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IN- VITRO ANTI-UROLITHIASIS ACTIVITY OF THE *HIBISCUS ROSA-SINENSIS L.* LEAVES HYDRO-ALCOHOLIC EXTRACT ON CALCIUM OXALATE CRYSTALLIZATION

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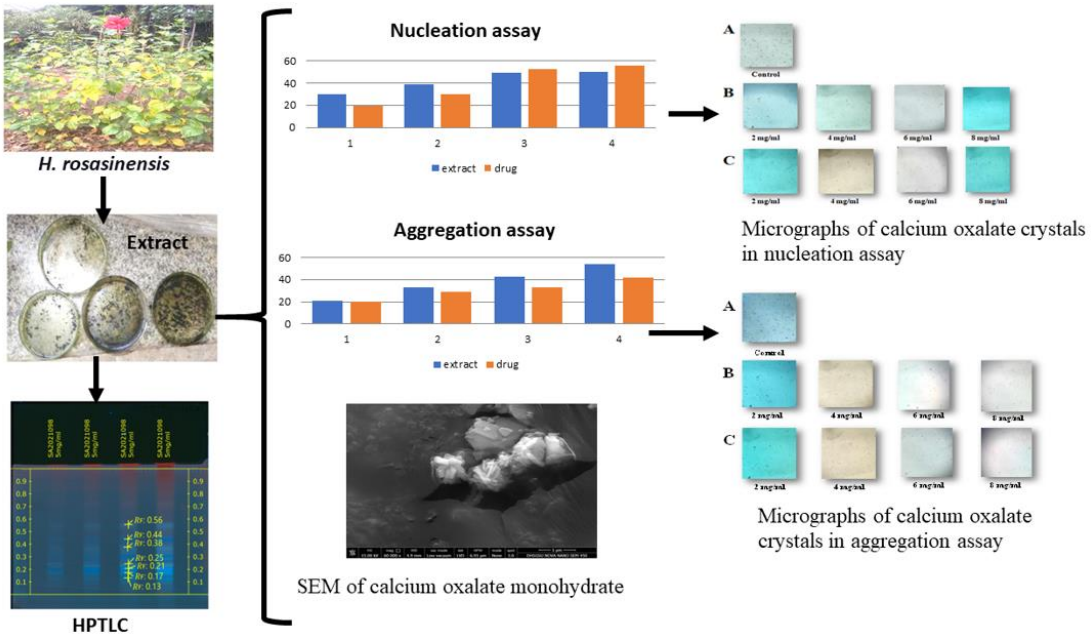
Abstract

The study examined how various amounts of *H. rosa-sinensis* hydroalcoholic extract prevented urolithiasis at different stone formation phases. The positive control was Cystone. The experiment examined how the extract affected calcium oxalate crystal formation in an unstable calcium chloride and sodium oxalate solution. A spectrophotometer at 620 nm monitored turbidity with time. The inhibition rate was calculated by comparing turbidity with and without the extract. A light microscope was used to examine the created crystals' number and shape in a random area. Phytochemical analysis and HPTLC were also performed on the extract. At all concentrations, *H. rosa-sinensis* significantly reduces crystal formation. *H. rosa-sinensis* extract inhibited nucleation by 29.98% to 50.45%, whereas cystone dosages inhibited it by 19.10% to 55.51%. *H. rosa-sinensis* extract inhibited aggregation from 20.88% to 53.66%, whereas cystone dosages inhibited it by 20.00% to 42.22%. Microscopic investigation revealed decreased crystal number and size. The hydro-alcoholic extract of *H. rosa-sinensis* shows anti-urolithiasis activities and might be studied further.

Keywords: Urolithiasis, Hibiscus Rosa-Sinensis, HPTLC, SEM, Herbal Medicine, Calcium Oxalate Crystals.

Graphical Abstract



1. INTRODUCTION

Urolithiasis is a condition that has been present since ancient times, affecting the urinary tract. It is a medical issue that affects about 12% of the world's population [1–3], with kidney stones being more common in men than women [4, 5]. Urinary tract stones are usually composed of calcium oxalate alone or mixed with calcium phosphate [6, 7]. The causes of urolithiasis can include being overweight or obese, having a family history of the condition, low fluid intake, poor diet, and certain medications [8, 9].

Kidney stones are formed when urine contains high levels of insoluble compounds such as calcium phosphate and calcium oxalate. This can happen due to dehydration or a genetic tendency to excrete these ions excessively in the urine. Kidney stones may cause severe cramping, urinary tract illness, back discomfort, bloody urine, infections, blocking urine flow, and dilatation of the kidneys (hydronephrosis). [10–12].

Numerous medicinal herbs have been found to prevent kidney stones in recent years. These plants change urine ion concentrations. They may reduce calcium ion excretion or enhance magnesium and citrate excretion. [13–15].

