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Inhibitory potential of *Ocimum tenuiflorum* L. on uric acid crystallization: An investigational study

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The enduring problem of kidney stone disease continues to afflict humans worldwide, with its recurrence being the most worrisome factor. Among the variety of stone-forming compounds, uric acid has received less attention despite being a significant contributor to the development of kidney stones. Throughout history, natural plant derivatives have served as a low-cost alternative for treating kidney stones. *Ocimum tenuiflorum* L. (Tulsi) is an indigenous medicinal plant primarily found in the Indian subcontinent and has gained recognition for its efficacy in managing various ailments, particularly kidney stones. The current investigation delves into the inhibitory capability of tulsi extract on *in vitro* uric acid crystallization. The addition of tulsi extract to synthetic urine resulted in a delay in the induction of uric acid crystallization and a decrease in the uric acid crystal size in a concentration dependent manner. At pH 5, a 19 fold increase in the induction time and a 3 fold decrease in the crystal size were observed when the concentration of tulsi extract was increased from 27.5 to 110 μg/mL. The current study effectively illustrates the inhibitory potential of tulsi extract in curbing uric acid crystallization.

Keywords: Holy basil, Kidney stones, Tulsi, Uric acid crystallization

Biomineralization is an evolutionary adaptation in living organisms for a variety of functions ranging from the formation of endo and exoskeletons to geotaxis and magnetotaxis[1]. However, biomineralization can also have pathogenic manifestations, including the uncontrolled formation of kidney stones. Calcium oxalate and uric acid are the two major contributors to the formation of kidney stones, with a plethora of research available on the former[2–6]. Despite contributing 10% of the total cases of kidney stone formation[7], uric acid kidney stones have received relatively less attention[8]. Apart from developing uric acid kidney stones, uric acid biomineralization has also been observed to contribute to gouty arthritis[9] and assist in the formation of calcium oxalate stones[10]. The fundamental mechanism of uric acid kidney stone formation is unknown, indicating a gap in our understanding of its pathogenesis.

Uric acid, also known as 2,6,8-trioxypurine, is a weak diprotic organic acid with the pK_{a1} value of 5.3[11]. It forms as the end product of purine metabolism in humans, and unlike other mammals, it does not get enzymatically degraded to a more soluble allantoin[12,13]. As a result, humans have comparatively higher levels of uric acid in their serum and consequently, in their urine and extracellular spaces, as compared to most mammals[14]. The solubility of uric acid is extremely poor in aqueous media and is a strong function of pH[15]. In blood and in extracellular spaces is around 7.4, where it primarily exists in the form of monosodium urate[14]. Excessive uric acid levels in serum result in the formation of monosodium urate crystals in the extracellular spaces (pH 7.4) with needle-type crystal morphology, and result in painful gouty arthritis[16]. In urine, where the pH can range from 4.5 to 8, pH in the acidic range (specifically $<pK_{a1}$ value of 5.3) promotes the protonation of urate ions to form poorly soluble uric acid. Excessive uric acid excretion through urine, with the urine pH lower than pK_{a1} (acidic urine) results in the formation of uric acid kidney stones[17].

The study of kidney stone pathology poses the question of how to manage or alleviate this condition. The process of kidney stone formation is mainly influenced by crystallization, with phenomena such as nucleation, crystal growth, and crystal agglomeration (Fig. 1) contributing to the formation of kidney stones[3]. Crystal growth modifiers are one area of research that is dedicated to this topic. Crystal growth

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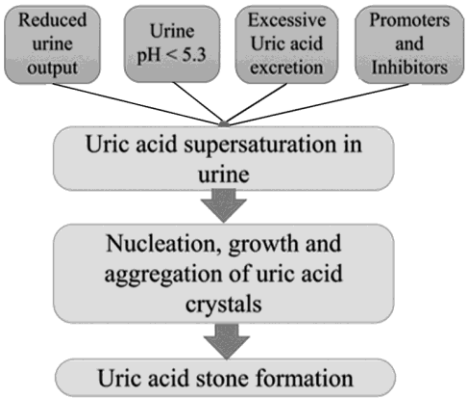

