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Inhibition and dissolution of calcium oxalate crystals and kidney stones by the extract of *Kalanchoe pinnata*

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Kalanchoe pinnata **leaves are used in South Asia as a natural kidney stone treatment.** ***In vitro*** **studies were conducted in supersaturated and artificial urine solutions to evaluate the antiurolithic properties of** ***K. pinnata*** **leaf extract on calcium oxalate monohydrate (COM) and surgically extracted kidney stones. Key organic acids present in the plant extract were also examined in artificial urine to investigate inhibition of kidney stone formation. Crystals harvested from inhibition and dissolution experiments were characterized by mass change, Fourier transform infrared (FTIR) spectroscopy, thermogravimetric analysis (TGA), scanning electron microscopy (SEM), redox titrations, and conductivity measurements.** ***K. pinnata*** **plant extract significantly inhibited crystal growth mechanisms depending on the extract concentration and supported dissolution. Crystal growth in supersaturated solutions and artificial urine was inhibited by 41 and 15 wt%, respectively, and COM and kidney stone mass decreased by 30 and 18 wt% after five extract washes. Active compounds identified** ***in K. pinnata*** **extract are malic, p-hydroxybenzoic, syringic, and caffeic acids, each demonstrated 2.70 to 4.65 wt% inhibition, which cumulatively accounts for the 15% inhibition observed in synthetic urine. This preliminary study demonstrates that the organic acid content of** ***K. pinnata*** **leaf extract is a crucial component for antiurolithic activity.**

Key words: Kidney stones, calcium oxalate monohydrate, *Kalonchoe pinnata* leaves, inhibition and dissolution effects, Ayurvedic.

INTRODUCTION

Kidney stone disease has shown a noticeable increase in prevalence over the last twenty years, and at present, it is reported to affect approximately 1 to 15% of the human population worldwide (Moftakhar et al., 2022). In the absence of preventive treatments, the reoccurrence rate of kidney stones increases with time after medical treatment and the chances of reoccurrence are approximately 20-45, 50, and 80% after one, five, and ten years, respectively (Jamal et al., 2023; Kavoussi et al., 2023). Kidney stone disease is more common in people,

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obesity, metabolic syndrome, and cardiovascular disease (Alelign and Petros, 2018; Ramaswamy and Shah, 2014; Wong et al., 2016). Kidney stones do not have a single definite cause, but several factors can increase the risk of stone formation. The risk of stone formation is much greater when the urine is highly saturated with kidney forming substances such as ions (e.g., Ca^{2+}, NH_4^+, $C_2O_4^{2-}$, CO_3^{2-}, PO_4^{3-}) and macromolecules (Kusmala and Riyanti, 2023; Mandal et al., 2016; Rimer et al., 2017), and so dehydration also increases the risk as the urine becomes more concentrated (Travers et al., 2023). Kidney stones are microcrystalline bio-mineralized solid aggregates found in the kidney, ureter, and bladder (Khan et al., 2016). Most human kidney stones contain calcium oxalate monohydrate (COM) as the main constituent which grows on a calcium phosphate nucleus (Xie et al., 2015). The formation of calcium stones is a complex process, and the detailed mechanisms of stone formation are still poorly understood. Crystal formation is known to proceed via three main steps: nucleation, crystal growth, and aggregation (Kachkoul et al., 2023; Zhang XZ et al., 2023). First, calcium oxalate precipitates from the urine solution when Ca^{2+} and $C_2O_4^{2-}$ ion concentrations exceed the super saturation levels to form a nucleus. The nucleus then acts as a substrate to induce more precipitation allowing for growth and subsequent formation of relatively large calcium oxalate crystals. Crystals can also aggregate together to form even larger stones. Stone size can vary from a few millimeters to several centimeters (Devbhuti et al., 2008; Huang et al., 2023). The next step in the biomineralization process is that the stones may attach to epithelial cells lining the renal tubules (Alelign and Petros, 2018; Basavaraj et al., 2007). Small stones may pass on their own causing little to no pain and without patients showing any symptoms (Johri et al., 2010; Tatliparmak et al., 2023), but large stones can block the flow of urine by occluding the urinary track causing severe pain and bleeding (Golzari et al., 2014).

Calcium stone formation is affected by molecular modifiers such as ions, molecules, and macromolecules (Alamani and Rimer, 2017; Chung et al., 2016; Jung et al., 2004; Phillips et al., 2015). Molecular modifiers act by promoting or inhibiting crystal growth and aggregation (De Yoreo et al., 2006; Li et al., 2022). Naturally occurring macromolecules such as certain proteins and organic molecules are known to act as inhibitors to alter the size, morphology, and mass of stones (Aihara et al., 2003; Grases et al., 2004; Thanasekaran et al., 2012). At present, very few therapeutic protocols are effective in dissolving calcium oxalate kidney stones found in humans (Gravina et al., 2005; Xie et al., 2015). Current therapies consist of a limited number of low efficacy dissolution agents and/or have associated risk factors (Ahmed and Al-Sayed, 2010; Gonzalez et al., 2012). As there are multiple mechanisms involved in stone formation and growth, to effectively inhibit growth and/or dissolve kidney stones, several active agents must be combined to act simultaneously on kidney stones. In this regard, medicinal plants have attracted much interest as they contain a wide variety of compounds with various therapeutic effects (Govender et al., 2023; Phien and Men, 2023).

Kalonchoe pinnata is a plant that has been used as a natural remedy to cure kidney stones in South Asia. The plant is an erect, succulent herb with simple opposite fleshy leaves, native to Madagascar, and is also found in West Indies, Asia, New Zealand, Australia, and Hawaii (Alok et al., 2013; Pattewar, 2012; Quazi Majaz et al., 2011). Multiple parts of the *K*. *pinnata* plant are rich in phytochemicals and exhibit medicinal value, including the leaf, root, and stem, and the plant has been used as an Ayurvedic medicine to treat infections, heal wounds, strengthen the immune system, and reduce fever (Dogra et al., 2022; Rajsekhar et al., 2016). The plant has antibacterial, antiviral, antiallergic, and analgesic properties and has been used to treat specific symptoms including vomiting, heartburn, diarrhea, earaches, diabetes, arthritis, hypertension, and upper respiratory infections (Ojewole, 2002; Okwu and Nnamdi, 2011). *K. pinnata* has also been used as a muscle relaxant and an insecticide (Kumar et al., 2023). The leaf juice is used to treat cholera and the leaves can be fire-roasted and applied to wounds (Coutinho et al., 2021; Fernandes et al., 2019). The aqueous extract of the leaves is given internally to treat kidney stones in certain parts of the world (Yadav et al., 2016). Shukla et al. demonstrated that *Bryophyllum pinnatum (synonym for K. pinnata)* is active for inhibiting and treating urolithiasis. According to the authors' findings, serum creatinine and blood urine levels were significantly improved while urine oxalate levels were reduced in rats with the administration of aqueous extract of *B. pinnatum* (Shukla et al., 2014). These investigations support *K. pinnata* leaf extract in treating kidney stones. For urinary problems, it is recommended to chew three leaves of *K. pinnata* daily, two times per day. It has been reported that paste composed of equal parts of *K. pinnata* and *Eclipta prostrata* leaves was effective in dissolving kidney stones when 1 g of the paste was given orally over several days (Yasir and Waqar, 2011). In another study, it has been revealed that one or two pills of 1 g of *K. pinnata* leaves mixed with the whole *E. prostrata* plant in equal proportion can dissolve kidney stones if taken twice a day orally for 20 days (Dinesh et al., 2013). A couple of studies have shown that *B. pinnatum (synonym for K. pinnata)* plant extract was effective at reducing the size of calcium oxalate monohydrate (COM) crystals and

supported the formation of calcium oxalate dehydrate (COD) crystals in rats (Surendra Patil et al., 2015). Excretion of COD crystals in the urine is much easier and does not cause damage to the epithelial lining of the urinary tract, unlike COM (Zhang et al., 2023; Zhao et al., 2014).

