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Inhibitory Effect of Alternanthera Sessilis, Cucurbita Maxima and Aerva Lanata on Whewellite Urinary Crystals

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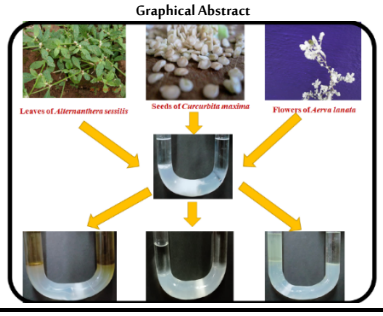
ABSTRACT

Phosphate stones, such as struvite (magnesium ammonium phosphate hexahydrate), brushite (calcium hydrogen phosphate dihydrate), and oxalates like whewellite (calcium oxalate monohydrate) are different kinds of urinary stones. Such kidney stones can be treated with herbs for natural healing and side effects-free results. These urinary crystals are traditionally created in the lab using the gel growth process and a silica gel medium at a specified pH. The inhibitory effects can be analysed by using the extracts of herbal plants. In the present study, ethanol extracts of leaves of Alternanthera sessilis, seeds of Cucurbita maxima and flowers of Aerva lanata are investigated for their inhibitory activity against the whewellite urinary crystals. They are characterized using Fourier transform infrared spectrum and their crystalline nature is examined through powder X-ray diffraction. Asymmetric and symmetric stretching unit of C=O in oxalate appears at 1620 cm-1 and 1400, 1310 cm-1 respectively. The monoclinic crystal system with a space group of P21/n is confirmed from the powder X-ray diffraction studies. The results indicated that among the three ethanol extracts, leaves of Alternanthera sessilis, seeds of Cucurbita maxima and flowers of Aerva lanata- the flowers of Aerva lanata exhibited inhibition towards whewellite crystals.

Graphical Abstract



KEYWORDS
Extract, flowers, herbs, oxalates, pH, seeds

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1. Introduction

The terms 'urinary stones', 'urinary lithiasis' and 'urolithiasis' refer to calculi formed anywhere within the urinary tract, from the renal tubules to and bladder. Additionally, the terms "kidney stones," "renal stones," and "nephrolithiasis" explain why people in contemporary affluent nations experience stones that largely originate in the kidneys (Coe *et al.*, 2019). Urinary stones can vary greatly in size, shape, colour, composition, and texture. The largest one known weighed 6.3 kg, which is virtually a "stone" in weight; the tiniest ones are crystals or crystalline aggregates around the size of a pinhead (Amara-Rekkab, 2023; Arthure, 1953). One to several hundred stones can be created at once, but single stones are considerably more typical than multiple ones. Polycrystalline aggregates have been used to describe urinary stones.

A genetic predisposition, metabolic disorders like diabetes, myeloproliferative disorders like leukaemia or hypocalcaemia, an unbalanced diet, inadequate hydration of the body, and bacterial infections like *Escherichia coli*, *Klebsiella*, *Staphylococcus*, or *Mycoplasma* can all contribute to stone formation. Men are far more likely to develop urinary stones due to their longer urethra compared to women. In addition, patients who finished all their treatment and removed all their existing stones have a higher risk of having urinary stones reappear. Calcium, oxalate, phosphate, magnesium and uric acid are all components of kidney stones, and the crystallisation process that results in urinary calculi comprises crystal nucleation, growth and agglomeration. Both homogeneous and heterogeneous stone formations are possible (Aggarwal *et al.*, 2013).

The most frequently examined stones are hydroxylapatite and calcium oxalate monohydrate (COM). These stones are capable of taking on various shapes, such as stars, that can cause excruciating discomfort as they move through the urinary tract. Although most of the examined stones had ovular, smooth shapes, several crystals had jagged, asymmetrical shapes. Uric acid, calcium oxalate combined with aspartate, calcium oxalate dihydrate combined with calcium phosphate, magnesium ammonium phosphate (struvite) and calcium oxalate combined with uric acid are additional typical calculi constituents (Canales *et al.*, 2010).

