ORIGINAL ARTICLE

In vitro antilithiasic activity of saponins rich fraction from the leaves of *Zizyphus lotus*

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Abstract

Introduction: A large number of medicinal plants have been used in Algeria since antiquity, which claims the efficient cure of urinary stone, one of these plants is the specie *Zizyphus lotus* (Family: Rhamnaceae). Leaves of this species are traditionally used for the treatment of many diseases. Nowadays, numerous clinical studies have provided reliable results on the effects of several hundred plants on several diseases such as the problem of urinary stones. The objective of this study is evaluated *in vitro* the activity antilithiasic of the extract saponins **created** fraction. **Materials and Methods:** Inhibition and dissolution of calcium oxalate (CaOx) were studied by the use of the techniques turbidimetric and gravimetric, using a ultraviolet–visible spectrophotometer and a precision balance. **Results and Discussion:** Qualitative secondary metabolites determination in the leaves of *Z. lotus* showed the presence of polyphenols, saponins, flavonoids, alkaloids, and tannins, and the yield of the fraction rich in saponins is equal to 1.41%. The absorbance decreased with the increase in the concentration of extract indicating that decreased the nucleation of CaOx particles with inhibition at 55.23 ± 1.23%. The weight of CaOx crystal decreased with the increase in the concentration of saponins, and the extract at 100% concentration possesses a maximum percentage of dissolution equal to 91.95 ± 0.72%. This effect could be attributed to Ca^{2+} chelation by the compounds that exist in this fraction. **Conclusion:** This study indicates that extract can be considered as feasible natural products to improve the efficiency of CaOx inhibition and dissolution.

Key words: Calcium oxalate, saponins, urolithiasis, *Zizyphus lotus*

INTRODUCTION

The North African species of the genus *Zizyphus* are known with the vernacular name "sedra," and its fruits are the edible parts of the plant by the local population.[1] *Zizyphus lotus* (*Z. lotus*) belongs to the Rhamnaceae family. This family includes regarding 135–170 species grow typically in arid and semiarid countries. In Africa, *Z. lotus* is extensively distributed in the Mediterranean regions, such as Algeria, Morocco, Tunisia, and Libya.[2] The leaves are the richest parts of Vitamins E, A, and C.[2] In Algeria, several parts (roots, leaves, pulp, or fruit) of *Z. lotus* have been used in traditional medicine to treat urinary troubles, diabetes, skin infections, fever, diarrhea, insomnia, bronchitis, and hypoglycemia,[3] these parts also have several properties such as anti-inflammatory, antidiabetic, anticancer, hypoglycemic, antispasmodic properties, antifungal, anti-ulcer, and analgesic.[4] Several biologically active secondary metabolites are isolated from this plant, for example, the leaves notably made in phenolic acids, flavonoids, tannins, and saponins notably jujuboside B, jujubogenin glycosides, and jujubasaponine IV.[5] Other compounds are identified within the leaves such as monosaccharides (glucose, galactose, rhamnose, arabinose, and xylose), a flavonol organic compound, and 3', 5'-diglucosylphloretin.[2] The urinary stones are named supported the chemical composition, are composed of inorganic

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Received: 20-06-2020
Revised: 18-07-2020
Accepted: 26-07-2020