Prior studies have been conducted on small quantities of calcium oxalate crystals which do not allow for the validation of these results in the bulk formation of kidney stones involving crystal nucleation, crystal growth, aggregation, etc. Furthermore, previous studies have failed to fully characterize the nature of the crystals formed. The current study aims to investigate the antiurolithic properties of *K. pinnata* leaf extract in bulk crystallization paying special attention to the comprehensive characterization of calcium oxalate crystals observed in inhibition and dissolution experiments.

MATERIALS AND METHODS

Chemicals

Sodium oxalate ($Na_2C_2O_4$), sulfuric acid (H_2SO_4), potassium permanganate ($KMnO_4$), sodium citrate ($Na_3C_6H_5O_7$), magnesium sulphate ($MgSO_4$), and sodium sulphate (Na_2SO_4) were obtained from Merck Chemical Company. Sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), and potassium chloride (KCl) were obtained from BDH Chemical Company. Calcium chloride dehydrate ($CaCl_2.H_2O$) was supplied from Sigma Chemical Company and calcium chloride ($CaCl_2$) was purchased from Cancaster Chemical Company. GPR grade chemicals were used as received without further purification. Syringic, caffeic, *p*-hydroxybenzoic, citric, and malic acids (purity 99+%) were purchased from Sigma-Aldrich.

Plant material collection and extract preparation

The leaves of *K. pinnata* were collected in Matara, Sri Lanka from July to December 2016. The identification of the plant was completed with the help of the Department of Botany, University of Ruhuna, Sri Lanka, and a specimen voucher was deposited with voucher number KP21. Leaves were harvested from plants which were about one year old. An aqueous extract was prepared from the *K. pinnata* leaves. Fresh leaves (8 g, approximately 3 leaves) were cut into small pieces and then ground using a grinder. Deionized water (200 mL) was added, and the mixture was stirred for 4 h using a magnetic stirrer. The solution was then filtered, the filtrate collected, and water was evaporated using a rotary evaporator to obtain the residual solids. This procedure was repeated five times, and the solids were combined to obtain a total of 0.3022 ± 0.008 g. These solids were then dissolved in 200 mL of deionized water to obtain the *K. pinnata* extract used for all experiments in this study. The solution concentration was 1500 mg of millet extract per liter, and from this *K. pinnata* extract solution, volumes were measured out to prepare different *K. pinnata* extract dosages for the inhibition and dissolution experiments. Note that calcium and magnesium ion concentrations, total lipid content, and total acidity of the plant extract were also determined (Supplementary Information, Section I).

Preparation of supersaturated and artificial urine solutions and formation of calcium oxalate crystals in the presence *K. pinnata* extract or extract active organic acid compounds

To prepare the supersaturated solution, 10 mmol of $Na_2C_2O_4$ was dissolved in 80 mL of deionized water with magnetic stirring. Separately, 10 mmol of anhydrous $CaCl_2$ was dissolved in 100 mL of deionized water and added at a rate of 5 mL/min to the aforementioned $Na_2C_2O_4$ solution. After adjusting the pH value to 7.3 by adding a few drops of 0.01 M HCl, this solution was used as the supersaturated solution. Volumes of *K. pinnata* extract solution (20, 40, 60, 80 and 100 mL) corresponding to 30, 60, 90, 120 and 150 mg *K. pinnata* dosages were added to aliquots of the supersaturated solution and $CaC_2O_4.nH_2O$ crystal formation was monitored. Control experiments were carried out by adding the same volumes of deionized water (20, 40, 60, 80 and 100 mL) to the supersaturated solution and all other study parameters were identical.

The composition of the artificial urine includes 0.634 g NaCl (sodium chloride), 0.0869 g NaH_2PO_4 (sodium dihydrogen phosphate), 0.2606 g $Na_3C_6H_5O_7$ (sodium citrate), 0.0489 g $MgSO_4$ (magnesium sulfate), 0.258 g Na_2SO_4 (sodium sulfate), 0.4500 g KCl (potassium chloride), 0.0890 g $CaCl_2.2H_2O$ (calcium chloride dihydrate), and 0.1610 g NH_4Cl (ammonium chloride) dissolved in 200 mL deionized water (Faragalla and Gershoff, 1963). The pH of the solution was then adjusted to 7.3 by adding a few drops of 0.01 M HCl. Then, 4 mmol of $CaCl_2$ was dissolved in 50 mL deionized water and 4 mmol of $Na_2C_2O_4$ were dissolved in 40 mL deionized water. These two solutions were injected at a constant rate (5 mL/min) into a bottle containing the artificial urine at 37 ˚C. *K. pinnata* extract (20.00 mL, 30 mg *K. pinnata* dose) was added to the artificial urine solution and the temperature was maintained at 37 ˚C for 24 h (Maurice-Estepa et al., 2000).

After 24 h, solutions were filtered using a Hartley filter funnel (Whatman filter paper, GF/C 47 mm) and the formed crystals were collected and dried at 100˚C for several hours before measuring the mass. The aforementioned experiment was repeated using 30, 60, 90, 120 and 150 mg *K. pinnata* doses (40, 60, 80, and 100 mL of the *K. pinnata* extract solution). The same procedure was carried out for the control experiments using deionized water in place of the *K. pinnata* extract solution. With the same artificial urine conditions, additional experiments were conducted with the addition of major organic acids present in *K. pinnata* extract. To test the effect of these organic acids as inhibitors, syringic, caffeic, malic, *p*-hydroxy benzoic, and citric acids were added individually at a 5 mg dosage. After filtration and drying, the formed crystals were also characterized to investigate whether crystals were comprised $C_2H_2CaO_5$ (COM), $CaC_2O_4.2H_2O$ (COD), or $CaC_2O_4.3H_2O$ (COT) using FTIR spectroscopy, thermal gravimetric analysis (TGA), and powder X-ray diffraction (XRD). Crystals were titrated against a standard $KMnO_4$ solution to determine the $C_2O_4^{2-}$ content and the solution ion mobility was measured using a conductivity meter.

Examining the effect of *K. pinnata* on COM and kidney stone dissolution

Approximately 1 g of COM crystals were placed in a conical flask, then 200 mL of *K. pinnata* extract (containing 0.3022 g of water-soluble mass) was added, and the solution was kept in an

electronic shaker for 4 h. The solution was then filtered using a Hartley funnel. Undissolved crystals were dried overnight at 100˚C and then weighed. The dried crystals were washed successively by adding freshly prepared 200 mL aliquots of the extract, and then the crystals were washed with de-ionized water followed by 95% ethanol before drying. Control experiments followed the same procedure but were performed using deionized water. This experiment was also conducted with kidney stones collected surgically from human patients.

Characterization

Ion mobility of the solutions was monitored using a conductivity meter (WGCON 1103, Wagtech). Fourier transform infrared (FTIR) spectra of pure calcium oxalate and calcium oxalate samples recovered from *K. pinnata* and water (control) treated samples were collected using a Thermo Scientific Nicolet IS10 spectrometer. Thermal gravimetric analysis (TGA) was performed on a TAQ 600 TGA under stream of air. The metal ion concentration was determined using an iCE 3000 AA015121001 vl-30 atomic absorption spectrometer (flame type: air-C_2H_4; burner height: 11.0 mm; wavelength: 422.7 nm; band pass: 0.5 nm; nebulizer uptake: 4 s). Powder X-ray diffraction was used to confirm the crystal type using an Ultima IV instrument, and crystal shape was determined by scanning electron microscopy (SEM) using a Hitachi SU 6600 with 35.0k magnification.

