

Assessment of Paronychia Argentea Extraction on Kidney Stone By Using Calcium Oxalate Method

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<https://dx.doi.org/10.13005/bpj/2049>

(Received: 09 July 2020; accepted: 30 September 2020)

Urinary calculi are stones (urolithiasis) that can form anywhere in urinary tract outside of the kidneys and mostly composed of calcium oxalate and phosphate, additionally with elevated throughout the last two decades in the world. Chemical composition plays a major part in nephrolithiasis. Therefore, the high concentrations of lithogenic substances in urine enhance the crystallization method in urine tract system. The most kidney stones form from calcium oxalate, the present study was inspected the effect of the crude aqueous extract as well as the fractionated methanol extract (ethyl acetate, isopropanol, acetone and methanol residue) of paronychia argentea on the crystallization of calcium oxalate salts. The effect of aqueous extract and fractionated methanol extract on the size, number, type of calcium oxalate crystals. Paronychia argentea both the crude aqueous and the fractionated extract, especially ethyl acetate fraction have antiurolithic activity via reducing crystal size as well as activate the formation of calcium oxalate dihydrate (COD) crystals out from calcium oxalate monohydrate (COM) with increasing concentration of extract. The shifting of crystallization process to producing calcium oxalate dihydrate (COD) rather than oxalate monohydrate (COM) and the reducing the crystal size and calcium ion concentration, in addition to the diuretic action of extract plays an important role in controlling urolithiasis.

Keywords: Urolithiasis; Calcium oxalate; *Prosopis farcta*; Urinary stone antioxidant.

Nephrolithiasis has no longer considered as isolated condition, because the hazard has been raised for chronic kidney disease. Kidney stones turn into one of the most prevalent disease and many studies have been shown the ratio incremented universal throughout the twenty years.

Also, kidney stones consider as markers of metabolic imbalance, presaging other comorbidities, due to their linke to the metabolic syndrome, hypertension, coronary artery disease, and reduced bone mineral density. (Lada Beara-

Lasic1 and David S. Goldfarb 2019). Therefore, prevention of stone recurrence offers possibility to keep away from painful, high priced stone episodes, while enhancing standard health via nutritional and pharmacologic interventions.

Kidney stones, urinary stones (aka urolithiasis) become one of the main diseases that affects 10-12% of population in developed countries (Sowers *et al.*, 1998).

The prevalence of the kidney stone, after an initial episode of nephrolithiasis of the first

stone, showed that the chance for recurrence the second stone within a year is about 15% and within ten years about 50%. Unlikely, nephrolithiasis literatures showed incremented in the number of individuals suffering from kidney stone in future decades. Due to, the cost of treatment will be raised globally. (Luyckx *et al.*, (2020)) is predicted to spend five billion dollars by 2030 on its treatment in USA. (Akram, M. and Idrees, M., 2019). (Antonelli *et al.*, 2014.)

Many factors play a major roles in increasing the risk of formation kidney stone, such as lifestyle, gender and geographic region, environmental and genetic factors contribute to their formation, and dietary, for example, type of food up take if it rich with protein continent the high risk to formation kidney stones. Also type of vegetable or fruits have been taken. A having a strong family history of urolithiasis, increase the risk of formation kidney stones (Luyckx *et al.*, (2020).

In other hand the gender factor showed that men have the high risk symptomatic stones compare with women. (Priti *et al.*, 2017).

There are several types of kidney stones based on the type of crystals of which are consist. The majority are calcium oxalate stones, observed by way of calcium phosphate stones. More rarely, Struvite stones ($MgNH_4PO_4$) are produced by urea splitting bacteria in people with urinary tract infections, and people with certain metabolic abnormalities may produce uric acid stones or cystine Stones. (Dyer and Nordin, 1967) and Singh *et al.*, 2010).

In general, it is recommended to have a dietary abundant with calcium and lower with oxalate, potassium and sodium contain to decrease the danger of having kidney stone. In contrast, excessive animal protein intake is the principle supply of acid in the human frame and lowers urine pH, which then will increase the risk for uric acid stones.