The whewellite's calcium atom forms bonds with the oxalate oxygen atom and the water molecules involved in crystallisation. Theoretically, a high level of calcium oxalate supersaturation (SS), a high oxalate/calcium ratio in urine at a given level of SS, a low level of crystal-growth inhibitors, an increased level of crystal-growth promoters and a low urinary pH are the causes of calcium oxalate stone formation. Citrate's and magnesium's propensity to form soluble complexes with calcium and oxalate, respectively, explains their inhibitory effects.

By binding certain negatively charged inhibitors and neutralising them, hypercalciuria raises the urine's saturation with calcium and

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reduces the inhibitory action of urine against the crystallisation of calcium salts. An increase in oxalate concentration raises the saturation of calcium oxalate due to a reduced drop in calcium activity resulting from complexation. Colloidal crystalline monosodium urate or uric acid crystallisation occurs when urine becomes supersaturated with uric acid, and the pH of urine approaches or falls below the dissociation constant of uric acid. The newly produced uric acid or urate may use the heterogeneous nucleation mechanism to cause calcium oxalate to crystallise (Robertson *et al.*, 1981; Tiselius, 1981).

There could be three distinct species of calcium oxalate. The kinetically preferred and most hydrated form of calcium oxalate, calcium oxalate trihydrate, is the first phase that may precipitate. It will mostly change into the thermodynamically favourable state, typically whewellite (COM). Weddellite, (Calcium oxalate dehydrate, COD), a thermodynamically less stable phase than COM, may also develop in the presence of crystallised foreign compounds at amounts that are expected to be present in human urine (Kok *et al.*, 1986; Rose & Sulaiman, 1982; Wickham & Buck, 1990). The presence of COM and COD in human urine is influenced by the molar ratio of calcium to oxalate.

Even with a great deal of urological study, urolithiasis remains a mystery. Despite advanced testing, research and other methods, the precise cause of urolithiasis remains elusive, and the illness persists. Modern medical care is not only costly, but also difficult for those who are impoverished and cannot afford it. In reality, there is currently no effective medication available in contemporary medicine for removing a stone. Therefore, doctors must continue to rely on complementary and alternative medicine to provide better comfort (Dehdari & Hajimehdipoor, 2018; Gupta & Shamsher, 2018; Khan *et al.*, 2019).

Compared to current medications, herbal remedies are more effective, have fewer adverse effects and lower the incidence of kidney stone recurrence. Although the full mode of action of these treatments is unknown, plant-based phytotherapeutic compounds are mostly utilised in urolithiasis medicine (Nagpal & Sharma, 2020; Nimavat *et al.*, 2022). Most plant-based therapies have been shown to be beneficial at several stages of stone pathophysiology, unlike allopathic medications, which exclusively target one element of urolithiatic pathophysiology (Chandel *et al.*, 2019).

The antilithogenic properties of plant-based medications work by changing the ionic makeup of urine, either by raising the excretion of magnesium and citrate or lowering the proportion of calcium ions. These treatments also exhibit lithotriptic activity or a diuretic impact. A medication with several protective action mechanisms could be one step towards reducing tissue damage in humans. Herbal remedies provide different phytoconstituents and work in several ways to alleviate urolithiasis (Jalal *et al.*, 2020; Stiani *et al.*, 2019).

The drugs used to dissolve kidney stones or facilitate their passage are in line with additional retention when used in conjunction with herbal treatment. To increase the volume of fluid passing through the kidneys and remove the deposits, diuretic action is also required. 'Lithotripsy' is the term used to describe the process of breaking, dissolving or disintegrating the produced stones. Certain medications cause the volume of urine to increase, which lowers the saturation of salts and inhibits crystal formation at physiological pH. Certain herbal remedies break down mucoproteins, which is what attaches the crystal to the renal cells (Saeidi *et al.*, 2012; Zhang *et al.*, 2014).

Stones develop when the chemistry of the urine causes concentrations of stone salts (phosphates, calcium and oxalates) to rise above the maximum metastability of the salts in solution, resulting in SS. A higher urine volume inhibits the crystal's formation at physiological pH by reducing the saturation of the ions. Every herbal remedy used to treat urolithiasis has a diuretic effect, and some of them are also known to alkalise urine (Harshita *et al.*, 2020).