and organic crystals, and also the majority of the urinary calculi are composed of calcium salts, oxalates, and phosphates.[6] This study is devoted to the form of calcium oxalate (CaOx) most frequent. Nowadays, many studies have provided reliable results on the effects of many secondary metabolites such as polyphenols, tannins, flavonoids, and saponins, on several diseases such as urinary stones.[7-9] Urolithiasis could be a sickness within which calcium salt crystals have a significant role, these crystals gift in several forms: Salt calcium oxalate monohydrate, calcium oxalate dihydrate, and also the rarer calcium oxalate trihydrate.[10] There are a series of complex physicochemical factors in the formation of CaOx stone starting with the nucleation step and crystal growth followed by crystal aggregation.[6] There is a growing interest in the global public for the use of herbal remedies, particularly in the treatment of urolithiasis, possibly due to the limited choice of pharmacotherapy or the high cost and side effects of the use of an extracorporeal shock wave lithotripsy.[11] The inhibitory effect of saponins in urolithiasis has recently been depicted in investigations of the many plants such as *Solanum xanthocarpum*, *Tribulus terrestris*, *Trachyspermum ammi*, *Hibiscus sabdariffa,* and *Achyranthus aspera*.[12] Some studies have shown that saponins, for example, α-amyrin, β-amyrin, and lupeol from various plants have demonstrated antiurolithiatic and diuretic activity,[13] on the other hand, an *in vitro* study of the saponin-rich fraction prepared from fruits of *S. xanthocarpum* has shown an effect of preventing nucleation and aggregation of CaOx crystals in a solution of artificial urine[14] not only for this reason, we chose this plant, but also on the basis of the work provided by the researcher Khouchlaa *et al*.[15,16] The objective of this study is to evaluate *in vitro* inhibition of the saponin-rich fraction of the leaves of this shrubby using the turbidimetric technique and the activity of dissolution of urinary stone by the use of the technique gravimetric. This study may be used for formulating the strategy for the prevention or dissolution of CaOx.

MATERIALS AND METHODS

Chemicals, Reagents, and Solvents

n-Butanol, ethanol, n-hexane, NaCl, $Na_2C_2O_4$, $CaCl_2.2H_2O$, as well as all the chemicals used in phytochemical studies were obtained by the faculty's chemistry laboratory using an approved supplier with these characteristics:

n-Butanol (99% pure) Sigma-Aldrich, ethanol (95.0% pure) Sigma-Aldrich, n-hexane (95% pure) BIOCHEM Chemopharma, sodium chloride (99.5% pure) Fluka, calcium chloride dihydrate (99% pure) Sigma-Aldrich, and sodium oxalate (99.5% pure) Sigma-Aldrich.

Plant Material

The leaves of *Z. lotus* were collected from in Elcheref-Djelfa, Algeria, in 2019, and identified in the Faculty of Science of Nature and Life in the University of Djelfa. The leaves were air-dried and then powdered.

Preliminary Phytochemical Screening

Preliminary phytochemical evaluation of the leaves of *Z. lotus* was carried out for the qualitative estimation of secondary metabolites.[17]

Preparation of the Plant Extract

Saponins rich fraction of leaves was prepared according Sana *et al*.[12] with some modification. The powder (8 g) of *Z. lotus* leaves was delipidated with stirring and at room temperature for 2 h with 50 ml of n-hexane. After filtration and removal of the organic phase, the residue obtained was dried and 3 g of delipidated powder was macerated in 100 ml of ethanol with magnetic stirring at room temperature overnight. The ethanolic phase was filtered and evaporated at 40°C with the rotavapor until a dry residue was obtained, this last was extracted three when with 60 ml of the mixture of distilled water/n-hexane (v:v) heated at 50°C in a water bath for 30 min. The phases obtained were decanted and the aqueous phases were mixed and extracted twice with 30 ml of n-butanol. The organic phase (butanolic) evaporated at reduced pressure at 40°C with the rotavapor, the dry residue was weighed (0.0425 g).

In Vitro Antilithiatic Activity

Inhibition of CaOx crystallization by turbidimetric method

Inhibition of CaOx crystallization was studied in a solution containing calcium chloride dehydrate (7.5 mmol/l) and sodium oxalate (2.5 mmol/l), these solutions were prepared using sodium chloride 0.15 mol/l as solvent. The study of crystallization based on Sweta *et al*. and Parveen *et al*.[7,18] with a slight modification.

Study without inhibitor

The crystallization begins when 7.5 ml of a solution of sodium oxalate is added to 7.5 ml of a calcium chloride solution at 37°C with magnetic stirring. The optical density (OD) of the solution was monitored at 620 nm after 30 min using an ultraviolet–visible spectrophotometer (Shimadzu 1240).