RESULTS AND DISCUSSION

Investigating the inhibition effect *K. pinnata* in supersaturated and artificial urine solutions

The conductivity of artificial urine solutions, with and without the addition of *K. pinnata* extract, was measured to examine for differences in ionic species mobility. Figure 1 shows the change in conductivity as a function of time for both solutions. In the control experiment, the conductivity declined rapidly during the first 50 min. Calcium oxalate (CaOx) precipitates quickly since the solution is supersaturated. Only a small 1-2 mS fluctuation in conductivity is observed between 5 and 150 min. A precipitation dissolution equilibrium was reached after ca. 150 min and the conductivity remained constant through the remainder of the experiment (> 400 min). In the presence of *K. pinnata* extract (90 mg dosage), there was a small drop in the conductivity, ~4 mS, initially, and after ca. 150 min the conductivity increased to a value ~4 mS above the starting value. These data show that in the presence of *K. pinnata* extract, the precipitation of CaOx from the supersaturated solution is more sluggish than in the control sample and that there is less precipitation resulting in higher concentrations of dissolved CaOx. With time, the conductivity of the *K. pinnata* treated solution increases, corresponding to precipitated CaOx crystals redissolving. The conductivity is higher than the initial conductivity at times >225 min indicating that not only has CaO_X not precipitated but perhaps a higher CaOx concentration is possible in the presence of *K. pinnata* extract. The conductivity measurements demonstrate the ionic concentrations are higher in the extract-treated sample than the water-treated (control) sample. The conductivity is directly related to the concentration of ionic species in the solution. These observations support the idea that the crystal deposition occurs to a smaller (and perhaps negligible) extent in the plant extract treated solution.

The conductivity and UV-Vis results can be further examined by measuring the mass of calcium oxalate crystals formed in the presence and absence of *K. pinnata* extract (Figure 2). Precipitation of CaO_X dropped by ~38% with the addition of 20 mL extract (30 mg dosage) in comparison to the control, deionized water. With the addition of subsequent extract dosages, from 60 to 120 mg, the crystal nucleation, aggregation, and growth continued to decrease stepwise by approximately ~3% for each additional 20 mL extract. The percentage of CaO_X crystals formed after the addition of 100 mL of *K. pinnata* extract (120 mg dose) was 50.2%. Crystal formation decreased steadily in the control samples with increased deionized water volumes, although not to the degree observed in the extract treated samples (~10% decrease with 100 mL added deionized water). The decrease in crystal formation for the control samples can be attributed to changes in dilution concentration. The crystal formation mass results show that the crystal deposition process is significantly inhibited by chemicals present in the *K. pinnata* extract.

Building on the findings in supersaturated solutions, experiments were designed to investigate the inhibition effect of *K. pinnata* extract in synthetic urine solutions. Figure 3 displays the percentage of crystals formed as a function of treatment solution volume and shows that *K. pinnata* extract is effective in preventing the formation of calcium oxalate crystals in synthetic urine. The inhibition effect of *K. pinnata* extract is consistent. At all added volumes, the *K. pinnata* treated samples have ~14% lower crystal formation than the control sample at the same added volume. The lower degree of inhibition observed for the artificial urine vs. the supersaturated solution may be due to the more complex composition of the synthetic urine solution. The harvested crystals were further characterized by FTIR, XRD, TGA, and redox titrations to investigate any differences in the type and structure of CaO_X crystals developed.

Chemical composition of the crystals was examined using FTIR spectroscopy. The spectra of the crystals harvested in the presence and absence of *K. pinnata* extract (60 mL, 90 mg dose) are shown in Figure 4. The FTIR spectrum for a neat sample of COM is shown in Figure 4a, and spectra for crystals harvested from

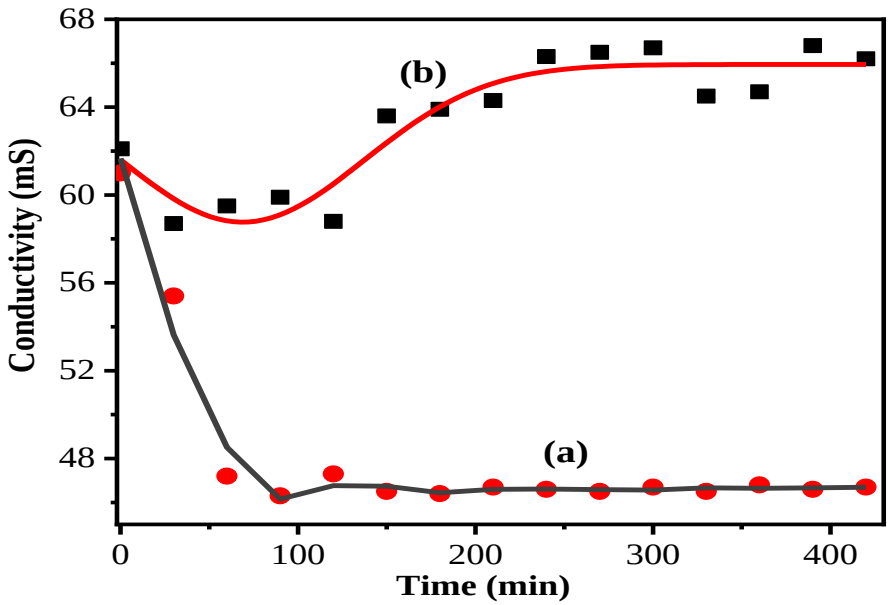

Figure 1. Conductivity over time for artificial urine solutions: (a) control; (b) 90 mg *K. pinnata* extract. Rapid drop in control conductivity is due to rapid CaOx precipitation from the supersaturated solution, which is mitigated in the presence of *K. pinnata* extract.
Source: Authors

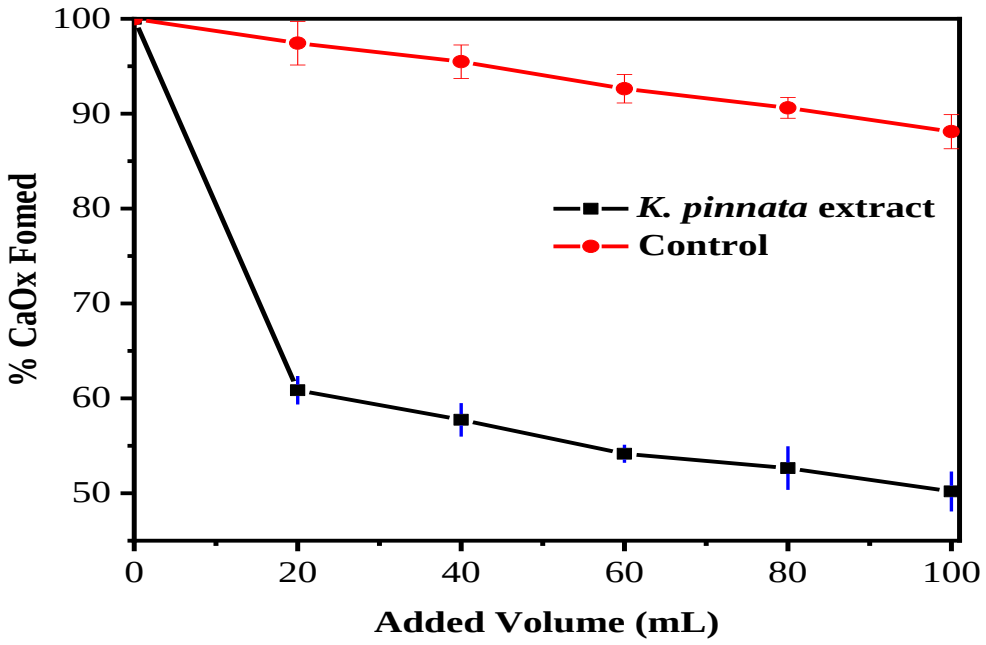

Figure 2. Percentage CaO_X crystals formed in supersaturated solution as a function of added volumes of deionized water (control) and *K. pinnata* extract (20 mL=30 mg; 40 mL=60 mg; 60 mL=90 mg; 80 mL=120 mg; 100 mL=150 mg). While the effect of dilution was observed in both samples (~10% with 100 mL volume addition), the effect of *K. pinnata* extract is significant resulting in an additional ~40% reduction in CaO_X.
Source: Authors