According to ancient literature, the leaves of the *H. rosa-sinensis* plant were used as diuretics and to treat kidney issues. This plant is originally from tropical Asia but has been spread worldwide. The *H. rosa-sinensis* plant is a member of the Malvaceae family. It is the oldest Indian herbal drug which is commonly known as Chinese hibiscus, shoeblack plant, rose mallow, Hawaiian hibiscus, and China rose and widely used by tribal people. The Ayurvedic system has already noticed the importance of this plant [16, 17]. It has several experimentally proven pharmacological activities, Its pharmacological effects include those of an antioxidant, immunomodulator, antidiabetic, fibrinolytic, hypolipidemic, memory-enhancing, cytotoxic, antimicrobial, antiparasitic, dermatological, urinary, anti-haemolytic, neuroprotective, hepatoprotective,and antitussive, among many others [18–20]. Based on the previously proven activity of *H. rosa-sinensis* flower as anti-urolithiatic, the present work is a prospective approach to evaluate anti-urolithiasis activity of leaves of *H. rosa-sinensis*.

2. MATERIALS AND METHODS

2.1 Plant collection and extract preparation

The *H. rosa-sinensis* leaves were gathered from Sagar, Madhya Pradesh in October 2019. The plant was identified and confirmed by Dr Pradeep Tiwari from the Department of Botany at Dr Harisingh Gour Vishwavidyalaya, Sagar, Madhya Pradesh. A voucher specimen was deposited under the Herbarium number BOT/H/06/203/03. After being let to dry in the shade, the leaves were ground into a powder using an electric grinder fitted with a No. 40 screen. Various leaf-to-solvent ratios (1:5, 1:10, and 1:15) were used in a Soxhlet's apparatus to yield a 70% hydroalcoholic extract (30:70 v/v) of *H. rosa-sinensis*. To get a solid extract residue, the resulting filtrate was then dried on a water bath set at 80°C for 6-8 hours and then filtered. The study found that the extract in a 1:5 ratio produced a maximum percentage yield of 10.3%. To ensure consistency, the concentration of the extract was standardized using qualitative phytochemical analysis. The extract in the aforementioned ratio contained the highest amount of phytoconstituents, making it suitable for use in the anti-urolithiasis study. The extract was stored in an airtight

container and refrigerated. The dried extract and reference medicine cystone were dissolved in distilled water at concentrations ranging from 2 to 8 mg/ml for each experiment.

2.2 Chemicals, reagents and solvents

The extraction, nucleation, and aggregation experiments used high purity grade solvents, reagents, and chemicals. The following chemicals were procured from Sigma-Aldrich: sodium oxalate, calcium chloride dihydrate, tris-buffer, chloroform, petroleum ether, and sodium chloride. Methanol was purchased from Hi-Media. The standard drug cystone was recieved from Pharma House stores in Sagar, Madhya Pradesh. The apparatus used for the experiments included Thermo Scientific Orion Aquamate 8000 UV-Vis spectrometer, Borosil Soxhlet with a capacity of 500ml, a benchtop pH meter, and a microscope with a sdigital imaging system with a camera. The morphology of calcium oxalate crystals was observed using NOVA NANOSEM 450.

2.3 In vitro study

2.3.1 Calcium oxalate crystals preparation

In order to create calcium oxalate crystals, a test tube was filled with 1 ml of 0.025M calcium chloride dehydrate and 2 ml of Tris buffer (0.05mol/l). Subsequently, a volume of 1 ml of a 0.025M solution of sodium oxalate was introduced into the same test tube to induce crystallization at 7.4 pH, and the following formula determined the expected crystallization:

$$CaCl_2 + Na_2C_2O_4 \longrightarrow CaC_2O_4 + 2NaCl$$

2.3.2 Nucleation assay

The production of stones commences with the presence of nuclei. The investigation of oxalate crystallization adhered to the previously established paradigm, with minor adjustments, as outlined in prior research [21]. The experiment involved testing various *H. rosa-sinensis* leaf extract concentrations and the standard compound cystone (at concentrations of 2, 4, 6, and 8 mg/ml). Initially, 1 ml of 0.025M calcium chloride dihydrate, 2 ml of Atari's buffer (0.05 mol/l), and 1 ml of plant leaf extract at varying concentrations were added for each sample. Subsequently, a volume of 1 ml of 0.025M sodium oxalate was introduced into a test tube maintained at room temperature (37°C) in order to investigate the extent of inhibition. The procedure was iterated six times for each concentration. The absorbance of the control, test, and standard samples was measured at a wavelength of 620 nm after a duration of 1 hour. The below equation was used to calculate the percentage inhibition of nucleation:

$$\textbf{Percentage inhibition of nucleation} = [\,(C-S)/C] \times 100$$

where C = turbidity without plant extract and S = turbidity with plant extract.