Herbal medicine spread through the world rapidly like Europe, United States, China and Hong Kong (Gohel and Wong, 2006; Yasui *et al.*, 1999). Many medicinal plants showed good effective antiurolithic activity such as *Phyllanthus niruri*, *Aerva lanata*, *Crataeva nurvala*, *Herniaria hirsuta* and indigenous plant (Kulaksizoglu *et al.*, 2008; Atmani *et al.*, 2003; Freitas *et al.*, 2002; Selvam

et al., 2001; Varalakshmi *et al.*, 1990; Patankar, 2013).

In this study, we will investigate the medicinal effect of *Prosopis farcta* in the treatment of kidney stones as alternative to surgically or ultrasound treatment of kidney stone, so to characterize effect of *Prosopis farcta* on kidney stone. In this paper study the effect of an aqueous extract of *Prosopis farcta* on calcium oxalate crystallization induced in vitro.

In different regions on the world, traditional medicinal have been used plants to cure urolithiasis. Herbel plants are containe different mixture of organic compounds, so, we must be digging deep by doing more researches to isolation and characterization of the active compounds and elucidation of the relationship between structure and activity. Combination of modern technology and traditional knowledge can produce invective cure for many diseases such as kidney Stones. (Patankar, 2013).

The aim of the study is to investigate the effect of crude aqueous extract and fractionated methanol extract of *paronychia argentea* on kidney stone.

MATERIALS AND METHODS

General experimental procedures

Retsch Laboratory Mill model 5657 (Haan, Germany) was used to grind *Paronychia argentea*. Thermo Electro Corporation forma orbital shaker equipped with HEPA Filter (Massachusetts, USA) was used to prepare the total methanol extract that was thereafter fractionated using Soxhlet apparatus. UV/VIS double beam Spectrophotometer SPUV-26 (Staffordshire, UK) was used to measure the absorbance of the samples. Flame Photometer Models PFP7 (JENWAY, UK) was used to determine the concentration of calcium ion. Krüss microscope connected to a Krüss optronic camera (Hamburg, Germany) was used to investigate the size and shape of calcium oxalate crystals.

Plant material

The aerial parts of *Paronychia argentea* was collected entire from the local Jordanian market within Karak governorate in May, 2019. The collected *Paronychia argentea* was cleaned then dried at 45°C overnight and afterwards ground to powder.

The plant was authenticated by Prof. Dr. Saleh Al-Qur'an at Department of Botany, Faculty of Science, Mu'tah University and a voucher specimen coded PA-201905 was kept at Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mu'tah University.

Preparation of plant extract and subsequent fractions

Through reflux method, 50 g of the dried ground the aerial parts of *Paronychia argentea* was exhaustively defatted with 400 ml n-hexane for 8 hr. The n-hexane extract was filtered out and concentrated under reduced pressure. The defatted plant was again refluxed using 400 ml methanol for 8 hr. The total methanol extract was concentrated till dryness under vacuum. Part of methanol extract residue was then fractionated against three different solvents, namely, ethyl acetate, acetone and isopropanol using Soxhlet apparatus. The different fractions together with the residual methanol extract were filtered out, concentrated, and dried under reduced pressure. Afterwards, they were all resuspended in distilled water, filtered through a 0.45 μm filter and used to prepare 1 mg/ml solution from each fraction in distilled water, also 100 mg/ml of aqueous solution for crude methanol extract prepared and different concentrations from it prepared as follow (1, 4, 8, 16, 32, 64, 80 and 100 mg/ml).

Phytochemical screening of the plant extract and subsequent fractions

Testing for carbohydrate

Molisch's test

To a 2 ml portion of the total extract and its subsequent fractions, 2-3 drops of α -naphthol solution in alcohol was added, shaken for 2 min and 1 ml of concentrated sulphuric acid was added slowly on the sides of the test tube. A deep violet colour at the junction of two layers indicates the presence of carbohydrates in all samples.