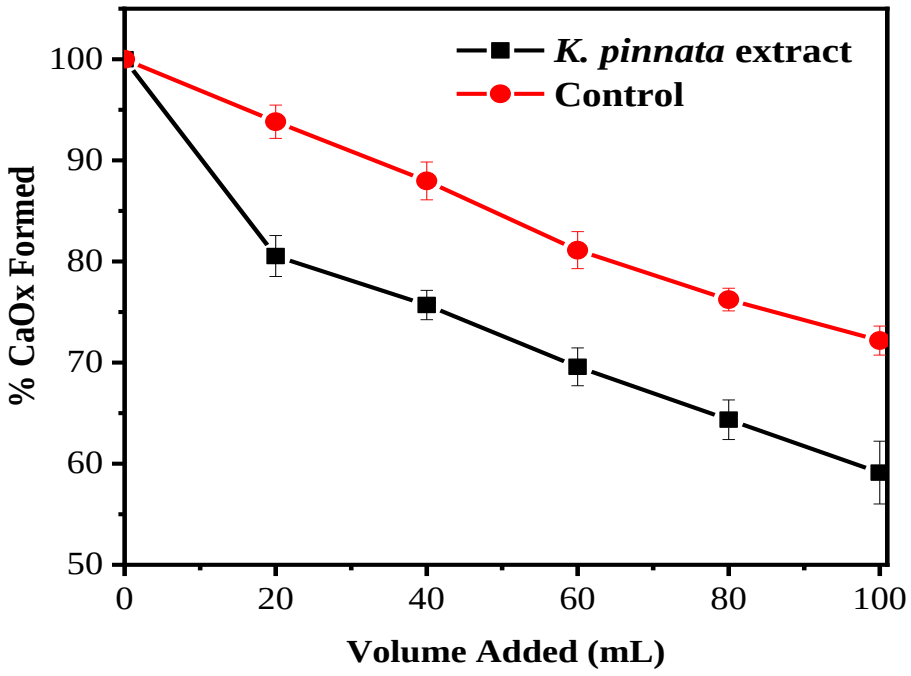

Figure 3. Percentage of crystals formed in artificial urine after the addition of deionized water (control) and *K. pinnata* extract (20 mL=30 mg; 40 mL=60 mg; 60 mL=90 mg; 80 mL=120 mg; 100 mL=150 mg). The effect of dilution is more pronounced in the artificial urine (~25% for 100 mL volume) than the supersaturated solution. *K. pinnata* extract provides ~25% additional reduction in crystal formation.
Source: Authors

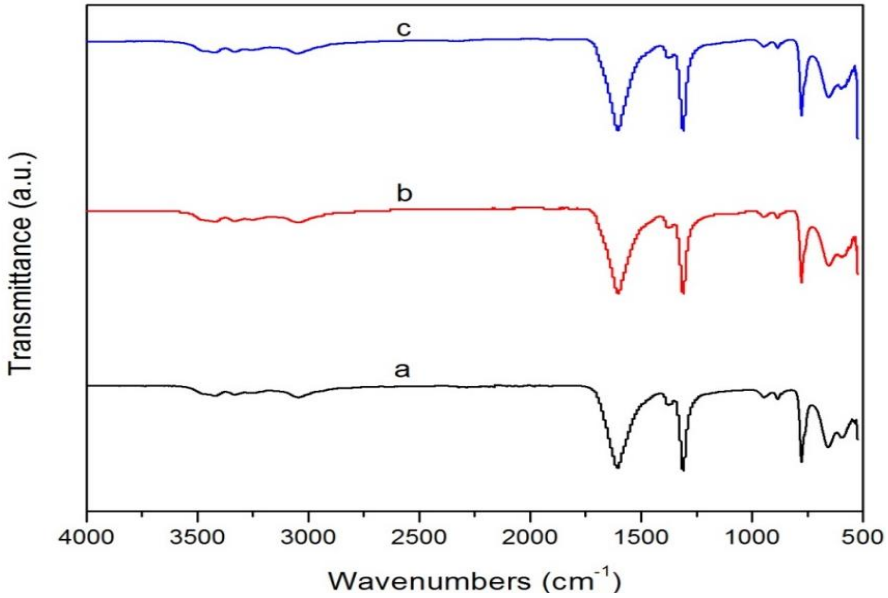

Figure 4. FTIR spectra of (a) neat COM and calcium oxalate samples formed in artificial urine (b) without and (c) with *K. pinnata* extract (60 mg extract in 40 mL water).
Source: Authors

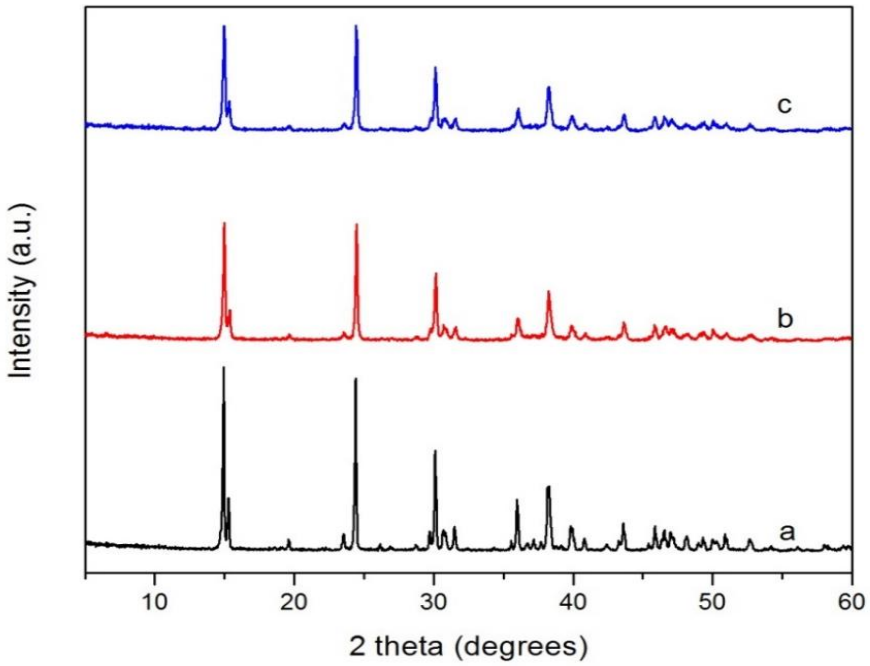

Figure 5. XRD patterns of (a) neat COM and crystals harvested from the artificial urine samples using (b) water (control) and (c) *K. pinnata* extract (90 mg extract in 60 mL water).
Source: Authors