Fig. 1 — Mechanism of uric acid kidney stone formation.

modifiers, a broad phrase, refer to chemicals that can influence several elements of crystal formation. These modifiers are compounds that can affect the size, form, and nucleation rate of crystals. One proposed alteration technique is to chelate one of the crystal's constituents in the solution, which reduces supersaturation[18]. Alternatively, the modifier may bond to the crystal's surface, decelerating or prohibiting crystal growth at the binding site and altering its shape[19]. After adsorption to the surface, the modifiers can impact the zeta potential around the particle, which affects the chance of agglomeration[20]. Modifiers can stabilize alternate polymorphs or hydrates using procedures such as templating[21,22]. Another option is that the additive gets incorporated into the crystal, causing a change in its behaviour and growth.

Many crystal growth modifiers have been studied for their ability to modulate or inhibit kidney stone development[23-25]. Many such studies, conducted both *in vitro* and *in vivo*, include the use of extracts of medicinal plants/herbs that are used in traditional antiurolithic therapy[26-29]. The most common *in vitro* models deal with nucleation and aggregation assays, and the dissolution of calcium oxalate stones[30-32]. The effectiveness of the medicinal herbs in dissolving or inhibiting calcium oxalate crystals can be attributed to the phytochemicals in the extract serving as crystal growth modifiers.

Ocimum tenuiflorum L., commonly called the Holy basil and locally Tulsi, has been used for thousands of years in southern Asia, particularly the Indian subcontinent, to treat a variety of diseases. It possesses medicinal properties such as antioxidant, anti-inflammatory, antimicrobial, antidiabetic, hepato-protective, and wound-healing[33]. Studies have been conducted to investigate the efficacy of different plant extracts against calcium oxalate crystallization[34,35], while the literature about the antiurolithiatic potential of tulsi is scanty. To the authors' knowledge, no studies have been reported that investigate the inhibitory potential of tulsi extract specifically for uric acid crystallization. The inhibitory influence of tulsi extract on uric acid crystallization is evaluated by determining its ability to delay the beginning of uric acid crystallization and reduce the size of crystals generated by the end of the experiment. Furthermore, the effect of the extract on uric acid crystals is examined by solid-state characterization.

Materials and Methods

Chemicals

The following reagents were used for this study without further purification. Uric acid was purchased from Sisco Research Laboratories Pvt. Ltd. Potassium chloride, ammonium chloride, sodium dihydrogen phosphate, and sodium sulphate were purchased from Merck Life Science Pvt. Ltd. Sodium chloride and sodium hydroxide were obtained from Thermo Fisher Scientific Pvt. Ltd., while magnesium sulphate heptahydrate was sourced from Avantor Performance Materials India Ltd. Hydrochloric acid was purchased from Finar Limited. All reagents used were of analytical grade.

Preparation of extract

The plant powder of *O. tenuiflorum* was purchased from an Ayurvedic shop in Nagpur, Maharashtra. The plant powder was prepared by gathering the *panchanga*, translating as the five parts of the plant. These five parts comprise of pushpa (flower), phala (fruit), tvaka (bark), mula (root), and patra (leaves). The *panchanga* was subjected to shade drying (~ 30-35°C) before grinding it to create the final powder. The method employed for the preparation of aqueous extract was decoction. The dried powder of *O. tenuiflorum* (25 g) was mixed with 100 ml of distilled water, and the mixture was poured into a three-neck round-bottom flask placed inside a heating mantle. The mixture was then heated at 100°C for 30 min. Throughout the extraction process, the flasks were continuously covered to reduce solvent evaporation. The extract was then refrigerated (4°C) for later use after being filtered through Whatman filter paper No. 42.

Phytochemical analysis

The *Ocimum tenuiflorum* extract was tested for phytochemicals such as phenols, flavonoids, alkaloids,

saponins, steroids, and glycosides using the standard procedure described in the literature. The procedure for conducting the ferric chloride test for phenols, Wagner's test for alkaloids, foam test for saponins, and Keller Kiliani test for glycosides was adapted from Hu *et al.*[36]. The method for the Salkowski test for steroids was taken from Hossain *et al*.[37] and the alcoholic ferric chloride test for flavonoids from Usman *et al* [38].