Molecules that lower crystal growth rate and aggregation, raise the SS necessary to initiate nucleation and prevent secondary nucleation are referred to as inhibitors. Promoters, on the other hand, lessen the supersaturated solution's formation product. Oxalate, calcium, cystine and uric acid are a few typical promoters, whereas citrate and magnesium are prominent inhibitors. It has been proposed that the production of urinary stones is more likely to result from an imbalance between urine-promoting and urine-inhibiting components than from a disruption of any one ingredient (Al-Yousofy *et al.*, 2017; Akhtar *et al.*, 2017; Nirumand *et al.*, 2018).

Urine contains a variety of crystalloids, including oxalate, uric acid, calcium and cystine. These crystalloids are retained in solution through the absorption process of colloids, such as mucin and sulphuric acid. Urinary stones arise when there is an imbalance in the crystalloid–colloid ratio, which can be caused by an increase in crystalloid levels and a decrease in colloid levels, or when the colloid loses its adhesive or solvent activity. The restriction of urine outflow by stones in the urinal system causes the glomerular filtration rate to drop in this circumstance. This causes waste products to build up in the blood, especially nitrogenous compounds like urea, creatinine and uric acid. Herbal treatment improves renal function through increased urea and creatinine excretion. Through the mechanism mentioned above, the majority of phytotherapeutic agents exhibit their antiurolithiatic activity (Cealan *et al.*, 2019; Cheraft–Bahloul *et al.*, 2017; Hayatdavoudi *et al.*, 2016).

The main aim in this area was to create a model system that allows for the evaluation of each factor separately or in combination. This model system can simplify the complexities found in biological systems, as it is challenging to ascertain the factors that either encourage or impede the formation of kidney stone crystals. A thorough investigation of the growth and function of inhibitors is now necessary given the current level of interest in the crystal aggregation process. As a result of this, a gel system was chosen to study the formation of whewellite urinary crystals, the impact of known inhibitors on their growth and the characterisation of the resulting crystals. The leaves of *Alternanthera sessilis*, seeds of *Curcurbita maxima* and flowers of *Aerva lanata* are selected for analysis because they are already used in traditional medicine for treating urinary infections. Therefore, a detailed analysis is required to predict the efficacy of these herbs in treating particular types of urinary stones.

2. Materials and Methods

All the apparatus and glassware used in this experiment are cleaned and completely dried in a hot air oven at 100°C for 30 minutes. All the chemicals used in this work are AR grade chemicals.

2.1. Crystal Growth:

U-shaped tubes of size 25*3 cm^2 were utilised as the vessel for crystallisation. Silica gel medium was prepared using sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$) of specific gravity of 1.03 g/cc. The gel medium of different pH levels ranging from 6 to 6.5 was prepared by adding an appropriate amount of 1.5 M glacial acetic acid to the sodium metasilicate. Once the specified pH is achieved, the U-shaped tube is kept corked for around 24 hours to allow for gelation. After the gelation period, one of the limbs was chosen to fill 20 mL of oxalic acid.

On the other limb, 12 mL of calcium chloride and 8 mL of magnesium

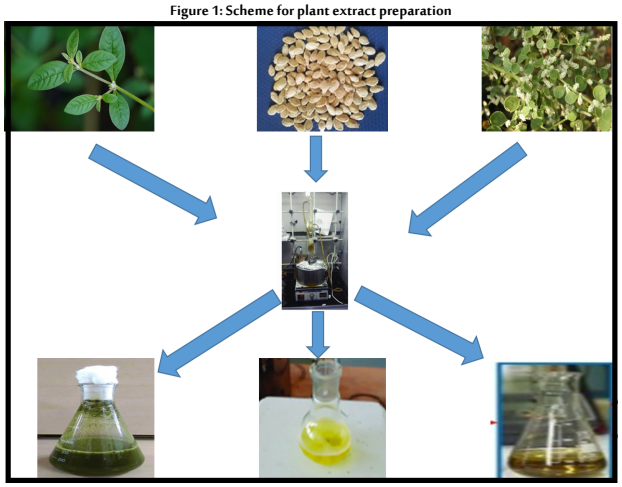
acetate were added carefully and made sure that the gel did not crack. crack. The tubes were again corked and kept at room temperature of around 29–35 °C. The supernatant solutions diffused through the pores of the silica gel, where they reacted with each other and initiated nucleation, yielding a disc-shaped assembly of whewellite crystals. Crystals of whewellite started to nucleate only after two to three days. After five weeks, the crystallisation of whewellite was completed, and tiny crystals were separated from the gel by washing it with distilled water and dried at room temperature. The following reaction is expected to take place:

$$H_2C_2O_4.2H_2O + (CH_3COO)_2Mg.4H_2O + CaCl_2.2H_2O \longrightarrow CaC_2O_4.H_2O + MgCl_2 + 2CH_3COOH + 7H_2O$$

2.2. Plant Extract Preparation:

In the next phase of the study, ethanol extracts of the leaves of the *Alternanthera sessilis*, seeds of *Curcurbita maxima* and flowers of *Aerva lanata*, which were prepared earlier using the Soxhlet apparatus, were again separately added to the freshly prepared gel to study the inhibitory activity of the extracts. The scheme for the plant extraction is given in Figure 1.

Figure 1: Scheme for plant extract preparation



Gel mediums with pH values of 6, 6.25 and 6.5 were prepared, with pH 6.25 found to be the optimal range for the growth of whewellite urinary crystals. Of each ethanol extract, 1 mL was separately added to each of the test tubes on both limbs, along with 20 mL of the supernatant solutions. The nucleation of whewellite crystals in this case was observed to be delayed. Nucleation was seen only after four days. After five weeks, the well-grown crystals were collected and characterised to study the inhibitory activity of the above extracts. In this case, the size of the disc assembly was found to have considerably reduced in comparison to the tubes with no extract addition.

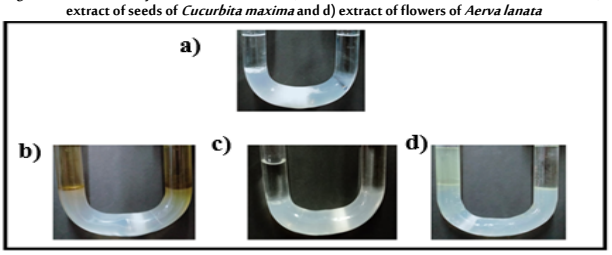
2.3. Characterisation

The Fourier transform infrared (FTIR) spectrum was recorded using SHIMADZU Model: FTIR-8400S and X-ray diffraction was recorded with Shimadzu XRD 6000 X-Ray Diffractometer.

3. Results

Figures of whewellite crystals at a pH of 6.25 before and after adding ethanol extracts are shown in Figure 2. Whewellite is formed as very tiny crystals of spherical shape in the pH range of 6.25, which is the perfect pH for their growth. Although a study at different pH was carried out, a pH of 6.25 was found to be effective for the growth of whewellite crystals.

Figure 2: Whewellite crystals without extract, b) with extract of leaves of *Alternanthera sessilis*, c) extract of seeds of *Cucurbita maxima* and d) extract of flowers of *Aerva lanata*



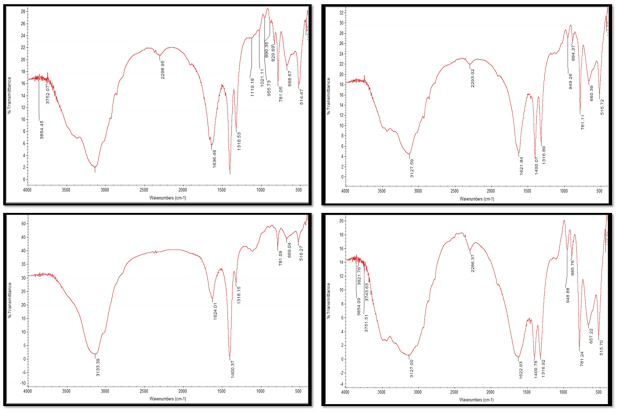
In the present study, the size of the crystals was measured and compared to demonstrate inhibitory activity. A reduction in crystal size indicates that components in the extract suppress crystal growth or hinder crystal formation by facilitating the elimination of small crystals through possible pathways. This approach aims to replicate the same effect within the kidneys.