Testing for flavonoids

NaOH test

A portion of the total extract and its subsequent fractions was separately treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids in all samples except isopropanol fraction.

Testing for saponins

Froth test

An aliquot of 30 mg from each tested fraction was dissolved in 2 ml distilled water by sonication then was vigorously shaken for 5 min each in graduated test tube. The solution is then allowed to stand for 15 min and the persistent froth height was then measured and taken as qualitative and quantitative indicator of the saponin content for each tested fraction, all fractions gave positive indication, according to the following order from the highest to lowest (ethyl acetate, acetone, methanol and isopropanol fraction).

Testing for tannins and phenols

Ferric chloride test

To a 3 ml aliquot of the total extract and its subsequent fractions, 3 ml of 5% (w/v) ferric chloride solution was added. The resulting blue-black colour indicates the presence of tannins and phenols.

Lead acetate test

To a 3 ml aliquot of the total extract and its subsequent fractions, 3 ml of 1% (w/v) lead acetate solution was added. The formed precipitate was filtered, dried and weighed where it given an indication about the presence of tannins and phenols.

As a result for above two tests all fractions showed presence of tannins and phenols except isopropanol fraction.

Crystallization assay in urine

A human urine samples were obtained from a 30-year old a healthy person in polypropylene bottles refrigerated immediately after collection. Urine sample was centrifuged at 5000 rpm for 8 min and the supernatant was decanted in clean test tube. After that Urine sample was divided into 14 portions (2 ml each); one as negative control, standard drug (cystone), ethyl acetate fraction, acetone fraction, isopropanol fraction and the residual methanol extract, and another eight portion for each concentration of crude extract as follow (1, 4, 8, 16, 32, 64, 80 and 100 mg/ml), to each urine sample was added 50 μl from the previously prepared 1 mg/ml solutions from each fraction and each concentration of crude extract as follow (1, 4, 8, 16, 32, 64, 80 and 100 mg/ml), followed by addition 50 μl of 0.1 M sodium oxalate and 50 μl of 0.1 M CaCl_2 to each test tube also.

The experiment was conducted in triplicate. All samples were then incubated with shaking at 37°C for 2 hours. Then, optical density (OD) to each concentration of crude solution was measured at 578 nm.¹³ Also, a 10 µl aliquot of the filtrate from all 14 samples was applied on a slide and examined under KRUSS microscope 151 and equipped with KRUSS optronic camera (Hamburg, Germany).

The percentage of the dissolution (inhibition) produced by the herb extracts was calculated using the following formula:

$$\% \text{ Dissolution} = \frac{[(\text{Absorbance of control} - \text{Absorbance of sample}) / (\text{Absorbance of control})] \times 100}{\dots(1)}$$

The description of calcium oxalate crystals with respect to hydration, size and shape, i.e. calcium oxalate monohydrate (COM) and calcium oxalate dehydrate (COD) were recorded. The concentration of soluble calcium in the samples was measured by using two methods as follows:

(a) Flame Photometric determination: To draw the calibration curve a serial dilution of a standard 100 mg/dl stock solution of anhydrous calcium chloride in deionized water was measured by using Flame Photometer JENWAY Models PFP7 (Staffordshire, UK). By measuring the emission of calcium ions, the concentrations of calcium ions for control and standard (cystone) and each fraction were determined by using calibration curve equation and correlation coefficient. The results shown in Table (1)

(b) Spectrophotometric determination of calcium ion: in order to measure calcium

ions in the samples. To each sample was added arsenazo III which was forming a colored complex that can be measured by UV/VIS double beam Spectrophotometer SCOTech SPUV-26 (Dingelstadt, Germany) at 650 nm using a Kit (Calcium-Arsenazo Biosystems). The concentrations of calcium ions in control and standard (cystone) and each fraction determined by measuring the absorbance, then comparing it with the standard in the kit. The results shown in Table(1).