Crystallization of uric acid

The method provided by Pradhane *et al.*[39] was followed to perform the crystallization of uric acid. Prior to each experiment, a freshly prepared uric acid stock solution was made by dissolving 0.2 g of uric acid in 95 mL of water and 5 mL of 1M NaOH. The formula for preparing synthetic urine was provided by Pradhane *et al.*[39]. Uric acid crystallization in the synthetic urine study was carried out in two jacketed flasks. Synthetic urine (160 mL) and uric acid (40 mL) were added to the jacketed flask, resulting in a final pH of 7.2, which was determined using a pH meter (Labman LMMP 30). A magnetic stirrer (REMI 1 MLH) was used to ensure uniform mixing of the solution, and the temperature of the solution inside the flask was maintained at 37°C. Continuous monitoring and pH control of the solution were achieved using a pH electrode. To achieve supersaturation of uric acid, the pH of the solution was regulated by adding 2-3 mL of concentrated HCl solution. As the solution's pH was adjusted, the timer was switched on to note the induction time. The induction time for crystallization was noted as the time required for the onset of turbidity in the solution. The crystallization of uric acid was conducted in the presence of *O. tenuiflorum* extract in a concentration range of 0-110 µg/mL. The resultant crystals were collected in microcentrifuge tubes and stored for further analysis.

Characterization

The images of uric acid crystals collected from the crystallizer were captured using a light microscope (Optscopes) equipped with a digital camera with a 40X objective lens. Subsequently, ImageJ software was used to calculate the size of the crystals, which was expressed as percent reduction in size. For characterization of uric acid crystals, a Rigaku Miniflex 600 X-ray diffractometer with Cu-K radiation was used, while the infrared spectra of samples were obtained within the wavenumber range of 500-4000 cm^{-1} using an IRSpirit (Shimadzu) spectrophotometer.

Statistical analysis

The statistical analysis of the data obtained during the experimentation was carried out with the help of Minitab statistical software (Version 17). The pH of synthetic urine (X_1) and the *O. tenuiflorum* extract concentration (X_2) were selected as the factors while the average crystal size (Y_1) and the average induction time (Y_2) were selected as the response. The statistical analysis was conducted by conducting an ANOVA test at a level of significance of P <0.05 and the F test[40]. The regression equation selected to describe the relationship between factors and response is given as follows

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + e_1 \quad \text{... (1)}$$

where Y represents the response, X_1 and X_2 represent the factors, β_0, β_1 and β_2 represent the coefficients and e represents the error.

Results and Discussion

Phytochemical analysis

The phytochemical analysis of the aqueous extract of *Ocimum tenuiflorum* was carried out, and the results obtained are shown in Table 1. The antioxidant property of *O. tenuiflorum* is attributed to the presence of phenols and flavonoids, which have the capability to donate electrons. Also, glycosides have the potential to serve as cardio stimulants[41], but the exact mechanism through which phytochemicals present in *O. tenuiflorum* contribute to the antiurolithiatic effect remains unknown and is a subject of future research.

Effect of *Ocimum tenuiflorum* extract on the size of uric acid crystals at different pH values

The effect of *O. tenuiflorum* extract on the size of uric acid crystals can be seen from the microscopic images (Fig. 2). The crystal size was measured using the Image J software by assuming the size of crystals to be spherical, and the calculated size of crystals is shown in Table 2. As shown in Fig. 2 and Table 2, there is a notable decrease in the size of uric acid crystals with increasing pH and *O. tenuiflorum* extract

Table 1 — Phytochemical analysis of *O. tenuiflorum* extract

Phytochemicals	Test	Ocimum extract
Phenols	Ferric chloride test	+
Glycosides	Keller Kiliani test	+
Saponins	Foam test	-
Flavonoids	Alcoholic ferric chloride test	+
Steroids	Salkowski test	+
Alkaloids	Wagner's test	-