2.3.3 Aggregation assay

Calcium oxalate crystals were synthesized by combining calcium chloride and sodium oxalate, both at a concentration of 0.025 M. The combination was thermally stabilized at 60°C in a water bath for a duration of one hour, followed by gradual cooling over the course of the night to a temperature of 37°C. The obtained crystals were collected using centrifugation for a duration of 5 minutes and then dehydrated at a temperature of 37°C. The crystals were then used at a concentration of 0.8 mg/ml in a Tris-HCl

buffer (0.05 mol/L) supplemented with NaCl (0.15 mol/L) at a pH of 6.5. The experiment was conducted at ambient temperature with the inclusion and exclusion of leaf extracts at different doses (2, 4, 6, and 8 mg/ml). The percentage of aggregation inhibition was determined by assessing the turbidity of both control and test extracts.

% inhibition = (1- (turbidity sample/ turbidity control) × 100

2.3.4 Microscopic assay

The conventional procedure was followed in order to produce the calcium oxalate crystals. Following centrifugation, the crystal residue was transferred onto a Petri dish and examined using a microscope equipped with a digital imaging equipment at a magnification of 10X. The observations were conducted under two conditions: without *H. rosa-sinensis* leaf extract and with varied concentrations (2, 4, 6, and 8 mg/ml) of the extract. The purpose was to investigate the process of crystal formation and dissolution.

2.4 Phytochemical studies

The extract was subjected to various qualitative phytochemical tests for the detection of various primary and secondary metabolites such as alkaloids, carbohydrates, glycosides, tannins and phenolic compounds, etc [22].

2.4.1 HPTLC fingerprinting

An investigation of the leaves extract of *H. rosa-sinesis* was conducted using HPTLC with a CAMAG automated TLC sample 4 (ATS4). The mobile phase included ethyl acetate, formic acid, glacial acetic acid, and water in a volumetric ratio of 10:1.1:1.1:2.6. Sample preparation was done with 25 mg/mL of ethanol. Merck TLC AI plates with silica gel 60 F254 were used as the adsorbent. The position of the solvent front was 70mm, and a scanning wavelength of 254 nm was used. The TLC plates were subjected to thermal treatment at a temperature of 150°C for a duration of 5-10 minutes and thereafter examined under visible light with a wavelength of 550 nm. The spraying agent used for spot detection on the TLC plate was a mixture of 0.5ml of anisaldehyde and 10ml of glacial acetic acid, with sulphuric acid. Spot detection was performed at wavelengths of 254nm, 366nm, and 540nm.

3. RESULTS

3.1 Effect of *H. rosa-sinensis* hydro-alcoholic Leaf extract on Calcium Oxalate nucleation

The graphs presented below show the inhibition capacity of *H. rosa-sinensis* leaf extracts on nucleation activities (**Table 1, Figure 1**). This was achieved by comparing the results of the extracts with those of the standard drug, cystone. The nucleation test was quantified by monitoring the variation in turbidity caused by the extract, with a solution devoid of extract acting as a control. The results showed that the turbidity in the solution containing the extract decreased dose-dependently compared to the control. However, the highest inhibition of nucleation activity was obtained from the standard drug, cystone. The study found that the anti-crystallization activity of cystone was highest at a concentration of 6mg/ml, with an inhibition percentage of 55.51%. This was 5.06% higher than the inhibition percentage of *H. rosa-sinensis* leaf extract, which was 50.45%. However, at lower concentrations (10mg/ml and 4mg/ml), the inhibition percentage of nucleation assay of *H. rosa-sinensis* leaf extract was higher

than that of cystone. At a concentration of 2mg/ml, the inhibition percentage of the extract was 29.98%, while that of cystone was 19.10%. At 4mg/ml concentration, the inhibition percentage of the extract and cystone were 38.48% and 30.09%, respectively.

Table 1: Effect of *H. rosa-sinensis* leaf extract on Calcium oxalate nucleation

Sample	Absorbance	% Transmittance	Optical Density	%Inhibition
E1	0.031	93.111	0.0214	49.5
E2	0.176	66.681	0.0299	29.48
E3	0.22	60.256	0.0331	21.93
E4	0.252	55.976	0.0357	15.8
D1	0.064	86.298	0.0231	45.51
D2	0.084	82.414	0.0242	42.92
D3	0.235	58.211	0.0343	19.1
D4	0.256	55.463	0.036	15.09
Control	0.327	47.098	0.0424	

3.2 Effect of *H. rosa-sinensis* hydro-alcoholic extract on Calcium Oxalate aggregation

The study aimed to determine the effect of inhibiting aggregation activities by comparing the rate of turbidity change in the presence of an extract to a control without the extract. The hydroalcoholic extract of *H. rosa-sinensis* was found to be more effective than the standard drug, cystone. *H. rosa-sinensis* extract inhibited aggregation by 20.88% to 53.66%, while cystone inhibited aggregation by 20.00% to 42.22%. The highest percentage of inhibition occurred at 6mg/ml of both cystone and *H. rosa-sinensis* extract. At this concentration, *H. rosa-sinensis* extract inhibited aggregation by 53.66%, while cystone only inhibited aggregation by 20.00%. Furthermore, at 10mg/ml, *H. rosa-sinensis* extract inhibited aggregation by 20.88%, which was 11.44% higher than cystone.