The degree of inhibition in the whewellite crystals could be observed by comparing the size of the crystal disc assemblies formed in the U-shaped tubes. That is, in the tubes to which extracts were added, the size of crystal disc assemblies was considerably reduced compared to those without extract. On comparing the three extracts, the size of the disc is much shorter in the gel medium with the extracts of flowers of *Aerva lanata*, indicating the strongest inhibition towards the crystals of whewellite. Quercetin and botulin present in *Aerva lanata* enhance the anti-urolithic activity.

3.1. Spectral Characterisation

The FTIR spectra of whewellite crystals grown in various controlled manners were recorded in the wave number range of 400–4000 cm^{-1}. The FTIR spectra of pure whewellite and whewellite grown with the addition of extracts from different substances are shown in Figure 3.

Figure 3: FTIR spectrum of a) whewellite crystals without extract, b) extract of leaves of *Alternanthera sessilis*, c) extract of seeds of *Cucurbita maxima* and d) extract of flowers of *Aerva lanata*



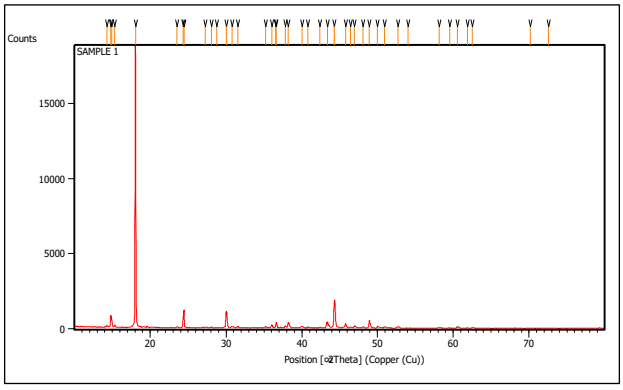
The coordinated outcomes suggested by the FTIR spectra are presented in Table 1.

Table 1: Analysis of FTIR spectrum of whewellite urinary crystals

Band Assignments	Pure whewellite cm^{-1}	Whewellite with extracts of *Alternanthera sessilis* cm^{-1}	Whewellite with extracts of *Cucurbita maxima* cm^{-1}	Whewellite with extracts of *Aerva lanata* cm^{-1}
Intermolecular and weakly H–bonded H-O-H stretching mode of water of crystallization	3752.07	3127.59	3133.39	3751.51
	3120.00	2293.02		3127.00
	2298.95			2286.37
Asymmetric C=O stretching mode in oxalate units	1636.46	1621.84	1624.01	1622.93
Symmetric C=O stretching mode in oxalate units	1400.00	1400.07	1400.37	1400.78
	1318.53	1316.89	1318.15	1316.92
Water librational vibrations (rocking, wagging, twisting)	890.35	949.26	-	948.88
	829.69	884.37		885.76
CO_2 in-plane deformation mode in oxalate units	781.05	781.11	781.09	781.24
Calcium-oxygen bond vibrations	668.67	660.39	669.04	657.22
CO_2 wagging mode in oxalate units	514.47	515.72	516.27	515.70

The powder X-ray diffraction was recorded for well-grown pure whewellite crystals between 2θ values 10 to 80° for well grown pure whewellite crystals. The X-ray diffractogram of pure whewellite is shown in Figure 4. The sharp peaks obtained confirm the crystalline nature of whewellite.