Study with inhibitor

The inhibitor (100%) is prepared by taking 0.0425 g of the dry residue with 60 ml of 1% ethanol. From this inhibitor, we have prepared numerous diluted inhibitor solutions of 1%, 10%, 25%, 50%, and 75% using solvent sodium chloride (0.15 M). Crystallization was started by adding 5 ml of sodium oxalate solution at 37°C and magnetic stirring. The temperature was maintained at 37°C. The OD of the solution

was monitored at 620 nm after 30 min. For each experiment, three replicates were taken. The percentage inhibition I (%) produced by the herb extract was calculated as follows:

% inhibition = (Absorbance of Control−Absorbance of Test)/ Absorbance of Control

Where, Absorbance test: Absorbance in the presence of inhibitor (extract), absorbance control: Absorbance without inhibitor (control negative).

Dissolution of CaOx by Gravimetric Method

Preparation of a precipitate of CaOx

The preparation of CaOx precipitate based on the references cited by Johannes *et al*.[19] and Anamarija *et al*.[20] with a slight modification.

A volume of 2 ml of 2.5 mmol/l sodium oxalate pH 7 (at 37°C) and 7.5 mmol/l calcium chloride pH 6 (at 37°C) was mixed in centrifuge tubes. The CaOx was allowed to precipitate for 30 min at 37°C. Then, the tubes were centrifuged at 6000 rpm using a centrifuge for 16 min, the supernatant was removed. Then, the precipitates were washed by adding 4 ml of distilled water and centrifuged again as described above. Finally, the supernatant was removed; the tubes were oven-dried at 70°C for 50 min and weighted again to calculate the mass of the precipitates.

Ability of the saponins fraction to dissolve the CaOx precipitate

We evaluated the effectiveness of the saponins fraction *in vitro* on CaOx dissolution using the method illustrated by Yachi *et al*.[21] and Kachkoul *et al*.,[22] with a slight modification.

A volume of 4 ml of the saponins fraction at different concentrations (1%, 10%, 25%, 75%, and 100%) were added to the CaOx precipitates and the tubes were incubated for 30 min at 37°C. Then, the tubes were centrifuged and the precipitates washed, dried, and weighed as described above. For each experiment, three replicates were taken. The dissolving activity (A %) was calculated with the following formula:

$$A\,(\%) = (W_{intial} - W_{final})/W_{initial}$$

Where: $W_{initial}$: The weight of the precipitate before the incubation with the saponins fraction (negative control). W_{final}: The weight of the precipitate after the incubation with the saponins fraction.

Statistical Analyses

The data were expressed as mean values of three independent experiments (each in triplicate) and we compared the resulting inhibition activity with inhibitor at different concentrations and without inhibitor using correlation and one-way analysis of variance (ANOVA) and Turkey's multiple comparison test. The values having $P < 0.05$ were considered as significant. Statistical analyses were performed with GraphPad Prism 7.00.

RESULTS

Preliminary Phytochemical Screening

Qualitative of secondary metabolites determination in the leaves of *Z. lotus* showed the presence of polyphenols, saponins, flavonoids, alkaloids, and tannins.

Inhibition of CaOx Crystallization by Turbidimetric Method

Table 1 shows the percentage inhibition of the crystallization of CaOx with different concentrations of extract of leaves of this species. We will take into consideration only the concentrations 10%, 25%, and 50% since these shines which give reliable results.

The percent inhibition was calculated using the above-mentioned formula.

Figure 1 displays the effect of the different concentrations of the extract of *Z. lotus* on the crystallization of CaOx. The increase in the concentration extract showed an increase in the inhibition of nucleation. Maximum inhibition is 55.23 ± 1.23% observed at a concentration of 50%.

Dissolution of CaOx by Gravimetric Method

After several tests of the concentrations, we are chosen the concentrations illustrated in Table 2.

