

Evaluating the efficacy of *Tribulus terrestris* on pH dependent uric acid crystallization under experimental condition

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The increasing incidence of kidney stone disease, particularly due to dietary and metabolic risk factors, highlights the urgent need for effective and natural therapeutic strategies. While most studies focus on calcium oxalate stones, uric acid stones have received comparatively less attention. *Tribulus terrestris* has been extensively documented in Ayurvedic literature for its effectiveness in preventing kidney stones, especially those formed from calcium oxalate. This study investigates the activity of *Tribulus terrestris* as a potential inhibitor of uric acid crystallization. The inhibition was evaluated by measuring the delay in induction time and reduction in mean crystal size. Crystallization of uric acid in the presence of aqueous *Tribulus terrestris* extract showed a notable delay in induction time and reduction in crystal size. At pH 4.5, increasing the extract concentration from 37.3 $\mu\text{g/mL}$ to 149.2 $\mu\text{g/mL}$ led to a 38-fold increase in induction time. A ~3-fold decrease in mean crystal size was observed when extract concentration increased from 0 to 37.3 $\mu\text{g/mL}$. Phytochemical analysis revealed the presence of phenols, flavonoids, saponins, and sterols. SEM and PXRD confirmed reduced crystallinity, while EDS analysis showed altered C, N, and O composition, suggesting phytochemical incorporation into the uric acid lattice, resulting in inhibition of crystallization.

Keywords: Antioxidant activity, Antiurolithiatic potential, Bioactive compounds, Crystallization, Uric acid inhibition

Nephrolithiasis, which is characterized by development and deposition of kidney stones¹; is a common and severe urological disorder affecting a significant proportion of global population². Nephrolithiasis is currently estimated to affect 10% of the global population with a potential increase to 30% by 2050³. It presents significant clinical problems due to its recurrence⁴ and accompanying consequences. The rate of recurrence is as high as 50% in adults, within the first five – ten years of their first kidney stone incidents⁵.

The development of any kidney stone begins with the crystallization of a particular urinary solute in urine, whilst it is in kidneys. Human urine has a complex composition with around 3000 molecules⁶, containing a variety of organic and inorganic compounds⁷. Among many compounds commonly found in kidney stones, calcium oxalate^{8,9} and uric acid^{3,10} are the two most common. A significant volume of research has already been carried out on calcium oxalate^{11,12}, while the uric acid kidney stones have received comparatively lesser attention³.

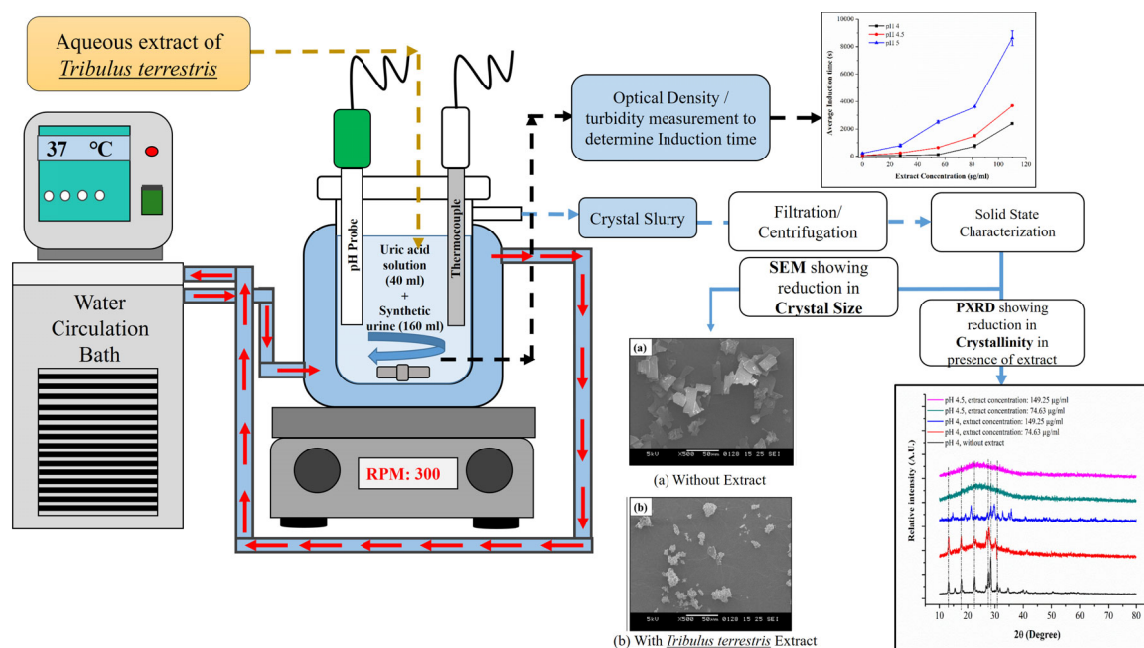
Uric acid, a metabolic by-product of purine metabolism^{2,10}, serves as a precursor for uric acid stone formation when the urine is supersaturated with it¹³. In its undissociated form, uric acid has extremely poor solubility in aqueous media, while its solubility improves when it loses a proton¹⁴. Its solubility is a strong function of pH, since it affects the protonation of uric acid³. Along with pH, hyperuricosuria¹⁵, characterized by elevated urinary uric acid levels, also predisposes individuals to uric acid stone formation. Inhibition of uric acid crystallization represents a pivotal strategy in the prevention and management of uric acid kidney stones, aiming to impede crystal nucleation and growth within the urinary system^{3,10}.

