RESEARCH PAPER

In Vitro Evaluation of the Anti-Urolithiatic Potential of Ensete Superbum Seed Ethanolic Extract: A Comprehensive Assessment of Calcium Oxalate Nucleation, Titrimetric Estimation and Antioxidant Activity

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ABSTRACT

Background: Urolithiasis is a common urological disorder characterized by the formation of urinary calculi, predominantly composed of calcium oxalate crystals. The search for plant-derived therapeutic agents with anti-urolithiatic properties has gained considerable attention due to the limitations of conventional treatment approaches. Ensete superbum (Roxb.) Cheesman, commonly known as cliff banana, is traditionally used for various medicinal purposes.

Objective: The present study aimed to evaluate the in-vitro anti-urolithiatic potential of the ethanolic seed extract of Ensete superbum against calcium oxalate crystallization and dissolution.

Methods: Seeds of Ensete superbum were collected, authenticated, and extracted using hydroethanolic maceration. The anti-urolithiatic activity was evaluated using spectrophotometric calcium oxalate estimation, titrimetric estimation, and calcium oxalate nucleation assay. Cystone was used as the standard reference drug. The concentration dependent inhibitory activity of the extract was assessed and compared with the standard.

Results: The spectrophotometric assay demonstrated concentration dependent calcium oxalate inhibition by the plant extract, with percentage inhibition increasing from 15.76% to 62.48% at concentrations ranging from 20 to 100 µg/mL. The calculated IC_{50} value was 78.31 µg/mL, compared with 66.47 µg/mL for Cystone. Titrimetric estimation and nucleation studies further supported the anti-urolithiatic potential of the extract.

Conclusion: The findings suggest that Ensete superbum seed extract possesses promising in-vitro anti-urolithiatic activity, potentially through the inhibition of calcium oxalate crystallization and promotion of crystal dissolution. Further phytochemical characterization, mechanistic investigations, and in-vivo studies are warranted to establish its therapeutic potential.

Keywords: Ensete superbum, Anti-urolithiatic activity, Calcium oxalate, Nucleation assay, Cystone

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INTRODUCTION

Kidney stone formation, also known as lithiasis, is a common and severe urological condition that causes significant discomfort and is often associated with changes in lifestyle and dietary habits. This condition involves the formation of calculi within the urinary system and is broadly classified into nephrolithiasis and urolithiasis. Nephrolithiasis refers to the formation of stones in the kidneys, while urolithiasis involves creation of stones in other parts of the urinary system, including the ureter and bladder. Normally, calcification occurs in a controlled manner to form bones and teeth; however, when body fluids become supersaturated, uncontrolled crystallization occurs, leading to kidney stone formation[1].

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Kidney stone disease is characterized by crystalline deposits within the kidneys and has become a growing global health concern. It affects about 12% of people worldwide and is linked to a higher incidence of kidney failure. Calcium oxalate kidney stones are the most prevalent kind and develop at Randall's plaques on the renal papillae. Stone formation is a complex physicochemical process involving Nucleation and supersaturation, crystal expansion, accumulation, and retention of crystals in tubular cells of the kidney. These processes occur due to an imbalance between promoters and inhibitors of crystallization in urine. Cellular damage caused by oxalate exposure activates p38 mitogen-activated protein kinase (MAPK) and other signalling pathways, leading to renal epithelial cell injury and promoting stone formation. Currently, effective medications for preventing recurrence remain limited[2].

PLANT PROFILE



Figure.1- *Ensete superbum* (Roxb.)

Table No:1- Common names of Ensete superbum (Roxb.) Cheesman[3]

Botanical Name	*Ensete superbum* (Roxb.) Cheesman
Common Name	Cliff banana
Synonym	Musa superba Roxb
Malayalam	Kalluvazha
Hindi	Jungli kela
Tamil	Kattu-valai
Plant part used	Seed

Taxonomical classification

Table No:2-Taxonomical classification of Ensete superbum (Roxb.) Cheesman[4]

Kingdom	Plantae
Division	Angiospermae
Class	Monocotylydonae
Sub-class	Commelinids
Order	Zingiberales
Family	Musaceae
Genus	Ensete
Species	Superbum

Geographical distribution

Ensete superbum is highly regarded for its remarkable adaptability and resilience in surviving dry spells. It thrives on moist, moss-covered rock edges, crevices, and desolate rocky landscapes, particularly in the Western Ghats, northeastern sub-Himalayan regions, and the arid deciduous forests of central and western India[51]. The

plant's medicinal properties, coupled with its robust, rain-dependent, and persistent nature, make it a perfect candidate for optimal utilization of Class 7 type land in India[5].