Antioxidant activity of plant extracts using DPPH radical scavenging assay

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical assay was carried out spectrophotometrically as described by Tepe and co-workers in 2005 (Tepe *et al.*, 2005). A 50µl from the various concentrations of each fraction extracts was added 5 mL of 0.004% methanolic solution DPPH. The samples were incubated for 30 min. at room temperature, the absorbance was determined using methanol as blank at 517 nm. All measurements were done in triplicate. The percentage of the Inhibition free radical scavenging activity was calculated by using the following equation:

$$\text{inhibition (\%)} = \frac{(\text{Abs. control} - \text{Abs. sample}) / \text{Abs. control} \times 100}{\dots(2)}$$

where Abs. control is the absorbance of the control reaction (containing all reagents except the tested compound) and Abs. sample is the absorbance of the tested compound with all other reagents. Extract concentration providing 50% inhibition (IC₅₀) was determined from a graph plotting percentage inhibition against

Table 1. Concentration of calcium ion in each fraction and cystone produced from dissolution of calcium oxalate measured by flame photometer and spectrophotometer.

Fraction	Concentration Ca ⁺² (ppm) Flame Photometry	Concentration Ca ⁺² (ppm) Spectrophotometry
Methanol	357.926±1.427	353.582±0.048
Acetone	360.829±0.755	363.911±0.039
Ethyl acetate	381.819±0.713	373.129±0.099
Isopropanol	343.095±0.756	335.404±0.195
Oxalate	246.699±2.854	241.629±0.179
Cystone	360.397±2.854	364.595±0.147

extract concentration. Trolox® [(±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] (final concentration 0 to 1.5 µg/ml) was used as a standard antioxidant drug for the construction of the calibration curve, and the DPPH radical-scavenging activities were expressed as mg Trolox® equivalents per gram of plant extract (Barros *et al.*, 2003). The results shown in Table(2).

RESULTS AND DISCUSSION

The in vitro kidney stone which composed from calcium oxalate salt, was artificially prepared

by mixing equall moles of sodium oxalate and calcium chloride.

Analysis of calcium oxalate by using dissolution method

The calcium concentrations were determined by two methods spectrophotometric by using calcium kit and flame photometric, as shown in Table 1. The statistical analysis by using anova single factor, to the result of both methods showed that no significant difference between two methods of measuring calcium concentration in Flame Photometry or in Spectrophotometry,

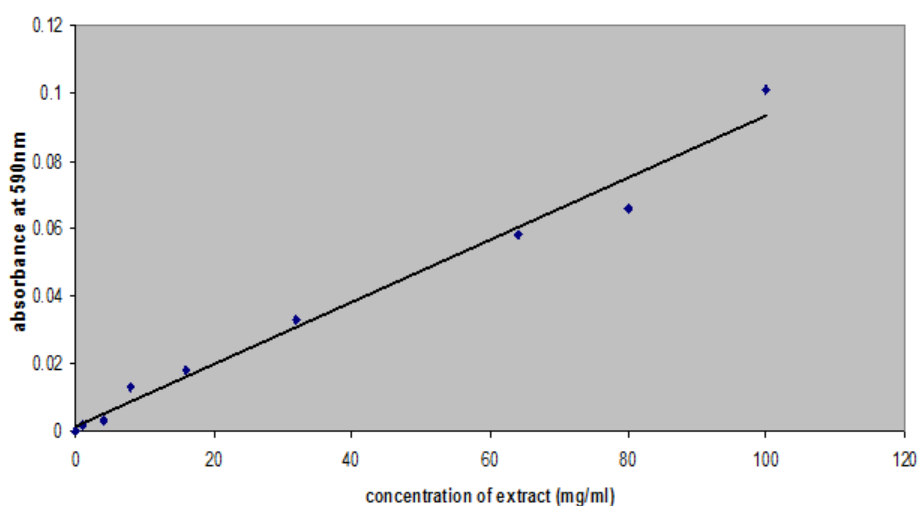


Fig. 1. Effect of increasing concentrations of *Paronychia argentea* extract on calcium oxalate crystallization measured absorbance at 590nm