The resurgence of interest in phytomedicine, driven by the attenuated side effect profile of herbal remedies, has catalyzed renewed exploration into natural therapeutics. Presence of phytochemicals mainly flavonoids, saponins, alkaloids and proteins render the diuretic and anti-lithiasis properties to *Tribulus terrestris*¹⁶. Therefore, *Tribulus terrestris* also known as Gokharu/Gokshur is used widely for treatment of kidney stones. Studies are mainly reported for calcium oxalate kidney stones. However, to the best of the authors' knowledge, scant literature is available on uric acid crystallization. This paper

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Suppl. data available on the respective page of NOPR



Graphical abstract

aims to investigate impact of *Tribulus terrestris* extract (hereafter referred to as *Tribulus* extract) on uric acid crystallization. Effect of *Tribulus* extract on uric acid crystallization will be determined by two parameters mean induction time and mean crystal size. A thorough solid-state characterisation will also be performed to determine the mechanism by which *Tribulus* extract prevents uric acid crystallization. Phytochemical analysis was also performed to determine the bioactive components in the extract.

Materials and Methods

Potassium chloride, ammonium chloride, sodium dihydrogen phosphate, and sodium sulphate were purchased from Merck Life Science Pvt. Ltd. and sodium chloride and sodium hydroxide were procured from Thermo Fisher Scientific Pvt. Ltd. Magnesium sulphate heptahydrate, uric acid and Folin-Ciocalteu reagent (FC) and hydrochloric acid were purchased from Avantor Performance Materials India Ltd., Sisco Research Laboratories Pvt. Ltd. and Finar, respectively. All reagents were of an analytical grade.

Preparation of plant extract

The *Tribulus terrestris* plant powder which is prepared from five different parts of the plant *i.e.* flower, fruit, bark, root and leaves was obtained from an Ayurvedic shop in Nagpur, Maharashtra. Each portion was shade dried and then milled into a fine powder. The *Tribulus* extract was made by weighing

approximately 25 g of plant powder in 100 mL of distilled water. The extract was prepared in a three-neck round-bottom flask by heating at 100°C (20 min) in a heating mantle. After filtration, the extract was stored in at 4°C.

Phytochemical analysis

The standard method for phytochemical (phenols, flavonoids, alkaloids, sterols, glycosides, and saponins) analysis was acquired from¹⁷. *Tribulus* extract's total phenolic content (TPC) was estimated by a modified Folin-Ciocalteu assay¹⁸, and total flavonoid content (TFC) was quantified using the aluminium chloride (AlCl₃) method¹⁹. A modified phosphomolybdate test was used to assess total antioxidant capacity (TAC)²⁰. The details of the analysis and the standard curves are included in supplementary material (sections 1, 2, and 3).