Table 2: Effect of *H. rosa-sinensis* leaf extract on calcium oxalate aggregation

Sample	Absorbance	% Transmittance	Optical Density	% inhibition
E1	0.121	75.6833	0.026	42.22
E2	0.180	66.0693	0.030	33.33
E3	0.212	61.3762	0.032	28.88
E4	0.265	54.325	0.036	20.00
D1	0.158	69.5024	0.028	38.66
D2	0.179	66.2217	0.030	33.66
D3	0.259	55.0808	0.036	20.00
D4	0.321	47.7529	0.041	8.88
Control	0.358	43.8531	0.045	

3.3 Phytochemical investigation

As shown in Table 3, qualitative analysis of *H. rosa-sinensis* extract showed the presence of Alkaloids, steroids, proteins, amino acids, phytosterols, carbohydrates, and saponins.

Table 3: Phytochemical screening of *H. rosa-sinensis* leaf extract

Chemical constituents	Test	Inference
Alkaloids	Wagner test Hager test Dregondorffs test	+ + +
Carbohydrates	Fehling test Molish test	- -
Glycoside	Anthraquinone test	_
Triterpenoids	Liebermann test	-
Proteins	Ninhydrine test	-
Steroids	Liebermann test	+
Tannine	5%Fecl3	-

3.4 HPTLC fingerprinting analysis

The results are shown in table 4 and figure 6.

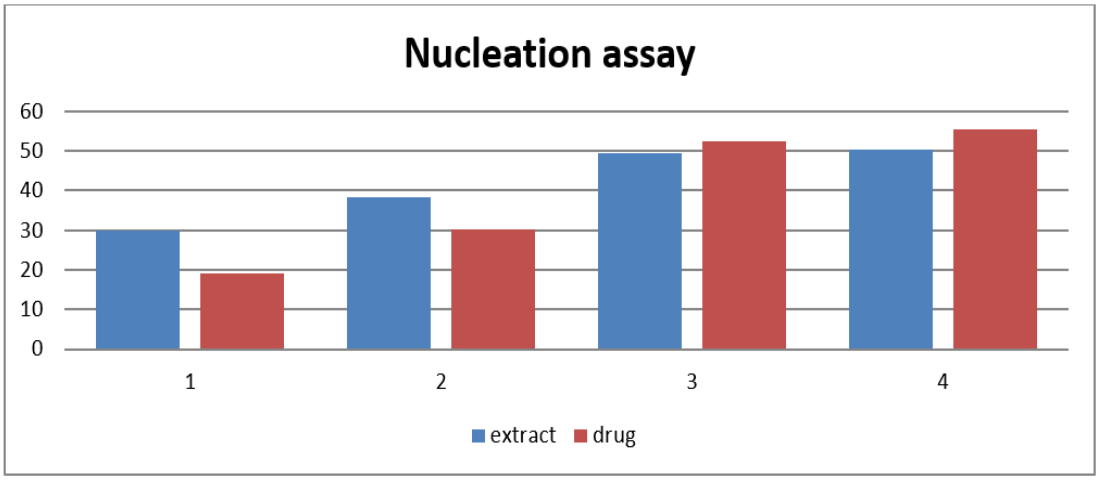


Fig 1: The effect of *H. rosa-sinensis* extract and cystone on the nucleation assay