artificial urine in the absence and presence of *K. pinnata* extract (60 mg extract in 40 mL water) are shown in Figure 4b and c. The peaks identified in all three samples are identical, and indicates that the crystals harvested from the artificial urine are composed of COM. The spectra show strong absorption bands at 1314 and 1604 cm^{-1} that can be attributed to the symmetric and asymmetric stretching modes, respectively, of carbonyl group (C=O) in oxalate (Asyana et al., 2016). The strong carbonyl peaks, along with the less intense peaks, observed in the IR spectrum of neat COM sample (Figure 4a) reveals that the spectral signature of the calcium oxalate samples formed in artificial urine are identical to those of COM. As determined by using FTIR spectroscopy, the chemical composition of samples collected from *K. pinnata* treated and control artificial urine solutions is identical to one another and to COM. The crystal structure of these samples was then examined using XRD. Powder XRD data was collected for neat COM and calcium oxalate samples harvested from artificial urine in the presence and absence of *K.* pinnata extract and is displayed in Figure 5. COM is a crystalline substance that shows intense reflections at 2θ values of 14.92, 24.38, 30.12, 35.96, and 38.28°. The *K.* pinnata treated sample showed several intense peaks having 2θ values of 15.00, 24.48, 30.20, 36.06, and 38.26°. These peaks correspond to the miller indices of (-101), (020), (-202), (301), and (130) for COM (Kesavan et al., 2012). While there are slight differences in the 2θ values for neat COM and the *K. pinnata* treated sample, these cannot be attributed to the presence of COD since reflections for COD occur at 2θ values of 14.32, 20.07, 22.72, 32.23, and 40.17°. Comparing the XRD reflections for COM and *K. pinnata* treated samples confirms that the harvested crystals are purely COM, which is thermodynamically more stable than COD. It should be noted that the minor differences in the XRD data could indicate slight differences between the crystal structure of COM and crystals harvested in the presence of *K. pinnata* extract. To examine possible structural differences, scanning electron microscopy (SEM) was used to image both COM and crystals harvested in the presence of *K. pinnata* extracts.

SEM images for neat COM and crystals harvested in the presence and absence of *K. pinnata* extracts are presented in Figure 6a to e. Neat COM crystals are relatively large, showing crystal lengths larger than 5 µm. In the presence of *K. pinnata* extract, the crystals formed are smaller in size. For the 40 and 60 mL *K. pinnata* treated samples (60 and 90 mg doses, respectively), 1-2

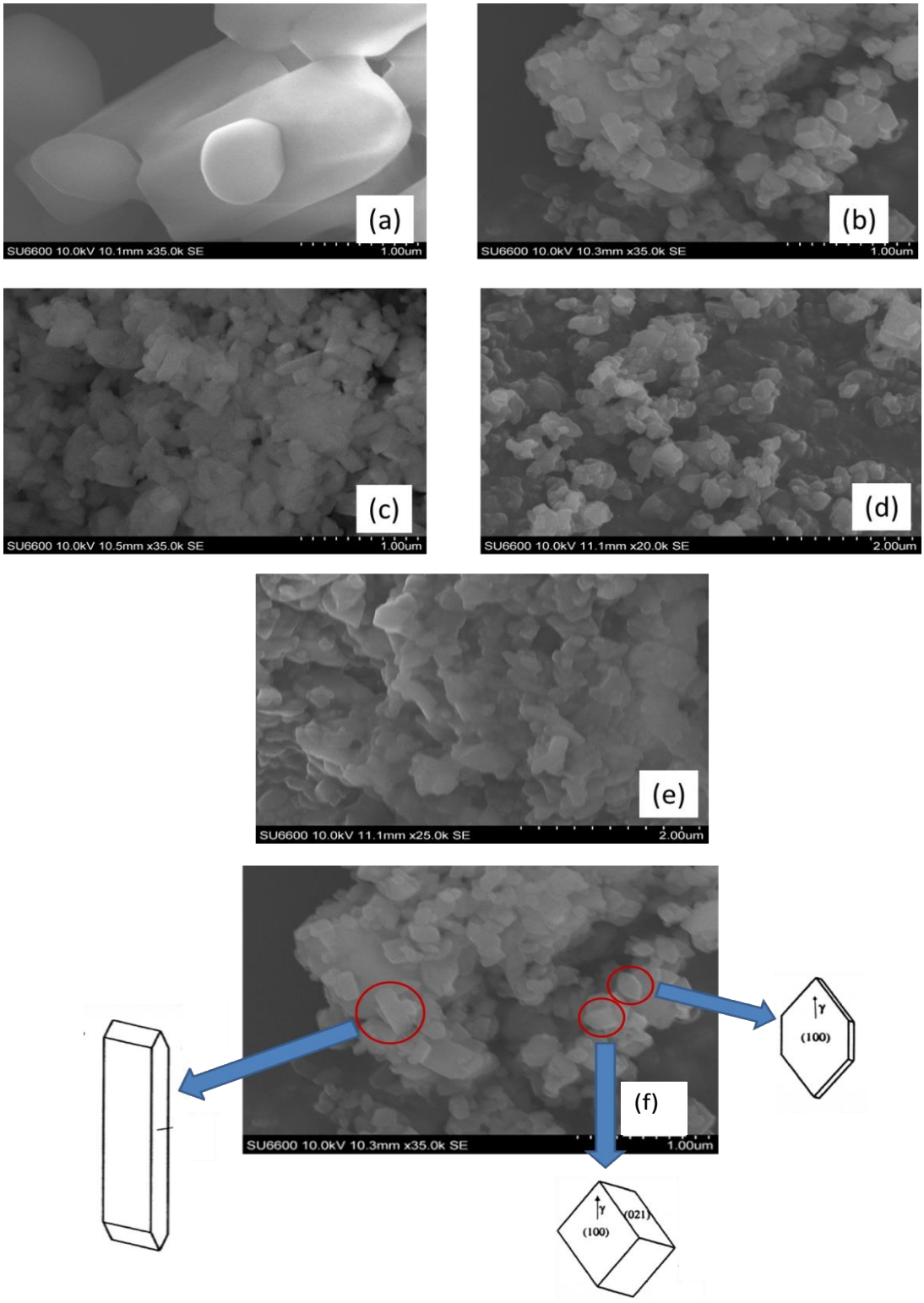

Figure 6. SEM images for (a) neat COM and crystals harvested from extract-treated samples: (b) 40 mL, (c) 60 mL, (d) 80 mL and (e) 100 mL. (f) Multiple crystal morphologies have been observed and identified in the SEM image from (b). The scale bar changes.
Source: Authors

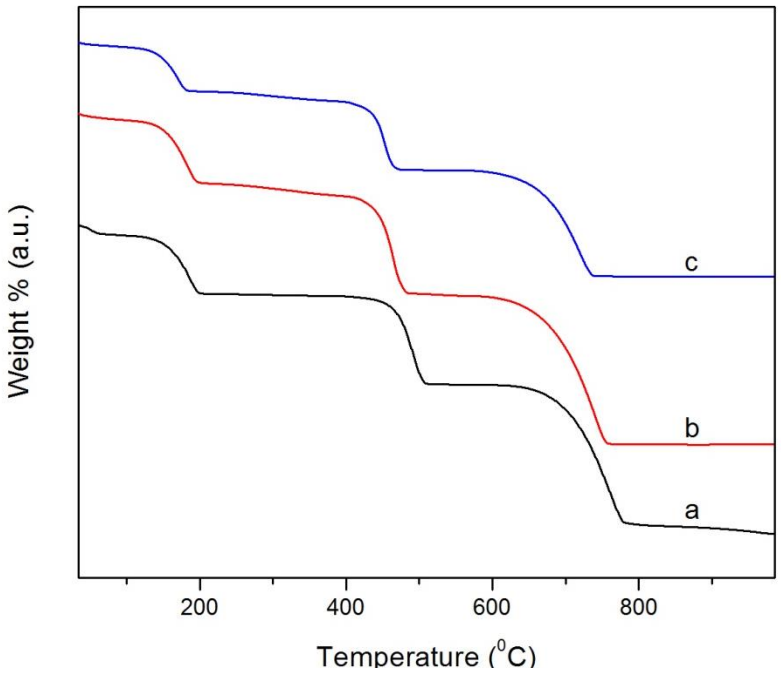

Figure 7. TGA curves of (a) neat COM and calcium oxalate crystals harvested from artificial urine (b) without and (c) with *K. pinnata* extract added.
Source: Authors

µm small particles are present along with larger 3-5 µm particles visible underneath the small particles. In crystal samples obtained from 80 mL and 100 mL *K. pinnata* extract solutions (120 and 150 mg dosages), there is a lower density of the 1-2 µm particles. Differences between the crystal sizes in these samples may be due to initially deposited small crystallites being re-dissolved when the dosage of the *K. pinnata* extract is higher. The smaller crystallites may also result from poor aggregation or growth mechanisms for the COM crystals when *K. pinnata* is present.