Crystallization of uric acid

Synthetic urine was prepared a fresh as per the composition reported elsewhere²¹. By taking 2 g of uric acid in 1 L of 0.05 M NaOH solution, a solution was prepared. Uric acid crystallization procedures were described elsewhere²¹. The crystallization was carried out in two jacketed vessels, each containing 160 mL of synthetic urine with 40 mL of uric acid solution leading to a final measured pH of 6.2 (using Labman LMMP 30, pH meter). The *Tribulus* extract in different volumes (0-1000 µL) was

introduced into the vessels in order to examine its influence on uric acid crystals (UAC) formation. The pH of the solution was then changed to 4, 4.5, and 5, respectively, with HCl solution. It was also possible to measure the induction time for crystallization, which is defined as the time when the solution first became turbid. Uniform mixing of the solution was done with a REMI 1 MLH magnetic stirrer and 37°C was maintained inside the flask over the course. Microcentrifuge tubes were used to harvest the crystals formed during the process and were stored in a refrigerator at 4 °C.

Characterization

Several solid-state characterisation methods, including Fourier transform infrared spectroscopy (FTIR), X-ray diffractometry (XRD), and scanning electron microscopy (SEM), were used to comprehend physicochemical characteristics of the crystals that were produced. Using a light microscope (Optscopes) fitted with a 40X objective lens and a digital camera, pictures of the UAC were taken. The size of the crystals was then measured using ImageJ software. The Rigaku Miniflex 600 X-ray diffractometer with Cu-K radiation was used to analyse the UAC characteristics. Using an IRSpirit (Shimadzu) spectrophotometer, the materials' infrared spectra was obtained within the wavenumber range of 500 to 4000 cm^{-1} . The morphology of the samples was determined by SEM (JEOL JSM-6380A) at a voltage of 5kV, while the EDS analysis was conducted at a voltage of 15 kV.

Results and Discussion

Effect of *Tribulus extract* on induction time of uric acid crystallization

It was observed that the average induction time increased with higher concentration of extract and increasing pH (Fig. 1). At pH 4.5, when the extract concentration was increased from 37.3 $\mu\text{g/mL}$ to 149.2 $\mu\text{g/mL}$, the average induction time increased by a factor of 38 compared to the absence of the extract. A similar trend was observed at pH values of 4 and 5, with a significant delay in induction time noted at pH 5. This delay is attributed to the enhanced solubility of uric acid at higher pH levels. The increase in the average induction time with increase in extract concentration proves the effectiveness of the *Tribulus extract* inhibiting the uric acid crystallization.

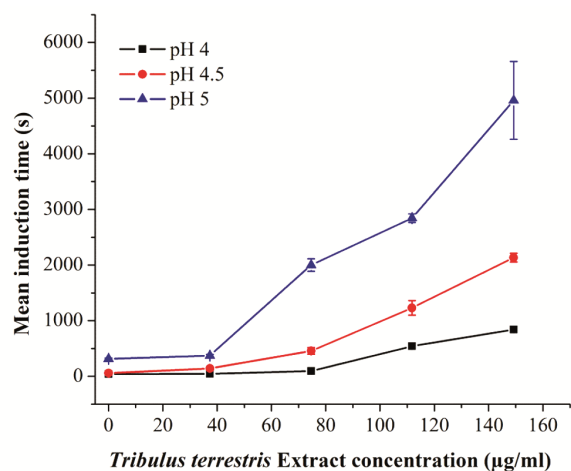


Fig. 1 — Effect of *Tribulus* extracton induction time of uric acid crystallization at three different pH values