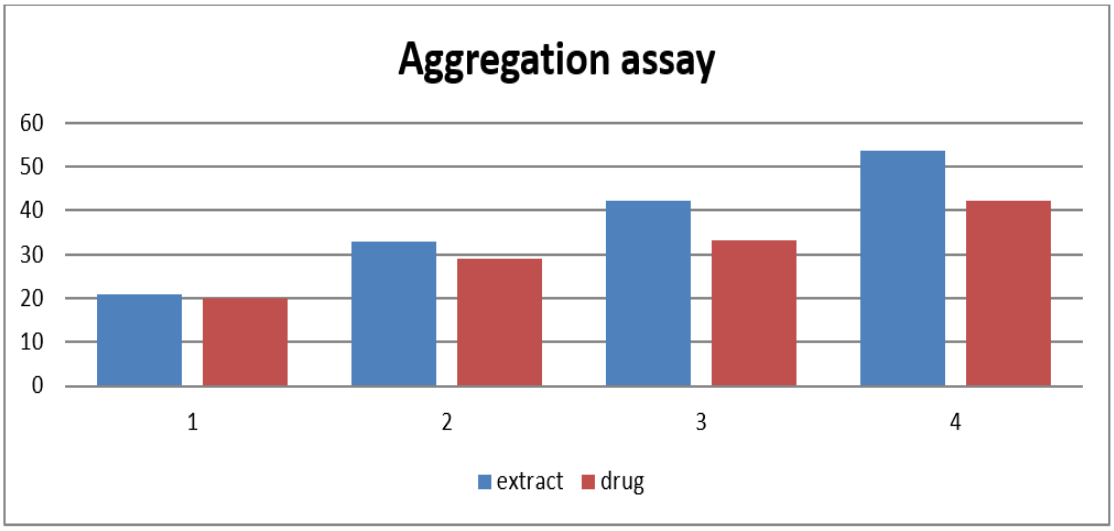


Fig 2: The effect of *H. rosa-sinensis*extract and cystone on the aggregation assay

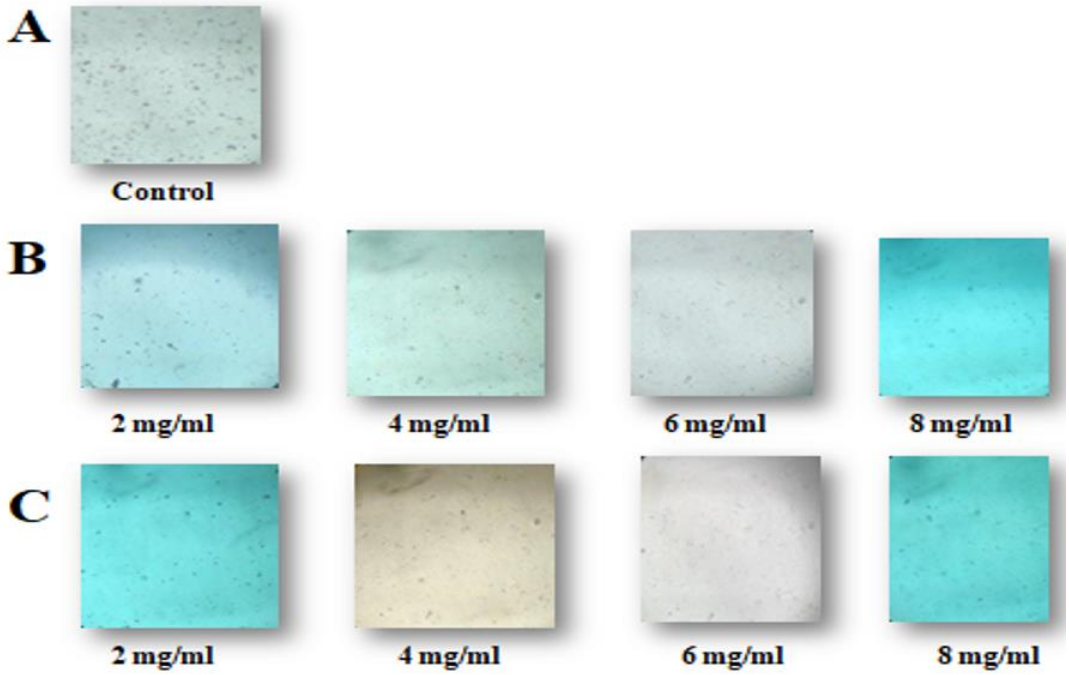


Fig 3: These micrographs depict calcium oxalate crystals seen during a nucleation experiment. Group A serves as the control group, while group B demonstrates the formation of calcium oxalate crystals when exposed to varying concentrations of cystone. Group C, on the other hand, indicates the presence of varied concentrations of *H. rosa-sinensis* hydro-alcoholic extract