In addition to the crystal size variation observed with SEM, several morphological features differ between COM and crystals formed in the presence of the plant extract. In Figure 6f, distinct crystal morphologies are visible (denoted with red circles), including pinacoid with cubical shape and crystal growth occurring on (021) plane, rhomboid geometry with (010) pinacoid, and monoclinic styloid (Bondada and Keller, 2012; De Bellis et al., 2019; Hartl et al., 2007), which implies crystal nucleation, growth, and aggregation occur via three different mechanisms. It is likely that the molecular modifiers present in the plant extract affect crystal growth differently. Terraces and steps have been identified for the crystals, and molecular modifiers such as carboxylic acids, proteins, and polysaccharides, may strongly interact with the terrace to inhibit the crystal growth over the terrace surface (Farmanesh, 2013). Small organic acids bind to the crystal surface to create a localized strain, and as a result, the crystal can collapse and become redissolved in the solution. As shown in Figure 6e, with the highest extract dosage (100 mL) almost all the COM are rhomboid pinacoid type (Hartl et al., 2007). At the highest concentrations, outer sharp edges are not prominent due to the dissolution of the *K. pinnata* plant extract. To further examine similarities and differences in structure and composition, the thermal decomposition of these samples was examined. The thermal decomposition of neat COM and crystals collected from the artificial urine testing was examined using TGA (Figure 7). The decomposition mechanism of calcium oxalate crystals involves these three steps (Frost and Weier, 2004):

$$CaC_2O_4.H_2O \longrightarrow CaC_2O_4 + H_2O$$
$$CaC_2O_4 \longrightarrow CaCO_3 + CO$$
$$CaCO_3 \longrightarrow CaO + CO_2$$

Theoretically predicted weight changes for these three steps are 12.33, 19.18, and 30.14%, respectively. TGA data was collected for neat COM and crystals collected

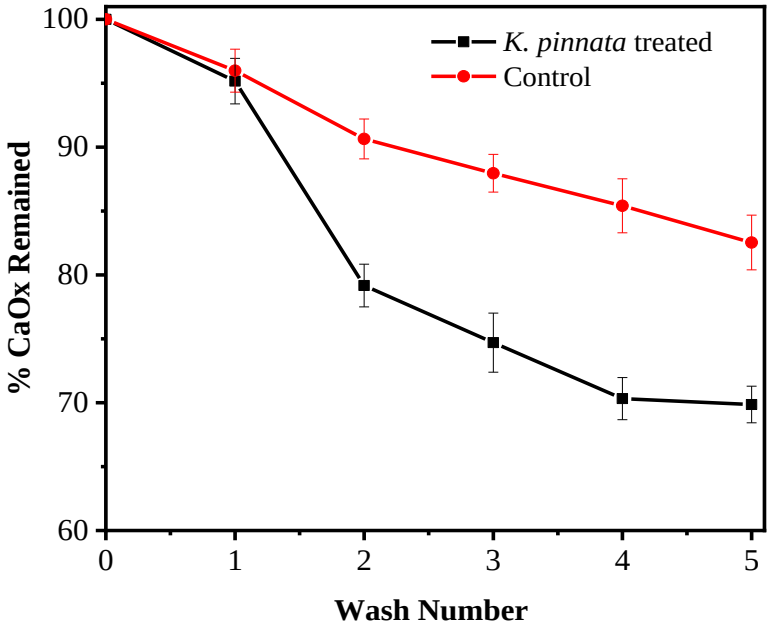

Figure 8. Mass percent of $Ca_2C_2O_4.H_2O$ remaining after successive washings with water (control) and *K. pinnata* extract solution.
Source: Authors

from the control and the *K. pinnata*-treated artificial urine. For COM, the three weight reductions were recorded as 12.46, 19.32, and 30.41%, respectively. *K. pinnata* treated samples showed weight losses of 13.85, 20.80, and 29.11%, and for the control these values were 14.47, 19.85, and 28.75%, respectively. The first weight change was slightly higher than theoretical in all samples, and this is expected generally due to the presence of small amounts of absorbed moisture. The second and third weight changes for all three samples closely agree with the theoretically predicted values, indicating that the harvested crystals are mainly composed of COM.

The oxalate content of the crystals obtained from select supersaturated and synthetic urine solutions was analyzed using oxidation reduction titration with a 0.002 M $KMNO_4$ solution. The samples examined with titration were the 90 mg *K. pinnata* treated sample, its control, and neat COM. The redox reactions that occur in the titration process are as follows:

$$C_2O_4^{2-}{}_{(aq)} \rightleftharpoons 2CO_{2(g)} + 2e$$

$$MnO_4^{-}{}_{(aq)} + 8H^+ + 5e \rightleftharpoons Mn^{2+}{}_{(aq)} + 4H_2O$$

$$5C_2O_4^{2-}{}_{(aq)} + 2MnO_4^{-}{}_{(aq)} + 16H^+ \rightleftharpoons 10CO_{2(g)} + 10Mn^{2+}{}_{(aq)} + 8H_2O_{(l)}$$

Stoichiometric ratio: $C_2O_4^{2-} : MnO_4^{-} = 5:2$

Titration results revealed that the oxalate content of the harvested crystals obtained from supersaturated solutions averages 60.42 ± 0.068%. For crystals harvested from the artificial urine samples, the oxalate content was measured to be 60.81 ± 0.36%. This slight difference between these two averages is not statistically significant, showing that the oxalate content of the formed calcium oxalate crystals from both solution types is identical. Calcium oxalate can be present as COM, COD, and COT, and the oxalate content for these three forms are 60.02, 53.62, and 48.32%, respectively. The mean oxalate percentages of the calcium oxalate obtained with and without *K. pinnata* extract are very close to the oxalate percentage in COM. Therefore, the titration results corroborate the prior analyses, showing that the harvested crystals consist of the more thermodynamically stable form, COM.

Dissolution effect of *K. pinnata* extracts on kidney stones

In dissolution experiments, initially 1 g of COM was successively washed five times with 200 mL *K. pinnata* extract containing 300 mg of water-soluble millet. A control experiment was carried out using 200 mL portions of deionized water. The mass remaining after each wash was measured and reported as a percentile (Figure 8).