Table 1: Variation of percentage inhibition, in terms of to the saponins fraction at different concentrations

CI (%)	00	10	25	50
OD (620 nm)	0.520±0.009	0.498±0.005	0.403±0.022	0.230±0.008
I (%)	00±00.00	4.03±0.96	22.53±3.26	55.23±1.23
CV (%)	3.33	1.83	9.46	6.63

CI (%) concentration of inhibitor, OD optical density at 620 nm, Cv (%) coefficient of variation of OD, I (%) percentage of inhibition. Density values are expressed as mean±standard error of the mean. ****P<0.0001 with F (2.6)=94.14. Percentage inhibition values are expressed as mean±standard error of the mean. ****P<0.0001 with F (3.8)=194.1

Table 2: Variation of dissolving activity in terms of the saponins fraction at different concentrations

C_{sf} (%)	00	10	25	75	100
W_{CaOX} (mg)	16.30±0.75	10.93±0.49	9.63±0.26	9.20±0.34	1.30±0.05
A (%)	00±0.00	33.12±0.13	41.10±1.15	43.90±0.47	91.95 ±0.72
C_V (%)	7.98	7.78	4.68	6.52	7.69

C_{sf} (%) concentration of saponins fraction, W_{CaOX} (mg) weight of calcium oxalate, Cv (%) coefficient of variation of weight, (A %) the dissolving activity. Values of weigh are expressed as mean±standard error of mean. ****P<0.0001 with F (4.10)=145, 2. Dissolving activity values are expressed as mean±standard error of mean. ****P<0.0001 with F (4.10)=2585

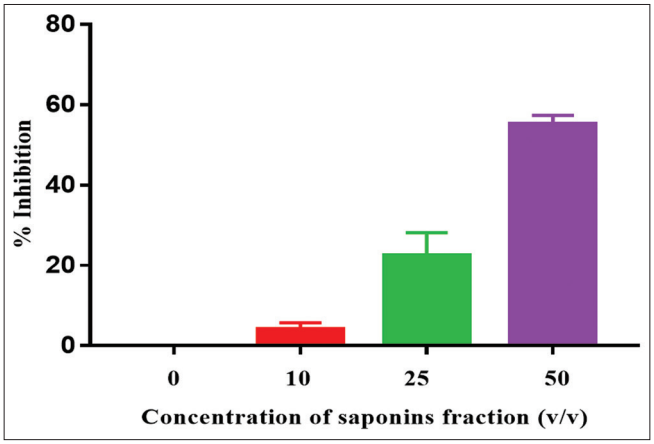

Figure 1: Effect of the extract of the extract of *Zizyphus lotus* on the crystallization of calcium oxalate

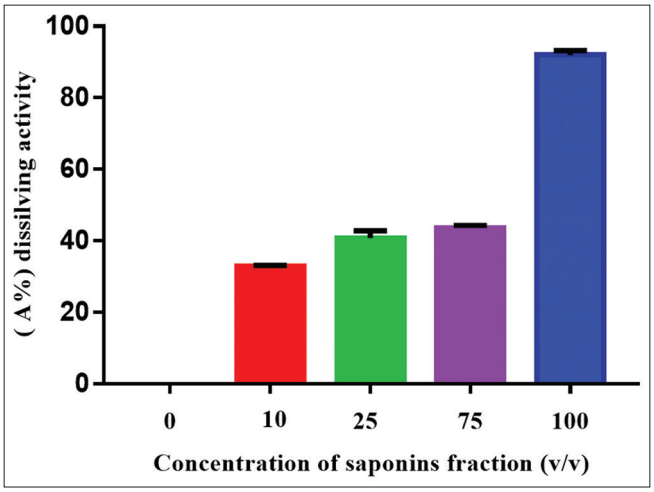

Figure 2: Effect of the different concentration of the extract of *Zizyphus lotus* on the dissolving of calcium oxalate

The results indicate the change of dissolving activity as a function of the concentrations of the saponins fraction, as shown in Figure 2.