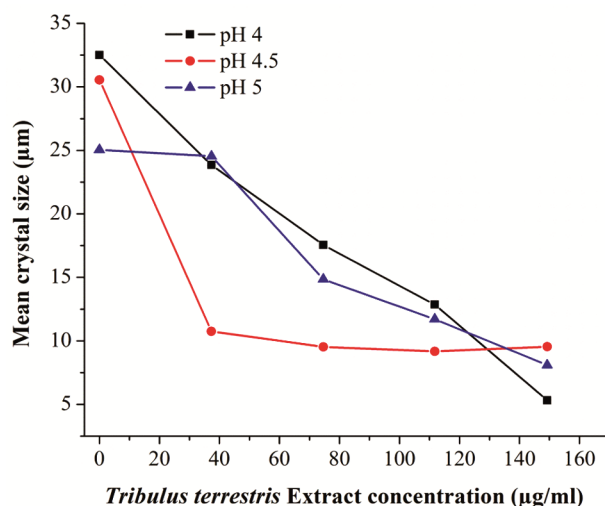


Fig. 2 — Effect of *Tribulus* extracton crystal size of uric acid crystallization at three different pH values

Effect of *Tribulus extracton* uric acid crystal size:

The effect of addition of *Tribulus* extracton the mean particle size of UAC is shown in (Fig 2). The mean crystal size reduced significantly with the inclusion of *Tribulus* extract in synthetic urineat all pH values. The trend of decrease of the mean crystal size at the three pH values was different. At pH 4, the mean crystal size decreased linearly, and a decrease of approximately 4.5 times was observed when the concentration of extract was increased from 37.31 $\mu\text{g/mL}$ to 149.25 $\mu\text{g/mL}$. At pH 4.5, in comparison to crystal obtained in absence of extract, a decrease of approximately 2.84 times was observed when 37.31 $\mu\text{g/mL}$ extract was added. At other concentration of *Tribulus* extract, the change in mean

crystal size was insignificant. At pH 5, there was an insignificant difference between mean crystal size of UAC in absence and in presence of 37.31 $\mu\text{g/mL}$. When the extract concentration was changed from 37.31 $\mu\text{g/mL}$ to 149.25 $\mu\text{g/mL}$, a decrease of ~ 3 times was observed. It could be inferred from (Fig 2) that the presence of *Tribulus extractis* resulting in the decrease in mean crystal size at all pH values.

Phytochemical analysis

A qualitative phytochemical analysis of *Tribulus* extract (Suppl. Table S1) revealed the presence of phenols, flavonoids, saponins, and steroids. The quantitative phytochemical analysis of the extract to evaluate total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant activity (TAC) revealed that TPC in extract was 77.5 mg GAE/g, while the TFC and AAC were 47.5 mg quercetin/g of extract and 63.5 mg AAE/g of extract respectively. As discussed earlier, the inclusion of extract in synthetic urine resulted in inhibition of uric acid crystallization by both delaying the induction of uric acid crystallization and reducing the average crystal size. The phytochemicals present in the extract could interact with the uric acid molecules, resulting in extract's inhibitory action. It has been reported earlier with uric acid crystals that different molecules tend to interact with different faces of uric acid crystals. The presence of theobromine is observed to interact with the $\{210\}$ face of the uric acid crystals¹⁰, while the interactions of methylene blue dye was localized within the $\{001\}$ and $\{201\}$ crystal faces²². Interaction of these phytochemicals with uric acid molecules can hinder the formation of nuclei and growth of crystals, both of which are important events in crystallization.

Alongside inhibiting the crystallization of uric acid, presence of above-mentioned phytochemicals can also provide health benefits. As anti-oxidative and anti-inflammatory agents^{23–25}, phenols and flavonoids are also known for scavenging free radicals and reducing stress in body cells, hence suggesting that *Tribulus* extract may reduce body oxidative stress²⁶.

Characterization

The FTIR analysis of UAC in the presence of extract is shown in (Fig. 3). The FTIR spectra of the crystals, both with and without the *Tribulus* extract, appear similar. The bands are represented by dashed lined and the results are almost similar with the FTIR result of pristine UAC. The FTIR analysis results indicate, C – C and C – N stretching at 1584 cm^{-1} ,