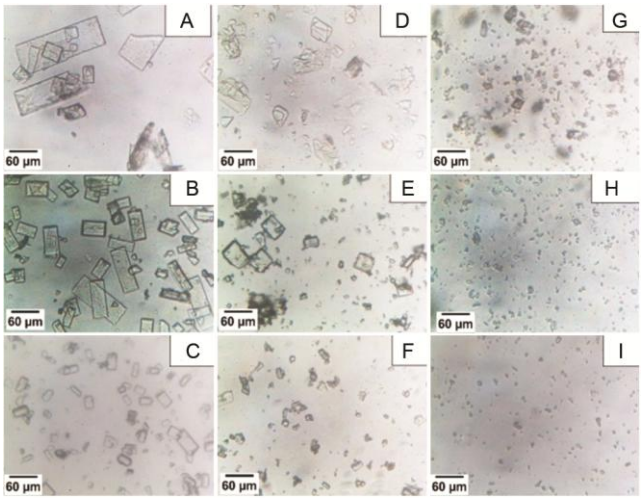

Fig. 2 — Microscopic images of uric acid crystals (A-C) in the absence of *Ocimum tenuiflorum* extract at pH 4, 4.5 and 5; (D-F) in the presence of extract concentration 55 µg/mL at pH 4, 4.5 and 5; and (G-I) in the presence of extract @ 110 µg/mL at pH 4, 4.5 and 5 µg/mL. [Images were captured with a 40X objective with a 5 MP camera mounted over the microscope].

Table 2 — Average crystal size of uric acid crystals and concentration of tulsi extract at different pH values

Conc. of *O. tenuiflorum* extract (µg/mL)	Average crystal size (µm)		
	pH 4	pH 4.5	pH 5
0	38.21	35.01	26.39
27.5	35.99	29.46	19.10
55	23.18	18.18	12.67
82.5	14.41	10.38	8.98
110	11.81	8.601	6.78

concentration. Statistical analysis of the results indicates that the decrease in crystal size with an increase in *O. tenuiflorum* extract concentration is more significant when comparing the effects of the two variables, pH and *O. tenuiflorum* extract concentration. The average crystal size decreased by 3 folds when the extract concentration was increased from 27.5 µg/ml to 110 µg/mL. In the absence of extract, a decrease of 1.4 folds was observed in the average crystal size when the pH was changed from 4 to 5. As evident in the microscopic images, the crystal morphology mostly remains unchanged.

Effect of *Ocimum tenuiflorum* extract on the induction time of uric acid crystallization

The effect of *O. tenuiflorum* extract on the induction time of uric acid crystallization is shown in Fig. 3. The average induction time of uric acid crystallization increased with both increase in pH and extract concentration. Within the investigated pH range of 4 to 5, the increase in the average induction time is more significant with increase in extract

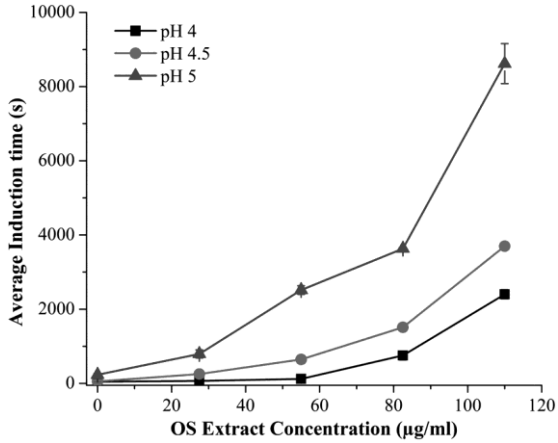

Fig. 3 — Effect of *Ocimum tenuiflorum* extract concentration on induction time in synthetic urine at different pH values.

concentration than with increase in pH. A two-fold increase in the average induction time was observed when the pH of the synthetic urine was increased from 4 to 5 in the absence of *O. tenuiflorum* extract. The increase in the average induction time at pH 5 can be attributed to the increased solubility of uric acid in aqueous media, thereby reducing uric acid supersaturation. The average induction time was observed to increase significantly with increase in concentrations of *O. tenuiflorum* extract in the synthetic urine. The most significant increase in the average induction time was observed at pH 5, where an increase of 19 folds in the average induction time was observed when the extract concentrations was increased from 27.5 to 110 µg/mL. The increase in the average induction time with increase in extract concentration proves the effectiveness of the *O. tenuiflorum* extract in inhibiting the uric acid crystallization.