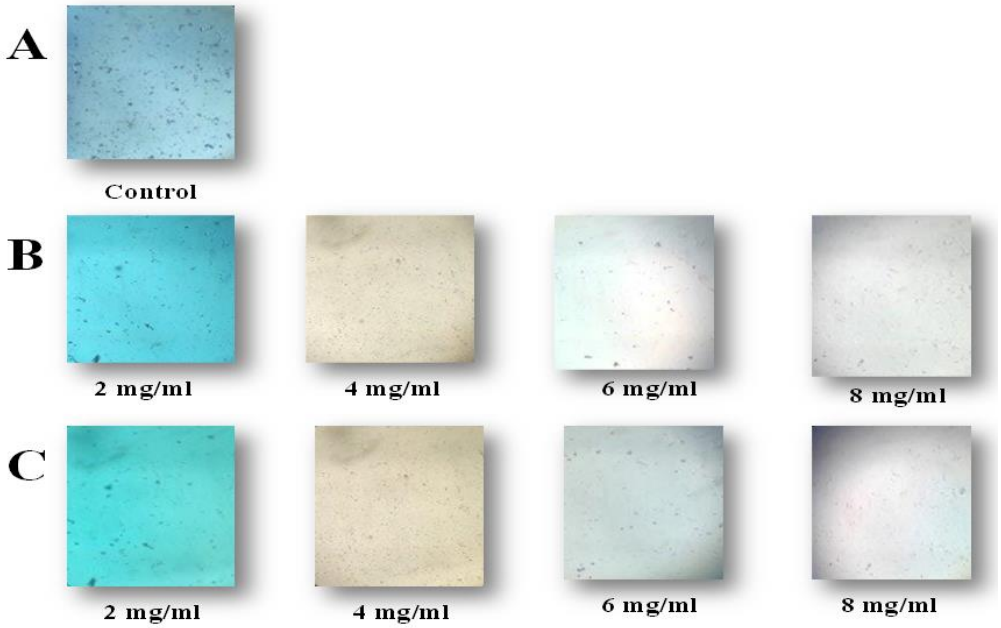


Fig 4: The micrographs depict calcium oxalate crystals seen in an aggregation experiment. Group A serves as the control group, Group B demonstrates the aggregation of calcium oxalate crystals in the presence of different concentrations of cystone, and Group C indicates the presence of different concentrations of the hydro-alcoholic extract of *H. rosa-sinensis*

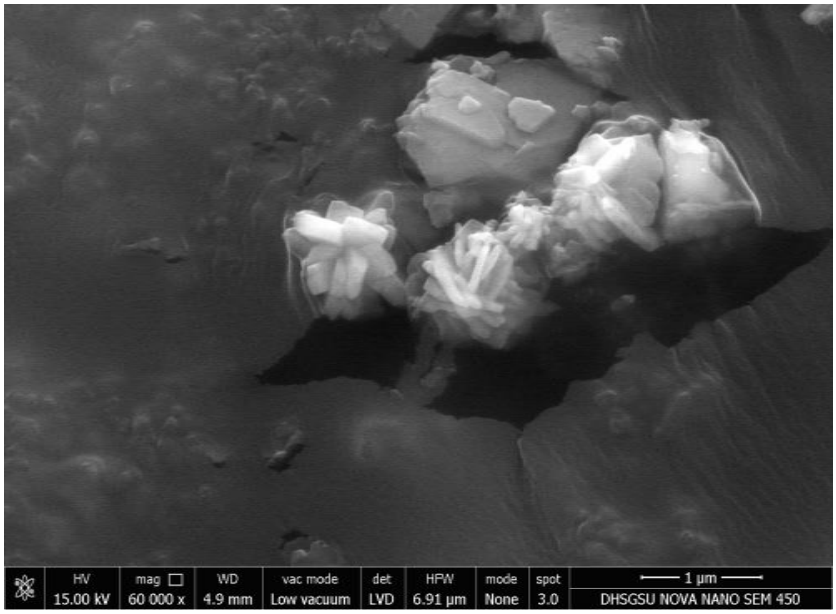
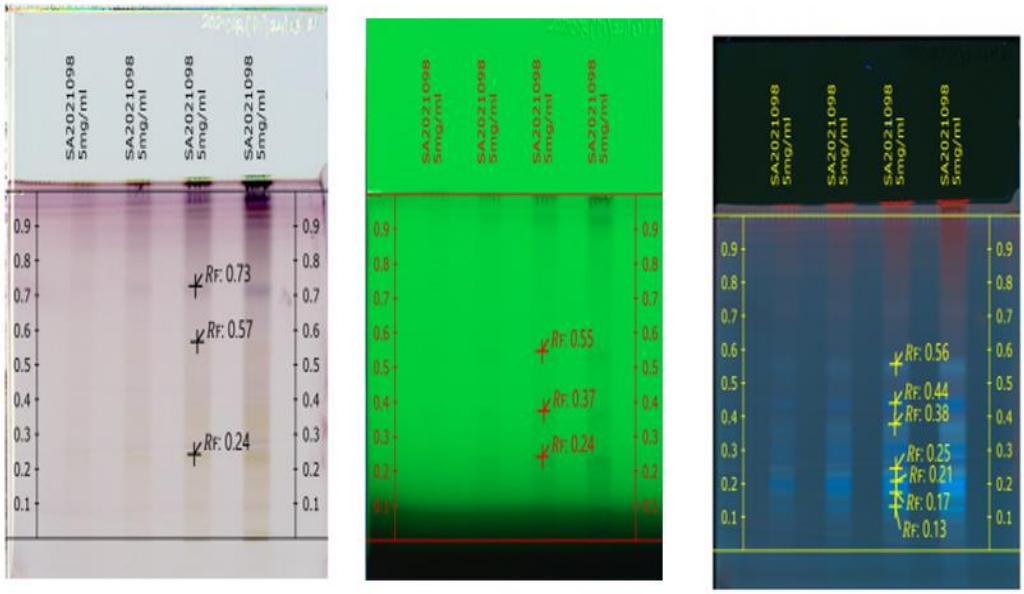