Figure 4: X-ray diffractogram of pure whewellite crystals



4. Discussion

In the FTIR spectrum, the peaks between 2200–3800 cm^{-1} corresponded to the intermolecular and weakly H-bonded H-O-H stretching mode of the water of crystallisation. Asymmetric C=O stretching mode in oxalate units appeared in and around 1620 cm^{-1}. The peaks around 1310–1400 cm^{-1} were due to symmetric C=O stretching mode in oxalate units. Water vibrations were found to be between 800–950 cm^{-1}. CO_2 in-plane deformation mode in oxalate units was at 781 cm^{-1}. The peaks around 660 cm^{-1} showed the presence of calcium–oxygen bond. CO_2 wagging mode in oxalate units appears at 515 cm^{-1}. The results interpreted from the FTIR spectrum confirmed the presence of calcium, oxalate units and H_2O. Powder X-ray diffraction proved the monoclinic crystal system, and the space group was found to be P21/n (JCPDS card number 77-1160). The list of plants used for the treatment of whewellite crystals is tabulated in Table 2.

Table 2: List of plants used for the treatment of whewellite crystals

S. No.	Botanical name	Family	Common name	Parts used	References
1	*Herniaria hirsuta L*	Caryophyllaceae	Rupture wort	Flowers	El-Habbani *et al.*, 2021
2	*Opuntia ficus-indica*	Cactaceae	Indian fig	Flowers	El-Habbani *et al.*, 2021
3	*Ammi visnaga L*	Apiaceae	Toothpick weed	Seeds	El-Habbani *et al.*, 2021
4	*Gypsophila struthium*	Caryophyllaceae	Baby's breath	Roots	Shirani *et al.*, 2020
5	*Duranta erecta*	*Verbenaceae*	Golden dewdrop	Whole plant	Agawane *et al.*, 2019
6	*Lygodium japonicum*	Lygodiaceae	Japanese Climbing fern	Whole plant	Nirumand *et al.*, 2018
7	*Ammodaucus leucotrichus*	Apiaceae	Nessoufa, Moudrayga	Whole plant	Beghalia *et al.*, 2015
8	*Terminalia arjuna*	Combretaceae	Arjuna	Whole plant	Mittal *et al.*, 2015
9	*Elettaria cardamomum*	Zingiberaceae	Cardamom	Seeds	Patel *et al.*, 2011
10	*Tribulus terrestris Linn.*	Zygophyllaceae	Puncture vine	Whole plant	Joshi *et al.*, 2005
11	*Bergenia ligulata Linn.*	Saxifragaceae	Prashanbheda	Whole plant	Joshi *et al.*, 2005
12	*Herniaria hirsuta*	Caryophyllaceae	Rupture wort	Whole plant	Atmani *et al.*, 2004

Comparing the list of plants, *Aerva lanata*, selected for the present study, is readily available on the roadside, abundant and available in all seasons.

5. Conclusion

Today, kidney stones, one of the most painful diseases, are common in India, affecting 12% of the population annually, with 50% of those affected getting kidney failure and renal damage. Among the various types of stones, the most common ones like whewellite urinary crystals were grown using silica gel as the growth medium to examine the inhibitory activity of ethanol extracts from the leaves of *Alternanthera sessilis*, seeds of *Curcurbita maxima* and flowers of *Aerva lanata*. This experiment was carried out in two phases.

In the first phase, pure whewellite crystals were grown in the silica gel medium under various pH ranges. It was found that pH 6.25 is the optimal range for the growth of whewellite urinary crystals. In the second phase of the work, ethanol extracts from the leaves of *Alternanthera sessilis*, seeds of *Curcurbita maxima* and flowers of *Aerva lanata* were prepared separately. The gel of pH 6.25 was made by incorporating glacial acetic acid. The same extracts were added to the top of the gel, along with oxalic acid in one limb and magnesium acetate and calcium chloride as the supernatant in another limb of the U-shaped tube. The inhibitory activity of the crystals was compared with respect to the three extracts.

The results indicated that, among the three ethanol extracts, flowers of *Aerva lanata* exhibited significant inhibition towards whewellite crystals. The crystals obtained in different environments were characterised by the FTIR spectrum. The spectrum confirmed the formation of the respective crystals. The X-ray diffractograms of pure whewellite confirmed its crystal nature. Future studies with different concentrations of flowers of *Aerva lanata* to prove the prevention effects on these crystals in vivo can be carried out.

Biographies

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