Statistical analysis

The regression equation relating the pH of synthetic urine (X_1) and the *O. tenuiflorum* extract concentration (X_2) as factors with average crystal size (Y_1) and the average induction time (Y_2) as responses, is as follows.

$$Y_1 = 77.70 - 9.94.X_1 - 0.2371.X_2 \quad \text{... (2)}$$

$$Y_2 = -11786 + 2501.X_1 + 40.52.X_2 \quad \text{... (3)}$$

The R^2 and the adjusted R^2 values for equation 2 are 0.9352 and 0.9244, respectively, while the same for equation 3 are 0.7121 and 0.6641, respectively. The R^2 values of more than 0.9 indicate a good fit of model (equation 2) with experimental data for percent size reduction as the response.

The results of ANOVA analysis for equations 2 and 3 are given in Table 3. The ANOVA evaluates the alteration in responses (average crystal size and average induction time) through the linear interaction of factors (pH and *O. tenuiflorum* extract concentration) and determines the statistical significance for each factor and the interaction [40]. For average crystal size as the response (Y1), pH (P <0.001, F = 28.08), *O. tenuiflorum* extract concentration (P <0.001, F = 145.11) and their linear interaction (P <0.001, F = 86.59) between the two factors (equation 3) were all statistically significant. When average induction time was considered as the response (Y_2), both pH (P = 0.012, F = 8.78) and *O. tenuiflorum* extract concentration (P = 0.001, F = 20.90), with their linear interaction (P = 0.001, F = 14.84) (Eq. 4) were found to be statistically significant.

Characterization

The FTIR spectra of uric acid crystals are shown in Fig. 4A. The FTIR analysis result shows the presence of C-C and C-N stretching at 1584 cm^{-1}, O-H deformation at 1300 cm^{-1}, ring vibration at 1110 cm^{-1}, N–H out-of-plane and in-plane bending at 880 cm^{-1}, skeletal ring deformation at 620 cm^{-1} [42]. It was noticed that no additional peaks were formed, but the intensity of existing peaks varied with the addition of extract. This can be a result of the interaction among phytochemicals present in *O. tenuiflorum* extract with uric acid molecules without significantly impacting their chemical composition. Thus, it becomes evident that the interactions between uric acid molecules and phytochemicals present in the extract are primarily physical in nature. The PXRD graph of uric acid crystals is shown in Fig. 4B. It was observed that there is a slight shift in diffraction peaks. The shifts in the characteristic peaks in PXRD patterns are attributed to the alteration in the arrangement of atoms within the crystal lattice, leading to lattice strain. As observed in Fig. 4B, a shift to lower diffraction angles and merging of peaks observed in the PXRD pattern indicates a decrease in the crystallinity of uric acid crystals[39], as a consequence of interactions of phytochemicals and uric acid molecules.

Kidney stones, a prevalent disease affecting people globally, are characterized by crucial factors such as urine supersaturation, which leads to nucleation, growth, and aggregation[29]. The expenses associated with treating this condition are significant. Over the course of history, medicinal plants have held a prominent role in the traditional healthcare system, and is still serving as a low cost source of pharmaceuticals for many individuals[3]. The present study focused on exploring the antiurolithiatic potential of *O. tenuiflorum* extract through an *in vitro* study.