MATERIALS AND METHODS[6]:

Collection of Plant Material and Extraction:

Ensete superbum seeds were collected from Nilambur, Malappuram Dt, Kerala, India during the month of November 2024. It was identified and authenticated by the head of the botany department at Farook College in Kozhikode, Kerala, is Dr. Kishore Kumar K.

The extraction process was done by maceration technique. Finely powdered seed (30g) is placed in a beaker with 70ml of ethanol and 30ml of water. For two to three days, the mixture is allowed to stand at room temperature. with occasional stirring, until the soluble matter has dissolved. Afterwards, the mixture is then filtered through filter paper. The filtrate was then concentrated in a China dish and used.

Preparation of the semi-permeable membrane from eggs[7]:

The eggs were pierced in the apex using a glass rod, allowing the complete contents to be squeezed out. The emptied eggs were then completely rinsed with distilled water before being placed in a beaker containing 4 mL of strong HCl diluted in 200 mL of purified water. The decalcification of the membrane that is semi-permeable occurred as the setup was left undisturbed overnight. The following day, the Carefully chosen semi-permeable membranes separated from the eggshells, thoroughly used distilled water to wash, and immersed in an ammonia solution to neutralize any leftover acid traces. Afterward, they were rinsed again with distilled water and stored in a moistened state inside a refrigerator, maintaining a pH of 7–7.4. The prepared membranes were then secured onto a double-sided, open-ended tube to enclose the components for incubation.

Synthesis of calcium oxalate by homogenous precipitation[8]:

The homogeneous precipitation method was used to create calcium oxalate by dissolving 1.34 g of sodium oxalate in 100 mL of 2N H_2SO_4 and 1.47 g of calcium chloride dihydrate in 100 mL of purified water. Calcium oxalate precipitated out when both solutions were added to a beaker in equal amounts while being constantly stirred. The precipitate was treated with an ammonia solution to remove any last remnants of sulfuric acid. After that, it was completely cleaned with distilled water and dried for two hours at 60°C.

Making a 0.02M KMnO4 solution[9]

0.32 g potassium permanganate was broken down in 100 mL of distilled water and brought to a boil for 30 minutes to create a 0.02M $KMnO_4$ solution. Any extra MnO_4 was removed by filtration after cooling.

Calculating Calcium Oxalate by spectrophotometric method

- Set I: 1 milliliter of purified water and 1 milliliter of calcium oxalate (1 mg/ml)
- Set II – One milliliter of calcium oxalate (1 mg/ml) plus one milliliter of cystone solution (400 mg/ml)
- Set III – 1ml of calcium oxalate (1mg/ml) added with 1 ml of ethanolic extract of *Ensete superbum* (20mg/ml).

The egg's semi-permeable membrane was carefully gathered, secured suspended in a 150 ml conical flask with a thread at one end of 0.1 M Tris buffer. The thread's other end was knotted. to a stick positioned across the flask's opening, which was then covered with aluminium foil. All samples were placed in a preheated incubator at 37°C for 4 hours and further incubated for three days. After incubation, the contents from each membrane pouch were transferred into individual test tubes. To each sample, 4 ml of 1N sulfuric acid (H_2SO_4) and 06–08 ml of 0.02M potassium permanganate ($KMnO_4$) were added and left undisturbed for two hours. Following this, a change in colour from deep pink to colourless was seen. At 620 nm, this shift in colour intensity was then measured using spectrophotometry.

Estimation of Calcium oxalate by titrimetric method[10]

A semi-permeable membrane was filled with precisely weighed 10 mg of extract, chemical, or standard and 1 mg of calcium oxalate. A conical flask holding 100 millilitres of TRIS buffer at 0.1 M was used to suspend the setup, while calcium oxalate alone was used as a negative control. For seven to eight hours, each flask was 37 °C for incubation. After transferring the contents of the membrane into test tubes, 2 millilitres of 1 N sulfuric acid were added, and 0.9494 N $KMnO_4$ was titrated until a light pink endpoint emerged. In this case, 0.1898 mg of calcium is equal to 1 ml of $KMnO_4$. To calculate the test substance's dissolution, the amount of undissolved calcium oxalate was deducted from the total.

Estimation of Calcium oxalate by Nucleation assay (Turbidity method)[11]

The Spectrophotometric technique was used to evaluate the extracts' inhibitory effect on the creation of crystals of calcium oxalate. To begin, 100 µl of 50 mM sodium oxalate and 100 µl of 4 mM calcium chloride were blended with 0.5 millilitres of buffered normal human urine consisting of 0.5 ml of 0.5 ml of a 0.15 mM NaCl solution and 0.05 mM Tris's buffer, maintained at 37ºC and pH 6.5. The last volume was brought to 2.6 millilitres of distilled water, initiating the crystallization process. The time taken for crystals to reach a detectable size, called the induction time, was recorded for both the extract and control samples. The nucleation rate was determined by measuring the optical density (OD) at 620 nm, and the following formula was used to get the % inhibition: (1 - OD (experimental) / OD (control))/100[13].