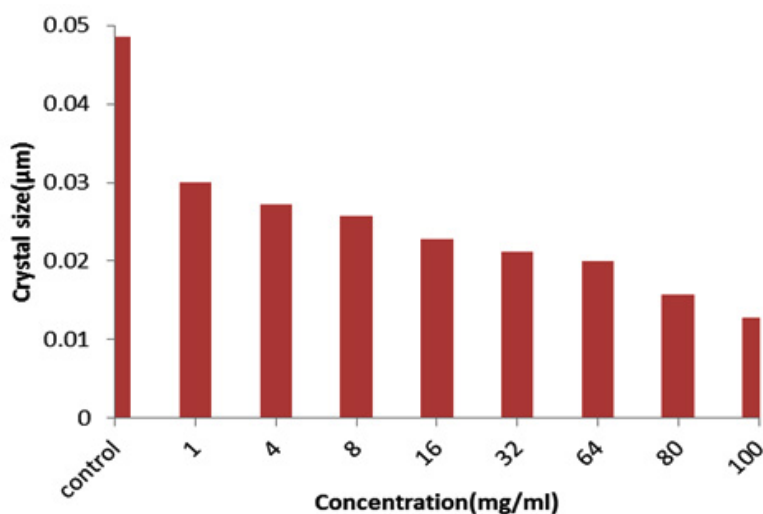


Fig. 2. The effect of increasing concentration of *Paronychia argentea* extract on calcium oxalate monohydrate (COM) crystal size

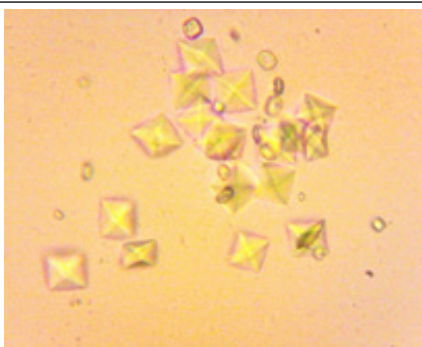
Table 2. *In vitro* antioxidant (DPPH) assay results

Fraction of coffee seeds and control and standard	IC ₅₀ (mg/ml)
Acetone	34.22
Methanol	465.93
Ethyl acetate	41.82
Isopropanol	99.08
Oxalate	No inhibition
Cystone	297.33

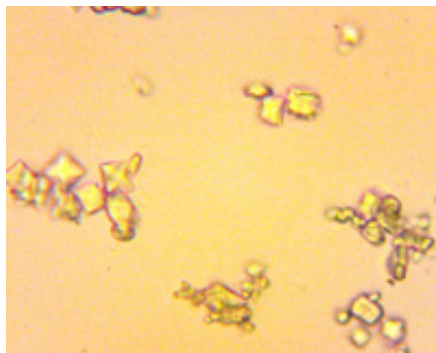
because F_{calc} less than F_{crit} , as shown in (Table 3).

The concentration calcium dissolved from calcium oxalate salt which was considered as control approximately around 246 ppm. Mean while the standard drug cystone showed 310 ppm after treatment with artificial calcium oxalate crystals.

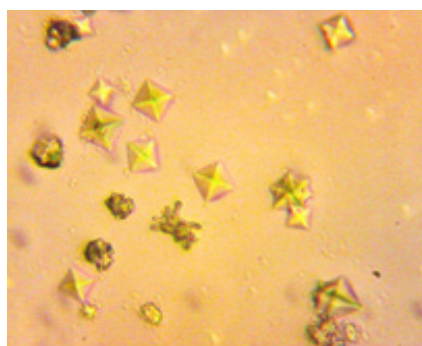
The aerial parts of *Paronychia argentea* was extracted by different solvent. These extracts



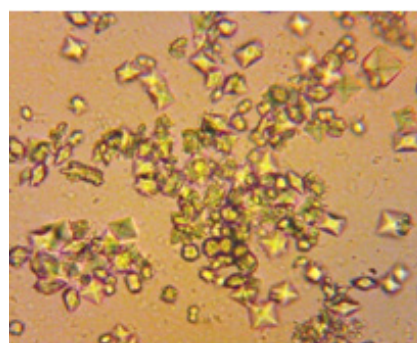
a) Oxalate(control)