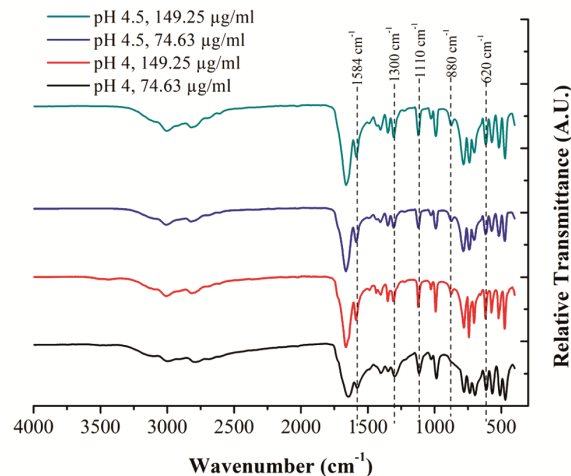


Fig. 3 — FTIR spectra of uric acid crystals obtained in the presence of different concentrations of *Tribulus* extract

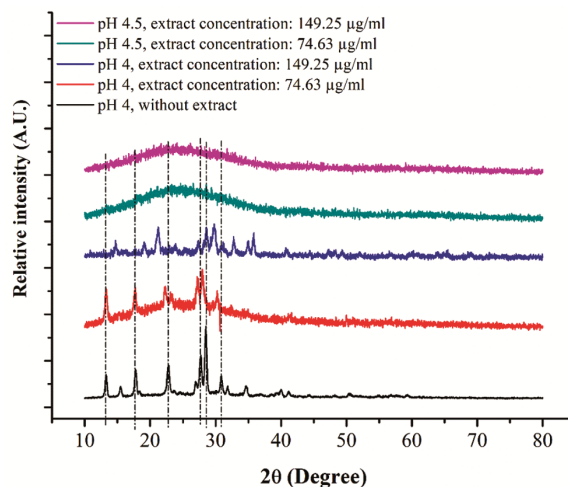


Fig. 4 — PXRD pattern of uric acid crystals obtained in the presence of different concentrations of *Tribulus* extract

O – H distortion at 1300 cm^{-1} , ring vibration at 1110 cm^{-1} , N – H out-of-plane and in-plane bending at 880 cm^{-1} , and skeletal ring modification at 620 cm^{-1} . No additional peaks were observed, but the intensity of existing peaks varied with the addition of the extract. This variation in intensity suggests interactions between the phytochemicals in the *Tribulus* extract and the uric acid molecules without significantly altering their chemical composition. Therefore, it is evident that the interactions between uric acid molecules and the phytochemicals in the extract are primarily physical in nature.

To examine the impact of adding *Tribulus* extract on the crystallinity of UAC, the samples were analysed using PXRD. The PXRD patterns of UAC in the presence of *Tribulus* extract is shown in (Fig. 4).

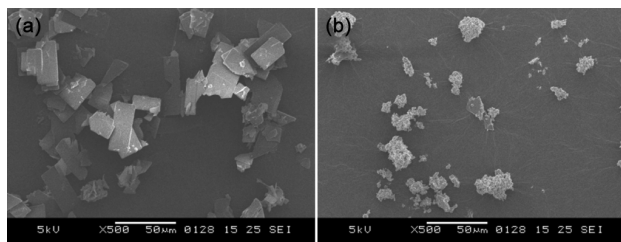


Fig. 5 — Scanning electron microscope images of uric acid crystals (a) in absence of extract and (b) in presence of *Tribulus* extract at pH 4

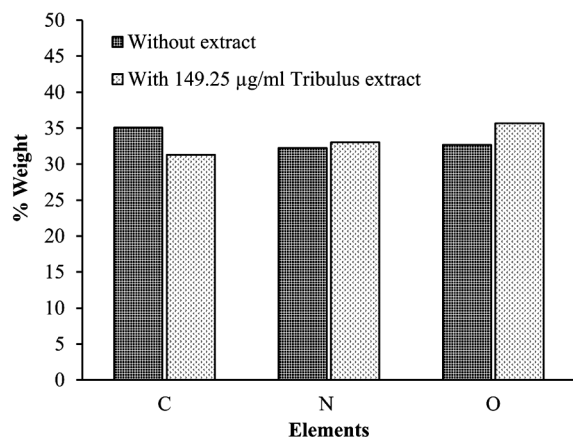


Fig. 6 — Weight % of Carbon, Nitrogen and Oxygen in uric acid crystals obtained in the absence and presence of 149.25 µg/mL *Tribulus* extract

For comparison, PXRD pattern of pristine UAC obtained at pH 4 is also shown. As evident in (Fig 4), the increasing concentration of extract results in decrease in the crystallinity of samples. The samples obtained at pH 4.5 were observed to be more amorphous than the samples obtained at pH 4. This decrease in crystallinity can be attributed to the inclusion of phytochemicals in the crystal matrix.