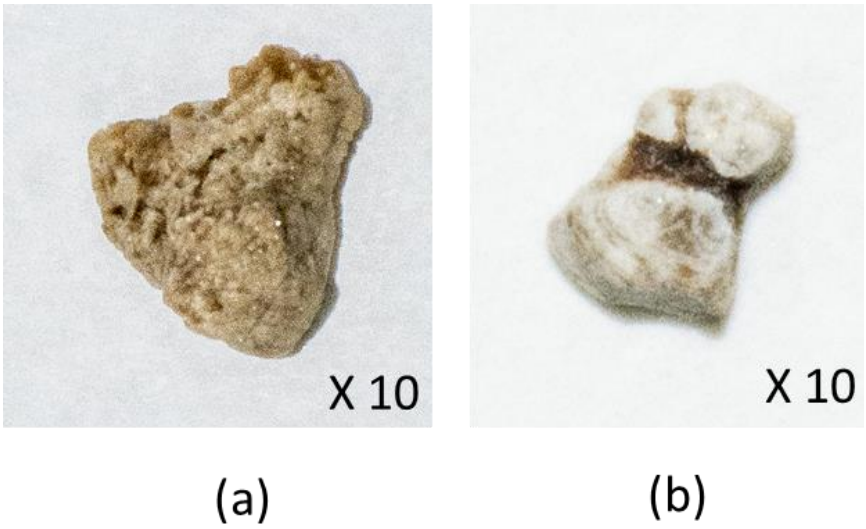

Figure 9. Images of a kidney stone (a) before and (b) treatment with *K. pinnata* extract.
Source: Authors

After the first wash, not much difference between the COM mass was observed. However, with the second wash, there was a significant dissolution effect from the *K. pinnata* solution. After five successive washes, ~30% of the crystal mass had been dissolved in the plant extract samples in comparison to ~17% for the control. These COM dissolution data were further confirmed by conductivity measurements (Supplementary Information, Section II Figure S-1). This observation provides additional evidence for the efficacy of the plant extract in dissolving COM, the most problematic form of calcium oxalate in the urinary tract. Therefore, the dissolution experiments were further extended to use actual kidney stones collected from a human patient.

The kidney stone showed 18% weight loss after five washings. The morphological changes of the kidney stones before and after five washings are as shown in Figure 9a and b. The outer surface of the kidney stone was washed away with the *K. pinnata* leaf extract; however, attempts to wash the actual kidney stones with deionized water (control) showed negligible weight loss.

Mechanisms of *K. pinnata* growth inhibition and dissolution on calcium oxalate crystals

To prevent large crystal formation in supersaturated urine in humans, the plant extract should contain active agents in sufficient quantities to inhibit crystal growth, aggregation, and crystal retention. On the other hand, to use the plant extract as a kidney stone dissolution therapeutic active agent must selectively extract Ca^{2+} and $C_2O_4^{2-}$ ions from calcium oxalate kidney stones. To investigate the growth inhibition and dissolution effects of *K. pinnata* plant extract, the chemical composition of the plant extract was evaluated using the available literature and characterization of the extract (Supplementary Information). *K. pinnata* leaf extract is a complex mixture and contains alkaloids, triterpenes, glycosides, flavonoids, steroids, bufadienolides, lipids, organic acids, phenols, tannins, free amino acids, saponins, and terpenoids (Fernandes et al., 2019; Rahman et al., 2019; Steyn and Van Heerden, 1998; Yamagishi et al., 1989). Many organic acids are contained in the leaf extract including ferulic, p-coumaric, isocitric, syringic, caffeic, malic, p-hydroxybenzoic, lactic, succinic, and aconitic acids (Faboro et al., 2016; Harlalka et al., 2007). The carboxylic acids present in the *K. pinnata* extract reduce the crystal growing rate by binding with Ca^{2+} ions as chelating agents. The saponins found in *K. pinnata* extract display crystallization inhibition properties by breaking up mucoprotein suspensions (Bhalodia et al., 2008), and the proteins present may display inhibition effects by attaching to the surface of the calcium oxalate crystals and acting as a barrier for the attachment of other calcium oxalate particles, thereby preventing aggregation and crystal growth. However, while saponins and proteins may also play a role in calcium oxalate crystal growth inhibition and dissolution, the organic acids present in the extract are water soluble compounds and therefore completely extracted to the aqueous phase. The amount of water extractable organic acids is significant,

Table 1. Summary of the inhibition effects on calcium oxalate crystals from the addition of select organic acids present in *K. pinnata* leaf extract.

Organic acid additive†	Major IR peaks (cm^{-1})	Major XRD reflections (2θ)	Crystal type(s)	Inhibition (%)
Malic acid	1604, 1314, 778	14.88, 24.40, 30.14	COM, S*, R*	2.70
p-hydroxybenzoic acid	1605, 1316, 779	14.84, 24.38, 30.18	COM, S*, R*	4.22
Syringic acid	1604, 1315, 778	15.02, 24.32, 30.12	COM, R*	4.65
Caffeic acid	1605, 1314, 779	14.88, 24.36, 30.08	COM, R*	3.35
Citric acid**	1603, 1314, 778	14.82, 24.48, 30.02	COM, R*	15.17
Control***	-	-	-	0.00

†Dosage of each acid = 5 mg/20 mL; S* = styloid; R*= rhomboid; ** = positive control; *** = negative control using 20 mL DI water
Source: Authors

as the titratable acidity was measured to be 4×10^{-4} mol dm^{-3}. Based on the acid content in plant extract, the inhibition effects of some select organic acids present in the plant extract were further investigated. Syringic, caffeic, *p*-hydroxybenzoic, and malic acids were each added separately to synthetic urine solutions to investigate their inhibition effect on kidney stones. (Chemical structures and pK_a values for the organic acids are provided in Supplementary Information, Section III. Figure S-2) Additionally, since citric acid has been used as an irrigating solution to treat kidney stones, it was also included in the current study as a positive control to better understand the performance of other plant extract acids. Deionized water was used as a negative control. The inhibition effects for each organic acid are summarized in Table 1.

Citric acid was the most active organic acid investigated. The total inhibition effect of four organic acids assessed in the current study was 14.92% which closely resembles the net inhibition effect of the plant extract observed in synthetic urine (~13-15%). Organic acids reduce the CaO_X crystal growth by binding to the growing crystal surface and creating a localized lattice strain. Due to this localized strain, the crystal collapses and CaO_X re-dissolves in the supersaturated solution preventing the precipitation of CaO_X. Small, anionic organic acid molecules can penetrate the cell-crystal interface resulting in a reduction of the attractive forces between the epithelial cells and crystal. Organic acids act as chelating agents to extract calcium ions or to change the surface charge by neutralization to detach CaO_X kidney stones from the epithelial cells.

This study demonstrated that in the presence of *K. pinnata* leaf extract, COM crystals form instead of the physiologically damaging COD crystals. Based on the results of this study, it seems that the plant extract contains more than one active agent to inhibit crystal growth and aggregation. Data from XRD, FTIR spectroscopy, TGA and titrations all clearly confirm the type of CaO_X found in the *K. pinnata* leaf extract inhibition and dissolution experiments are mainly the thermodynamically stable COM. The plant extract is shown to have the potential to treat kidney stones, as the organic acids and water-soluble compounds (in the aqueous extract) are readily absorbed by the body and transported into the urine and can act to dissolve kidney stones (Keyfi et al., 2017; Kishida and Matsumoto, 2019). Phytochemical constituents, present in ethanol and water extraction of the *K. pinnata* leaf are displayed in Supplementary Information, Table S-1. TLC experimental results for the ethanolic crude extract of *K. pinnata* leaf are shown in Section IV Figure S-3.

Conclusions

This current study provides solid evidence for the antiurolithic properties of *K. pinnata* leaf extract in CaO_X bulk crystallization. This plant extract shows a significant inhibition effect and reduction in CaO_X precipitation, even at supersaturated concentrations. Native urine concentrations are generally lower, and the *K. pinnata* leaf extract is even more effective under these conditions. The dissolution effect was studied on human kidney stones with a ~18% mass reduction after washing five times with the dilute plant extract. In addition, the outer surface of the kidney stones was dissolved by the plant extract solution allowing for the potential for positive outcomes related to the detachment of stones from kidney epithelial cells. Dissolution reduces the size of the kidney stones which makes them more likely to be passed with the urine. String evidence showed that the antiurolithic properties of *K. pinnata* leaf extract result from organic acids present in the plant extract.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY MATERIALS

Characterization of the chemical composition of *K. pinnata* extract is presented.