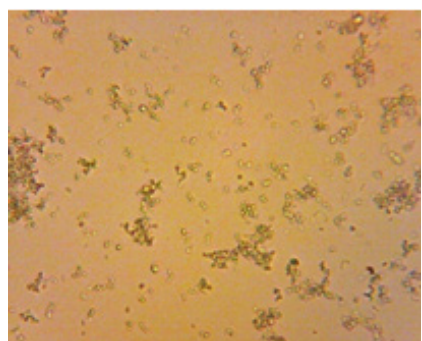
b) Cystone(standard)



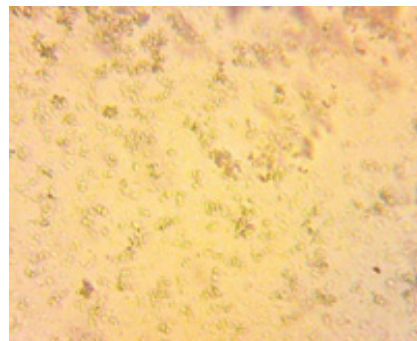
c) isopropanol



d) Methanol



e) acetone



f) Ethylacetate

Fig. 3. Light microscopy of calcium oxalate crystals induced *in vitro*, (a) control(oxalate) (b) standard(cystone) (c) isopropanol (d) methanol (e) acetone (f) ethyl acetate at 400x magnification

were used to dissolve the calcium oxalate crystals. Ethyl acetate fraction was observed to have the highest rate of dissolution; because ethyl acetate fraction showed the maximum calcium ion concentration around 381ppm, Table 1. Which means the dissolution is the highest rate. The less amount of calcium oxalate dissolution was observed in isopropanol extract, which showed the calcium ion concentration around 343 ppm. Even though the isopropanol extract shows the less dissolution rate compared with other solvent extract of the aerial parts of *Paronychia argentea*, it shows higher dissolution rate compared with the standard drug cystone which shows less dissolution rate compared with isopropanol extract. In other hand the calcium ion concentration of both methanol and acetone extracts 358ppm, this result exhibited methanol and acetone extracts have approximately the same dissolution rate.

Phytochemical screening

Phytochemical screening of the different fractions of the total methanolic extract prepared from *Paronychia argentea* areal parts revealed that all the fractions possessed carbohydrates among their contents depend on Molisch test results. All the fraction give positive results for tannins, phenols and flavonoids tests except isopropanol fraction. Interestingly, ethyl acetate fraction displayed the highest and the most persistent froth indicating its relative high content of saponins compared to other fractions. Atmani., *et al.*, 2006 found that the fraction rich in saponins of methanolic extract has the highest potency on treatment of kidney stones in

vitro and *in vivo*. While other studies showed that the fractions of the total extract has high potency in treatment of kidney stones (calcium oxalate and calcium phosphate) mainly fractions contains non polar and semipolar compounds of this plant (Tork. *et al.*, 2018).

DPPH radical scavenging assay

The *in vitro* antioxidant (DPPH) activity of the extracts of *Paronychia argentea* areal parts were determined by measuring the DPPH radical scavenging activity at 517nm, as shown in Table 2. acetone and ethylacetate fraction showed the highest ability to scavenge the DPPH radical with IC_{50} values of 34 mg/mL and 42mg/mL respectively. In other hand, both methanolic fraction and standard drug cystone have lowest ability to scavenge the DPPH radical with IC_{50} of values 466 mg/mL and 297 mg/mL respectively, than acetone, ethyl acetate and isopropanol extracts.

Nucleation

In this study, it was found that the extracts of areal parts of *Paronychia argentea* increased the crystallization process of calcium oxalate salt in a concentration dependent manner (Fig.1, $R^2 = 0.9822$). The result showed the turbidity of the solutions directly proportional to concentrations of extract, as a result of that more but smaller size of (COD) produced from dissolving of (COM) crystals as shown in (Fig. 2).

