it has been observed that methanolic soluble fractions of *Humulus lupulus* containing sugar alcohols and organic acids have high ability to dissolve calcium oxalate crystals; insoluble fractions in methanol which contain only sugar derivatives have also differences in morphology of crystals.[27] Atmani and Khan in 2000 showed that extract of *Herniaria hirsuta* increases the number of calcium oxalate crystals and reduces their size *in vitro*. In addition, it reduces the accumulation of calcium oxalate crystals that may be effective in the prevention of kidney stones formation.[28] In the present study, dissolution of stones by *K. odoratissima* Mozaff. is reported; however, microscopic studies are required to evaluate its effect on the number and size of crystals.

As well as, the *in vitro* study of Chaudhary *et al.* in 2010 indicated that fractions of *Terminalia arjuna* extract are more effective in inhibition of calcium oxalate and calcium phosphate stones formation, compared to total extract that these results are consistent with the current study.[29] According to the study of Wesson *et al.* in 1998, Pinirui extract is effective in the treatment of urinary calcium oxalate stones by increasing the ratio of calcium oxalate dihydrate to calcium oxalate monohydrate. This is due to the greater affinity of calcium oxalate monohydrate to cell membranes of the kidney and urinary tract.[30]

In this study, the effect of *K. odoratissima* Mozaff. extract on the treatment of urinary stones showed that total extract and fractions of this plant had significant potential for the dissolution of kidney stones (calcium oxalate and calcium phosphate). These findings substantiate the traditional use of *K. odoratissima* Mozaff. in the treatment of urinary stones and kidney problems.

The study on the effect of *K. odoratissima* Mozaff. *in vivo* and with human subjects in the future can confirm that this plant can act as a supplement to the treatment of kidney stones. The results of isolation and fractionation of the total extract of this plant support on the effective role of nonpolar and semipolar compounds of this plant in dissolving the studied kidney stones. Besides that, the phytochemical investigations on this plant comprise a good basis for finding potential molecules or compounds that serve as the leading contributors to dissolving kidney stones.

CONCLUSION

Results of this study showed that *K. odoratissima* Mozaff. extract and its fractions could dissolve urinary stones. Therefore, it can be effective in prevention and treatment of kidney stones in people who are prone to the formation of calcium oxalate and phosphate stones. However, it is hoped that this plant can be used in future studies as a medicinal supplement or lead compounds for designing of the effective pharmaceutical product in the treatment by determining the mechanism and investigating in future animal and human studies.

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Conflicts of interest

There are no conflicts of interest.

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