Fig 5: Scanning electron micrograph of calcium oxalate monohydrate

Table 4: HPTLC analysis of *H. rosa-sinensis*

Rf	color	Intensity
0.13	Blue	Medium
0.17	blue	High
0.21	blue	High
0.25	Sky blue	Medium
0.38	blue	Medium
0.44	blue	high
0.56	Sky blue	medium



a

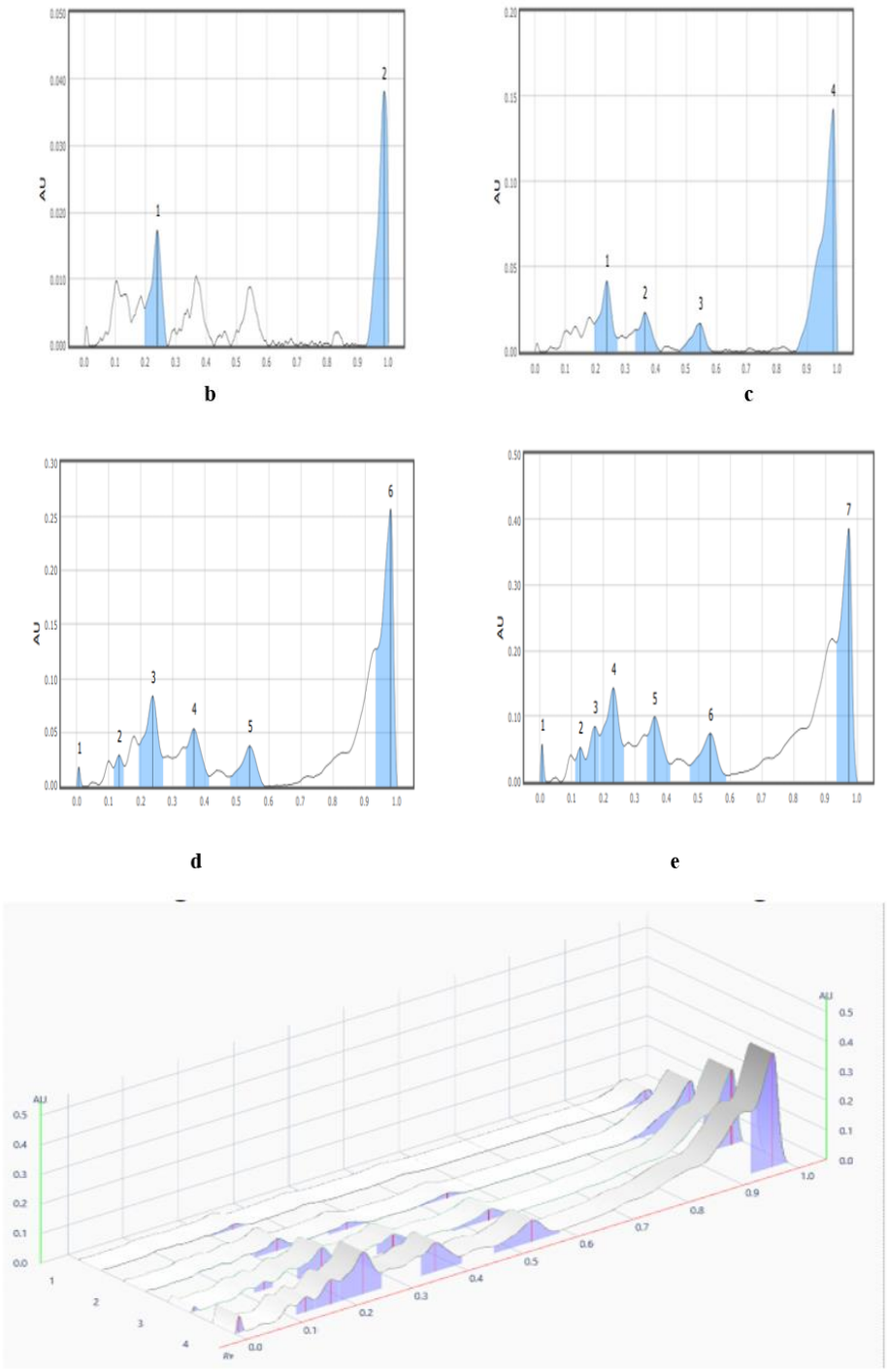


Fig 6: An analysis of the *H. rosa-sinensis* using high-performance thin layer chromatography to create a fingerprint and monitor its characteristics. A depicts the high-performance thin layer chromatography of *H. rosa-sinensis* conducted at various wavelengths. B to E represent distinct tracks of *H. rosa-sinensis*. The numbers 1, 2, 3, 4, 5, 6, and 7 in the figure correspond to the fingerprinting conducted at different wavelengths. The 3D chromatogram is shown by F