Table 3 — ANOVA for average crystal size (Y_1) and average induction time (Y_2) as responses

Source	DF	Adj. SS	Adj. MS	*F* Value	*p* Value
Average crystal size (Y_1)					
Regression	2	1522.4	761.18	86.59	< 0.001
pH (X_1)	1	246.8	246.81	28.08	< 0.001
O. tenuiflorum extract conc. (X_2)	1	1275.5	1275.55	145.11	< 0.001
R^2	0.9352				
Adjusted R^2	0.9244				
Average induction time (Y_2)					
Regression	2	52880752	2644.38	14.84	0.001
pH (X_1)	1	15637502	15637502	8.78	0.012
O. tenuiflorum extract conc. (X_2)	1	37243249	37243249	20.90	0.001
R^2	0.7121				
Adjusted R^2	0.6641				

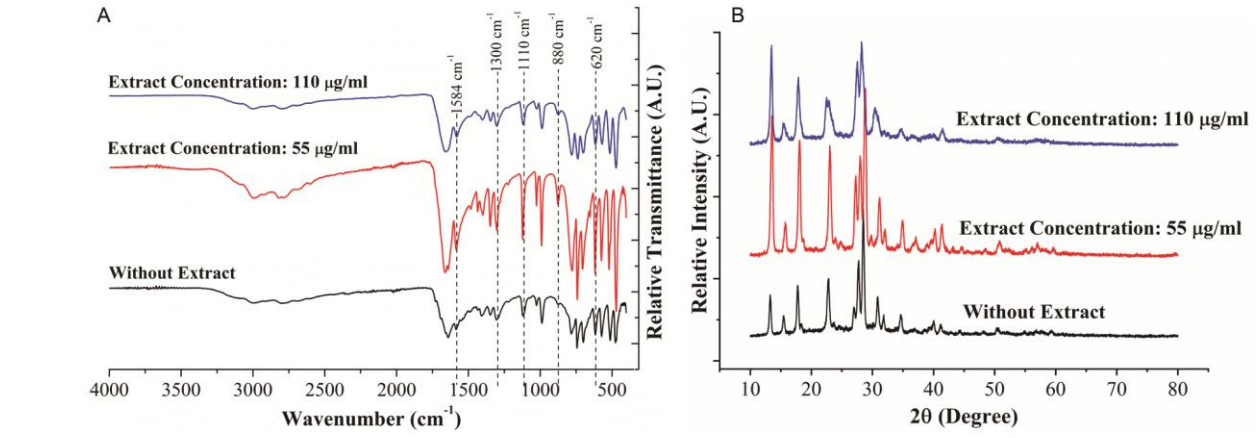

Fig. 4 — (A) FTIR spectra; and (B) XRD spectrum of uric acid crystals prepared in the absence and the presence of *Ocimum tenuiflorum* extract. The characteristic bands for uric acid are indicated by dashed lines in FTIR spectra.

The pharmacological effects of a medicinal formulation are primarily influenced by specific phytochemicals present in the plant. Therefore, a preliminary phytochemical assessment of the *O. tenuiflorum* extract was conducted, and it showed the presence of various secondary metabolites such as phenols, flavonoids, glycosides, saponins and steroids and showed negative results for alkaloids. The active constituents in *O. tenuiflorum*, particularly eugenol (1-hydroxy-2-methoxy-4-allylbenzene), have been identified as the major contributors to its therapeutic potential[43]. It is also reported that the leaves and seeds of *O. tenuiflorum* exhibit notable hypouricemic and uricosuric effects, as well as the potential of *O. tenuiflorum* to reduce the uric acid formation in the body[44].

The experimental results showed that the *O. tenuiflorum* extract could inhibit uric acid crystallization. The addition of extract led to a delay in the induction time, indicating that specific phytochemicals present in the extract have the capability to impede uric acid nucleation. This inhibitory effect increased with the increase in the concentration of extract. At higher concentrations of *O. tenuiflorum* extract, the time required for the formation of crystals increased. The increase in induction time caused by the extract was observed to be higher at a higher pH due to the increase in solubility of uric acid, thereby decreasing the supersaturation. The results have shown that the change in urine pH can significantly affect the solubility of uric acid, thereby impacting its tendency to crystallize, thus highlighting the importance of pH regulation in preventing uric acid stone formation. Additionally, the increase in the average induction time at pH 5 with higher concentrations of *O. tenuiflorum* extract highlights the synergistic effect of pH and extract concentration on uric acid crystallization kinetics. This observation underscores the importance of considering both pH regulation and the use of *O. tenuiflorum* extracts in the development of preventive strategies against kidney stone formation.