Section I

The chemical composition of the *K. pinnata* extract was examined to understand the mechanism of inhibition and dissolution on calcium oxalate kidney stone formation. Calcium and magnesium concentrations were determined by carrying out ethylenediaminetetraacetic acid (EDTA) titrations with *K. pinnata* extract. Total lipid content was 0.1925% and the total acidity of *K. pinnata* extract was 4×10^{-4} mol dm^{-3}.

Section II

The weight percentage of crystals remaining was lower in the *K. pinnata* treated sample than the water treated sample. Conductance measurements collected over treatment time for samples treated with *K. pinnata* extract and deionized water (control) are as shown in Figure S-1.

Section III

K. pinnata extract is a complex solution. Some of the water-soluble compounds present in the extract are organic acids. The chemical structures and pK_a values for those acids are as shown in Figure S-2.

Section IV

Thin layer chromatographic (TLC) experiments were conducted on the *K. pinnata* leaf ethanolic extract. TLC measurements were attempted with different ratios of the following solvent pairs: hexane: methanol, hexane: dichloromethane, acetone: methanol, chloroform: methanol, toluene: chloroform.

None of the aforementioned solvent pairs showed satisfactory separation of the TLC spots or bands. The best solvent system identified for the *K. pinnata* leaf ethanolic extract was 3:2 ratio of hexane:ethyl acetate. TLC results for different ratios of the hexane:ethyl acetate solvent system are as shown in Figure S-1 for the ethanolic crude extract of *K. pinnata* leaf.

Table S-1. Phytochemical constituents present in ethanol and water extraction of the *K. pinnata* leaf.

Phytochemical constituents	Ethanol	Aqueous
Alkaloids	+++	+
Cardiac glycosides	++	-
Flavonoids	+++	+
Saponins	+	+
Steroids	+	-
Tannins	++	-
Terpenoids	+	-
Carbohydrates	+++	+
Protein and amino acids	+++	+

Key: +++ high; ++ moderate; + low; - absent

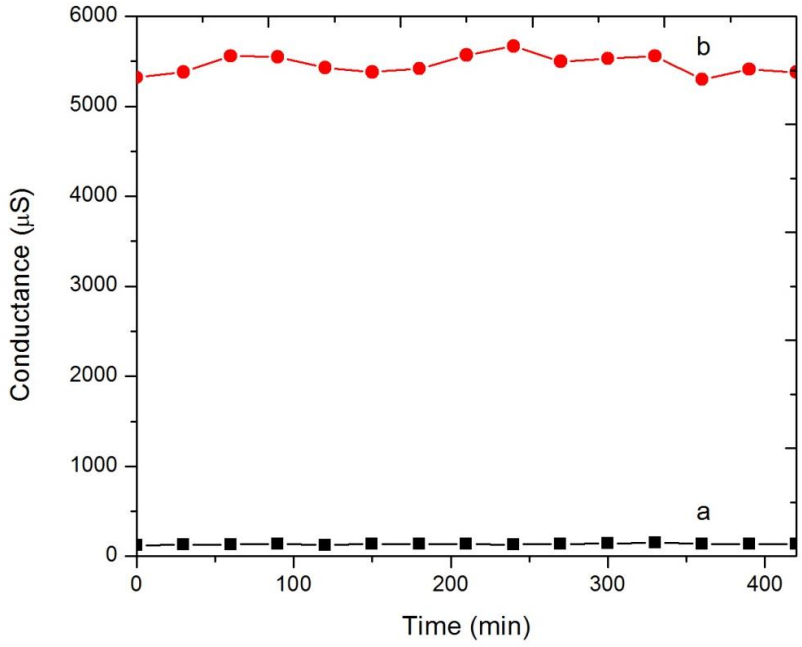

Figure S-1. Conductance as a function of treatment time with (a) water (control) and (b) *K. pinnata* extract.

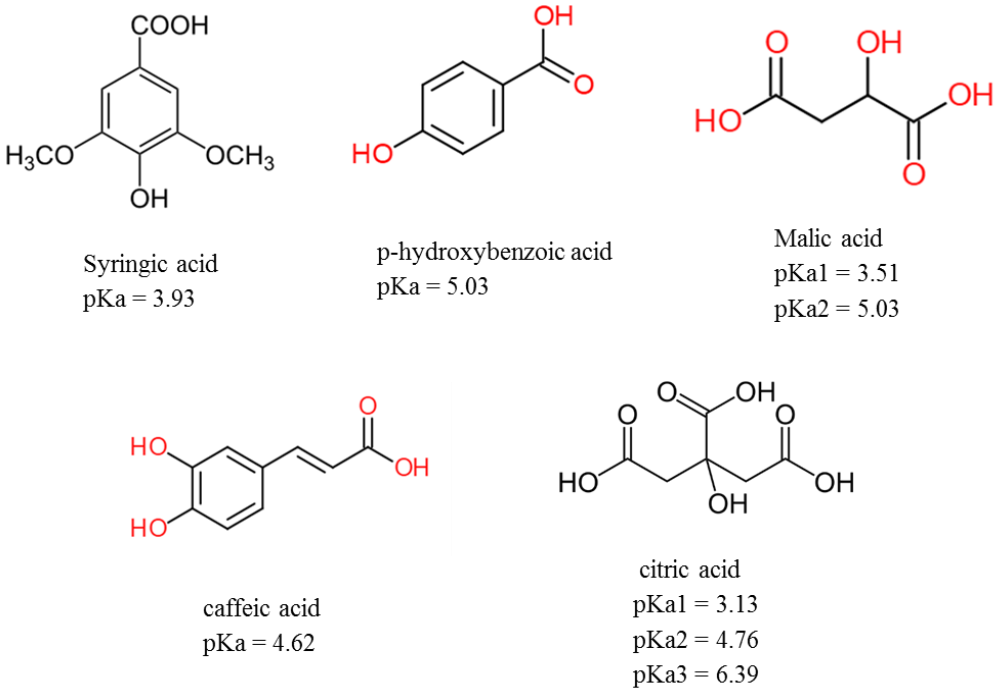

Figure S-2. Chemical structures of organic acids present in *K. pinnata* extract, plus citric acid (positive control).

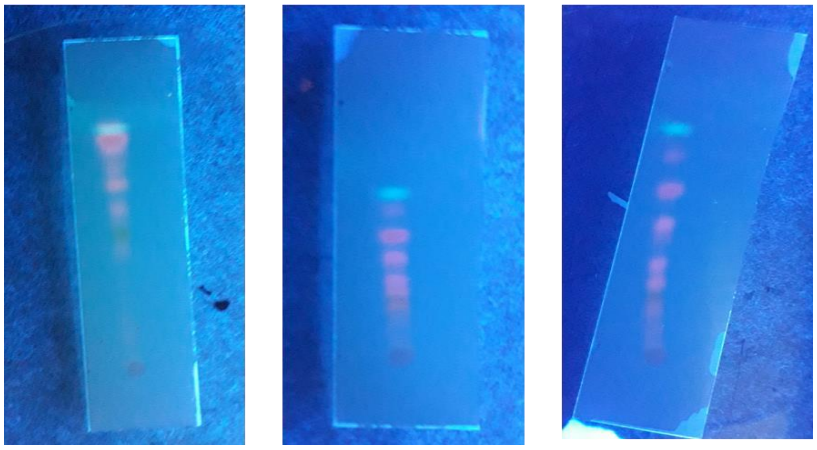

Figure S-3. Different ratios of hexane:ethyl acetate as the solvent system were evaluated for TLC of *K. pinnata* leaf ethanolic extract with the 3:2 ratio of hexane:ethyl acetate (far right image) showing the largest distance between the components and satisfactory separation.