4. DISCUSSION

All parts of a *Hibiscus rosa sinensis* have been widely used in traditional medicine since many earlier times. It has been known to be used as an anti-tumour, anti-infertility, anovulatory, antispasmodic, anti-implantation, antiviral, antipyretic, anti-inflammatory, antifungal, analgesic, antibacterial, antiestrogenic, spasmolytic, hypoglycaemic, hypotension, juvenoid activity and CNS depressant [23].

Previous studies found that *H. rosa-sinensis* flower extract effectively inhibited crystal nucleation, growth, and aggregation at various concentrations [24, 25]. This preliminary scientific evidence suggests that *H. rosa-sinensis* leaf extract may also have lithotripsy properties, which can be beneficial in treating urolithiasis. The development of calcium oxalate stones involves a series of stages, including nucleation, crystal growth, aggregation, and retention. The development of stones is influenced by the equilibrium between crystalloids and the substances that prevent stone formation in urine, with nucleation being the first stage in the production of kidney stones [26].

The hydroalcoholic solvent is used to extract plants. The extract obtained had a yield of 10.3%, was dark brown in color and had a semi-solid consistency. The qualitative phytochemical analysis of *H. rosa-sinensis* hydroalcoholic extract showed the presence of several compounds like alkaloids, steroids, proteins, amino acids, phytosterols, carbohydrates, and saponins. Cysteine served as a positive control in this investigation to assess the differences in the suppression of nucleation and aggregation activities. Cystone is a herbal supplement used to treat renal calculi illness and urolithiasis. It is made by combining three powerful herbs known to support the health of the urinary tract; Small caltrops (Gokshura), Pasanabheda (Saxifraga ligulata), and Shilapushpa (Didymocarpuspedicellata) [27].

Nucleation is the initial stage in forming calcium oxalate crystals, which then grow and form aggregates. The effect of *H. rosa-sinensis* leaf extract on calcium oxalate crystallization was evaluated by measuring turbidity. The result of various concentrations of hydroalcoholic extract of *H. rosa-sinensis* showed nucleation preventing action and aggregation preventing action. A saponin-rich fraction of *Musa acuminate* × *balbisianacolla cv* 'Awaklegor' pseudo stem extract was found to be a potent inhibitor of calcium oxalate crystal formation in vitro [15, 28]. The extract of *H. rosa-sinensis* leaf also contains saponin, which inhibits the growth of calcium oxalate crystals. In a nucleation assay, the hydroalcoholic extract of *H. rosa-sinensis* reduces the turbidity of the solution in a dose-dependent manner. The cystone showed the highest inhibition at 40mg/ml, i.e., 55.51%, whereas the inhibition shown by extract at 4mg/ml was 50.45%. On the other hand, at lower concentration i.e. 10mg/ml and 20mg/ml the inhibition of turbidity by the extract was higher than the standard drug cystone. Aggregation is the process of crystals in free solution agglomerating to form larger multi-component particles. In the aggregation assay, the hydroalcoholic extract of leaves from *H. rosa-sinensis* demonstrated higher inhibition when compared to the standard drug cystone. The extract's inhibition percentage ranged from 20.88% to 53.66%, whereas the inhibition of cystone ranged from 20.00% to 42.22%.

Light microscopic images of the crystals in the nucleation assay showed an abundance of dehydrated type crystals in the control group. The number of crystals was directly proportional to the absorbance. However, the images with *H. rosa-sinensis* extract and cystone showed a reduction in the size and number of crystals in

a dose-dependent manner. The images of the control sample in the aggregation assay showed numerous large monohydrates and oval-shaped crystals. In contrast, the microscopic pictures of samples treated with cystone and extract showed a little aggregation of crystals and a decrease in crystal size, with a decrease in the number of crystals as the concentration rose. SEM observations indicated that almost all the calcium oxalate crystals formed in the nucleation assay and aggregation assay had super- and hyper-twinned calcium oxalate monohydrate crystals, forming flower-like aggregates. The crystal habits of the super/hyper twins tended to exhibit rounded edges. These results support the potential of hydroalcoholic extract of leaves from *H. rosa-sinensis* to prevent urolithiasis and protect nephrons.

5. CONCLUSION

Calcium oxalate crystal nucleation and aggregation were shown to be significantly impacted by the extract obtained from *H. rosa-sinensis* leaves in the present research. The extract shown significant efficacy in suppressing the formation of calcium oxalate crystals. However, further studies are required to confirm these results in living organisms and develop a potent anti-urolithiasis medication.

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