The effect of *O. tenuiflorum* extract on the size of uric acid crystals was also studied. The size of the uric acid crystals obtained from the *in vitro* study, which ranges between 30 and 40 µm, provides valuable insights into their potential implications for the urinary system, particularly regarding the dimensions of the ureter and nephron. The ureter, the tube for transporting urine from the kidneys to the bladder, typically has a diameter ranging from approximately 3 to 4 mm. In contrast, nephrons, the kidney's functional units, are minute structures with diameters averaging about 15 to 30 µm[45]. Therefore, if the size of the uric acid crystals falls within that range could potentially obstruct urine flow through the nephron and potentially compromise kidney function. In the presence of extract, the crystal size was reduced to 75% of its original size. By reducing the size of the crystals with the addition of extract, it may facilitate easier passage through the urinary system, thereby lowering the risk of urinary tract obstruction and pain. The size of crystals was also observed to decrease with increasing pH. Hence, the use of extract along with alkalinisation can be an effective strategy for dealing with uric acid kidney stones.

The samples were analysed using characterization techniques, including FTIR spectroscopy and PXRD to understand the effect of *O. tenuiflorum* extract on uric acid crystals. Based on FTIR analysis, it was observed that no new peaks were formed, but the intensity of the peaks varied, showing that the presence of the plant extract may have altered the arrangement of the uric acid molecules within the crystals. This alteration in intensity could indicate changes in crystal size, orientation, or packing. It implies that the plant extract likely interacts with the uric acid crystals, affecting their morphology or organization without causing significant changes in their chemical composition. Hence, it becomes apparent that the interactions between uric acid molecules and phytochemicals within the extract are predominantly physical. The PXRD pattern of uric acid crystals showed a slight shift in diffraction peak and merging of peaks at higher concentrations of extract, which could be due to the modification in the crystallinity of uric acid crystals and decrease in size.

The experimental findings clearly indicate that the *O. tenuiflorum* extract demonstrates antiurolithiatic properties. However, there are challenges that need to be addressed for further advancement in research. Firstly, the *in vitro* approach may not fully replicate the complexities of uric acid crystallization *in vivo*, potentially limiting the applications of these findings to clinical trials. Moreover, the use of synthetic urine as a medium may not accurately mimic the composition and properties of real urine, thereby

affecting the generalisation of results. Additionally, ensuring the consistency and standardization of *O. tenuiflorum* extract presents a challenge due to variations in extraction methods and chemical composition. Although translation of these findings into clinical practice requires rigorous validation through clinical trials, the study sets a solid groundwork for exploring the efficacy of *O. tenuiflorum* extract as a potential therapeutic agent. Furthermore, while challenges remain in understanding the underlying mechanisms and pharmacokinetics of *O. tenuiflorum* extract, is essential to elucidate the mechanism through which *O. tenuiflorum* extract inhibits uric acid crystallization. Overall, overcoming these challenges will be crucial in ensuring that the study's findings are credible and applicable for managing uric acid-related disorders.

Conclusion

Results of this study highlight the significant inhibitory potential of *Ocimum tenuiflorum* extract on uric acid crystallization. Experimentation showed that the inclusion of *O. tenuiflorum* extract effectively delayed the induction time and reduced crystal size in a concentration-dependent manner indicating its potential as a cost-effective therapeutic agent in managing conditions associated with uric acid crystallization. The utilization of *O. tenuiflorum* extract as a natural remedy aligns with the growing interest in herbal medicine, offering a promising alternative to conventional treatments. However, our study focused on synthetic urine, and it is imperative to acknowledge the complexity of real urine, which contains various ions, proteins, and compounds that may influence uric acid crystallization differently. Therefore, future research should prioritize investigating the effects of *O. tenuiflorum* extract in real urine to better understand its efficacy and potential clinical applications. Furthermore, exploring the bioavailability and metabolism of the active compounds present in *O. tenuiflorum* extract is essential for evaluating its therapeutic potential. By elucidating the mechanism of action and pharmacokinetic properties, we can better assess the feasibility of *O. tenuiflorum* extract as a therapeutic agent for treating uric acid-related conditions.

Conflict of interest

Authors declare no competing interests.

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