It is well known scientific fact that kidney stone formation process is a result of crystallization that happened in supersaturated

Table 3. Statistical analysis (ANOVA single factor)

Groups	Count	Summary		
		Sum	Average	
Column 1	6	2032.25	338.7083	2429.324
Column 2	6	2047.862	341.3103	2302.381

Source of Variation	SS	df	ANOVA			
			MS	F	P-value	F crit
Between Groups	20.31121	1	20.31121	0.008585	0.928007	4.964603
Within Groups	23658.52	10	2365.852			
Total	23678.83	11				

F crit	<F calc
4.964603	0.008585
0.928007	P-value

urine (Barros *et al.*, 2003), so to treat and forbid of urolithiasis, is necessary to change and control the crystallization process using some substances in order to modification the products that affect on kidney stone formation (Veronika B and Khan SR., 2009). The medicinal plants that traditionally used are the best alternative substances for treatment of kidney stone. Insertion of the extract of *Paronychia argentea* in the crystallization process of calcium oxalate in the human urine has shown influence effect on the calcium oxalate crystal size and number. The extract has the ability to preventing urinary stone formation by inducing large number but smaller size calcium oxalate crystals which were easy to excretion from the kidney and prevent them from retention in the urinary tract.

Furthermore, the extract enhance the induce (COD) by dissolving of (COM) crystals. This property of *Paronychia argentea* extract lead to preventing kidney stone formation. Because the COD crystal less bounded to epithelial cell comper with COM crystal (Wesson *et al.*, 1998).

It is known that control urine sample contains mixture of COD and COM calcium oxalate crystals (Fig. 3). Incorporation of *Paronychia argentea* extract has shown a good effect by changing the morphology of crystals from COM form to COD form that change the ratio in the direction to get less COM that has high affinity for cell membrane damages the epithelial cells than COD and high activation effect on kidney stone formation (Yamaguchi *et al.*, 2005).

Paronychia argentea areal parts extract was significantly more effective in inhibiting the nucleation and aggregation of calcium oxalate monohydrate (COM) crystals than was cystone. Moreover, the extract induced more calcium oxalate dehydrate (COD) crystals, with a significant reduction in the number and size of COM crystals.

Microscopic results (Fig. 3) indicated that the crystals in the control oxalate had the hexagonal shape related to COM. When the different fractions were added, the COM crystals lost their crystalline nature and they were converted to octahedral shape, as indicated by the dispersed, lesser, very smaller octahedral shape COD particles compared with control in 400× magnification. By comparing different fractions of methanol extract of *Paronychia argentea* areal parts extract, it was

found that ethyl acetate fraction (Fig. 3) have the the highest ability to reduce the size and shape of calcium oxalate monohydrate crystals compared with other fractions and standard drug cystone under the same magnification power. This notion reflects the influence of the natural products in ethyl acetate fraction that inhibited the crystal growth of calcium oxalate monohydrate, which according to the phytochemical screening results might be attributed to saponins and polyphenolics such as flavonoids and tannins featuring potent antioxidant activities.

CONCLUSION

The present study demonstrated that extract of *Paronychia argentea* areal parts showed an ability to inhibit the calcium oxalate crystallization process in vitro and possess a high antioxidant activity. Beside that, the *Paronychia argentea* areal parts extract has the ability to reduce the crystal size and change the morphology of calcium oxalate monohydrate (COM) from hexagonal to tetrahydral a calcium oxalate dihydrate (COD) crystal. That might be attributed to its high polyphenolic contents including saponins mainly. However, further in vivo studied using animal models of urolithiasis is needed to evaluate the potential antiurolithic activity. Future work will be continued to characterization and isolation of the major active compounds from this plant.

ACKNOWLEDGMENTS

The authors are expressing their appreciations to all those who have assisted in the research, namely the staff of Pharmacy College, the Mutah' University, and researchers at the Medical Research Centre at College of Pharmacy, Mutah' University, Jordan.

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