To verify the change of crystallinity, samples obtained at pH 4 were subjected to scanning electron microscopy. Sample obtained at pH 4 without extract (Fig. 5a) were more crystalline than the sample obtained at same pH with extract concentration of 149.25 µg/mL (Fig 5b). The crystal size, as previously discussed (Fig 2), was observed to decrease with increasing extract concentration. To check for the incorporation of any phytochemicals in the crystals, samples were subjected to EDS, at a magnification of 1000 x a voltage of 15 kV. From the EDS data (Fig. 6), it was evident that the weight percentage of C, N, and O changed when extract was used during the crystallization of uric acid. As C, N, and O are the key elements present in most of

the phytochemicals, it can be concluded that the phytochemicals are incorporated in the crystal lattice of uric acid, when it crystallizes in the presence of extract. The incorporation of phytochemicals in UAC lattice was also evident from the transition of colour of uric acid samples from colourless to light brown.

Conclusion

The inclusion of aqueous *Tribulus* extract in synthetic urine delayed the induction of uric acid crystals. At a fixed pH (4.5), the induction time of uric acid increased (38 folds) with increasing the extract concentration (from 37.3µg/mL to 149.2µg/mL). The inclusion of aqueous *Tribulus* extract also reduced the mean size (by 3 folds) of UAC. When extract concentration was changed from 0 to 37.3 µg/mL at pH 4.5. FTIR analysis suggests that the interaction of phytochemicals in extract with uric acid molecules is purely physical. The PXRD patterns and the SEM images displayed the reduction in the crystallinity of UAC in the presence *Tribulus* extract. EDS analysis suggests the same as a change in the percentages of C, N, and O in UAC samples. The presence of certain bioactive compounds in the *Tribulus* extract was confirmed by the phytochemical analysis and it also showed significant amount of phenol, flavonoid and total antioxidant activity. These results show the potential of *Tribulus* extract as a natural inhibitor of uric acid crystallization.

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Conflicts of interest

All authors declare no conflict of interest.

References

- 1 Wigner P, Grębowski R, Bijak M, Szymraj J & Saluk-Bijak J, The molecular aspect of nephrolithiasis development. *Cells*, 10 (2021) 1.
- 2 Krishnan AS, Pradhane AP, Dhamole PB & Methekar RN, Inhibitory potential of *Ocimum tenuiflorum* L. on uric acid crystallization: An investigational study. *Indian J Exp Biol*, 62 (2024) 496.
- 3 Pradhane AP, Methekar RN & Agrawal SG, Batch Crystallization of Uric Acid: Modeling, Simulation, and the Impact of 3, 7-dimethylxanthine. *Crystal Res Technol*, 57 (2022) 2200125.
- 4 Khan SR, Pearle MS, Robertson WG, Gambaro G, Canales BK, Doizi S, Traxer O & Tiselius HG, Kidney stones. *Nat Rev Dis Primers*, 2 (2016) 16008.