DISCUSSION

Urolithiasis is a multifactorial urological disorder common all over the world and affects up to 10–15% of the population.[23] By chemical analysis, we can say that for all types of kidney stones, CaOx is the most common composition with a frequency of more than 80%.[24] Referenced studies[2,5]

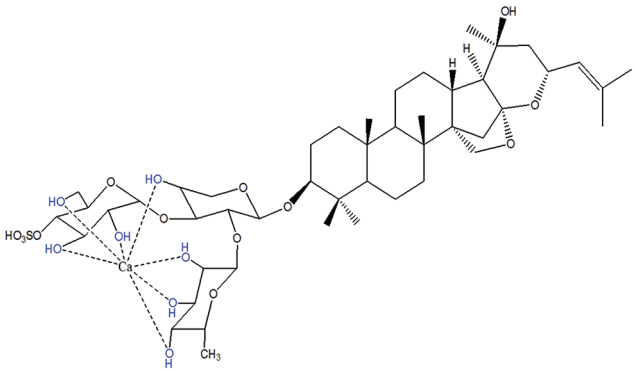

Figure 3: Ca^{+2} - Jujubogenin glycosid complex, this structure is inspired by Hamed *et al*.[30]; $^{+2}$exhibitor in calcium

say that the leaves of *Z. lotus* contain several secondary metabolites such as flavonoids, polyphenols, saponins, tannins, and alkaloids; this result is in accordance with what has been obtained. The yield of the fraction rich in saponins is equal to 1.41% w/w; other study gives a yield of 0.34% this difference is due to the climatic conditions and to the extractions techniques.[25,26] In the first study, we are interested to assess the effectiveness of the saponins fraction in inhibiting the formation of CaOx crystals. Nucleation is the formation of a solid crystal phase in a solution. It is an important step in nephritic stone formation. The inhibitory efficiency of the species *Z. lotus* was tested on the step of nucleation. The absorbance decreased with the increase in the concentration of saponins rich fraction indicating that decreased the nucleation of CaOx particles. The OD was highest (0.520 ± 0.009) in the absence of extract, and it was lowest (0.230 ± 0.008) at the highest concentration of saponins fraction (50%). Recent studies have shown that extract saponins contribute significantly to the antiurolithiatic activity,[13,24] this property of plants may be important in preventing kidney stone formation. In this second study, we specifically tested the efficacy of saponins fraction to dissolve CaOx. The extract of *Z. lotus* at 100% concentration has been shown to be the most effective in dissolving CaOx stone in terms of weight reduction. This effect could be attributed to a higher rate of ionization and Ca^{2+} chelation by these agents, thereby increasing the solubility of CaOx crystals.[27] The comparison of the results obtained by the gravimetric technique with the study[16] shows that the aqueous extract of the leaves of *Z. lotus* has an ability to dissolve the CaOx stone with a percentage of

7.65%. Saponins are the glycosides of triterpenes or steroids and include the group of cardiac glycosides and steroidal alkaloids[28] and the presence of hydroxyl groups in the saponins of the leaves of *Zizyphus*[2] allowed us to say that the presence of a part glycoside increases the complexing capacity of Ca^{+2}, so the inhibitory properties of crystal formation are high,[29] as shown in Figure 3. However, the highest dissolution properties were observed for a maximum of the hydroxyl groups.[29] Since in recent years, many data have appeared to indicate that sugars have high potency for calcium binding.[29]

CONCLUSION

Fraction saponins of *Z. lotus* leaves are a natural agent that able to prevent the formation of CaOx crystal *in vitro*. On the other hand, the extract of this species can be used to dissolve CaOx kidney stones. Structural identification and characterization of these saponins and the studies *in vivo* constitute the main objective of our next study.

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Source of Support: Nil. **Conflicts of Interest:** None declared.