- 5 Tasian GE, Kabarriti AE, Kalmus A & Furth SL, Kidney Stone Recurrence among Children and Adolescents. *J Urol*, 197 (2017) 246.
- 6 Schlittenbauer L, Seiwert B & Reemtsma T, Matrix effects in human urine analysis using multi-targeted liquid chromatography- tandem mass spectrometry. *J Chromatogr A*, 1415 (2015) 91.
- 7 Bouatra S, Aziat F, Mandal R, Guo AC, Wilson MR, Knox C, Bjorn Dahl TC, Krishnamurthy R, Saleem F, Liu P, Dame ZT, Poelzer J, Huynh J, Yallou FS, Psychogios N, Dong E, Bogumil R, Roehring C & Wishart DS, The Human Urine Metabolome. *PLoS One*, 8 (2013) e73076.
- 8 Khan SR, Crystal/cell interaction and nephrolithiasis. in *Arch Ital Urol Androl*, 83 (2011)1.
- 9 Abou-Elela A, Epidemiology, pathophysiology, and management of uric acid urolithiasis: A narrative review. *J Adv Res*, 8(2017) 513.
- 10 Grases F, Rodriguez A & Costa-Bauza A, Theobromine Inhibits Uric Acid Crystallization. A Potential Application in the Treatment of Uric Acid Nephrolithiasis. *PLoS One*, 9 (2014) e111184.
- 11 Ramaswamy K, Killilea DW, Kapahi P, Kahn AJ, Chi T & Stoller ML, The elementome of calcium-based urinary stones and its role in urolithiasis. *Nat Rev Urol*, 12 (2015) 543.
- 12 Kesavan M, Kaliaperumal R, Tamilmani E & Shanmugam K, In vitro evaluation of calcium oxalate monohydrate crystals influenced by *Costus igneus* aqueous extract. *Scand J Urol Nephrol*, 46 (2012) 290
- 13 Pradhane AP, Methekar RN & Agrawal SG, Batch Crystallization of Uric Acid: Modeling, Simulation, and the Impact of 3, 7-dimethylxanthine. *Crystal Res Technol*, 57 (2022) 2200125.
- 14 Königsberger E & Wang Z, Solubility of uric acid in salt solutions and artificial urine. *Monatsh Chem*, 130 (1999) 1067.
- 15 Arowojolu O & Goldfarb DS, Treatment of calcium nephrolithiasis in the patient with hyperuricosuria. *J Nephrol*, 27 (2014) 601.
- 16 Saeed M, Munawar M, Bi JB, Ahmed S, Ahmed MZ, Kamboh AA, Arain MF, Naveed M & Chen H, Promising phytopharmacology, nutritional potential, health benefits, and traditional usage of *Tribulus terrestris* L. herb. *Heliyon*, 10 (2024) e25549.
- 17 Agidew MG, Phytochemical analysis of some selected traditional medicinal plants in Ethiopia. *Bull Natl Res Cent*, 46 (2022) 1.
- 18 Berker KI, Ozdemir Olgun FA, Ozyurt D, Demirata B & Apak R, Modified Folin-Ciocalteu antioxidant capacity assay for measuring lipophilic antioxidants. *J Agric Food Chem*, 61 (2013) 4783.
- 19 Ling YY, Fun S, Yeop A, Yusoff MM & Gim bun J, Assessment of maceration, ultrasonic and microwave assisted extraction for total phenolic content, total flavonoid content and kaempferol yield from *Cassia alata* via microstructures analysis. *Mater Today Proc*, 19 (2019) 1273.
- 20 Saeed N, Khan MR & Shabbir M, Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. *BMC Complement Altern Med*, 12 (2012) 1.
- 21 Pradhane AP, Methekar RN & Agrawal SG, Investigations on melamine-based uric acid kidney stone formation and its prevention by inhibitors. *Urolithiasis*, 51 (2023) 1.
- 22 Sours RE, Fink DA & Swift JA, Dyeing uric acid crystals with methylene blue. *J Am Chem Soc*, 124 (2002) 8630.
- 23 Maleki S J, Crespo J F & Cabanillas B, Anti-inflammatory effects of flavonoids. *Food Chem*, 299 (2019) 1.
- 24 Jomova K, Alomar SY, Valko R, Liska J, Nepovimova E, Kuca K & Valko M, Flavonoids and their role in oxidative stress, inflammation, and human diseases, *Chem Biol Interact*, 413 (2025) 111489.
- 25 Sobhani M, Farzaei MH, Kiani S & Khodarahmi R, Immunomodulatory; Anti-inflammatory/antioxidant Effects of Polyphenols: A Comparative Review on the Parental Compounds and Their Metabolites. *Food Rev Int*, 37 (2021) 759.
- 26 Tyagi P & Ranjan R, Comparative study of the pharmacological, phytochemical and biotechnological aspects of *Tribulus terrestris* Linn. and *Pedalium murex* Linn: An overview. *Acta Ecologica Sinica*, 43 (2023) 223.