RESULTS

***In-vitro* studies**

Spectrophotometric estimation of calcium oxalate:
The spectrophotometric estimation of calcium oxalate showed that the percentage of undissolved calcium oxalate decreased with increasing concentration of plant extract and standard drug. The GraphPad Prism plot demonstrated a dose-dependent calcium oxalate dissolution, indicating anti-urolithiatic activity.

Undissolved calcium oxalate by standard drug (Cystone).

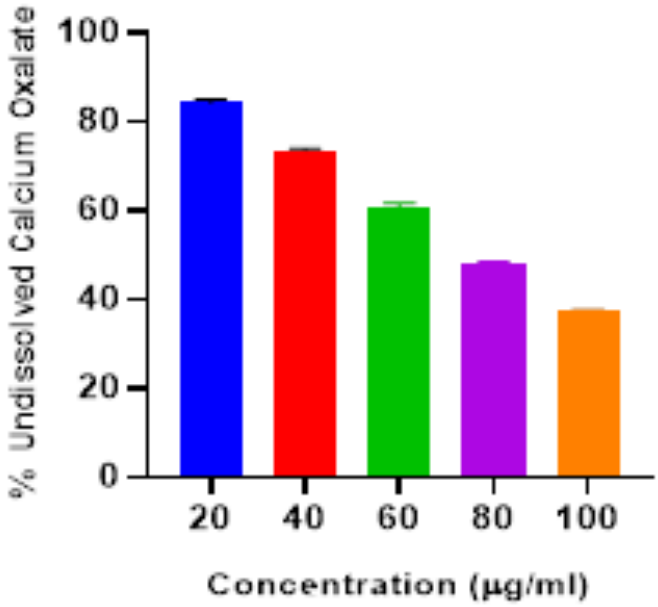


Figure 2: Dose-dependent reduction in percentage of undissolved calcium oxalate by standard drug.

Undissolved calcium oxalate by plant seed extract (*Esteem superbum*).

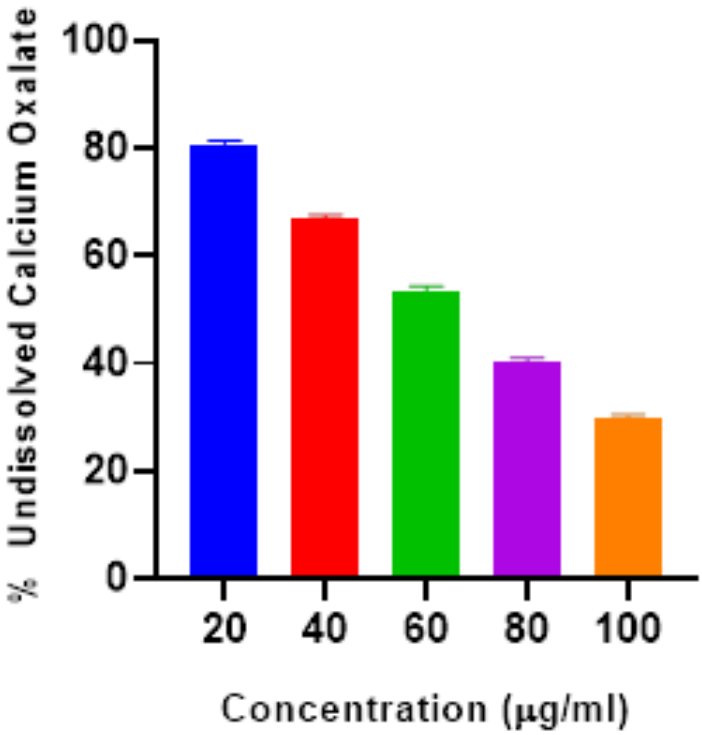


Figure 3: Dose-dependent reduction in percentage of undissolved calcium oxalate by plant extract (*Esteem superbum*).

Dose–response curve showing percentage inhibition of calcium oxalate by plant extract and standard drug (Cystone) at different concentrations (20–100 µg/ml). The graph demonstrates a concentration-dependent increase in percentage inhibition with high linearity (R^2 = 0.998 for Cystone and R^2 = 0.9989 for extract). The standard drug exhibited higher inhibition compared to the plant extract, indicating stronger calcium oxalate dissolution and anti-urolithiatic activity

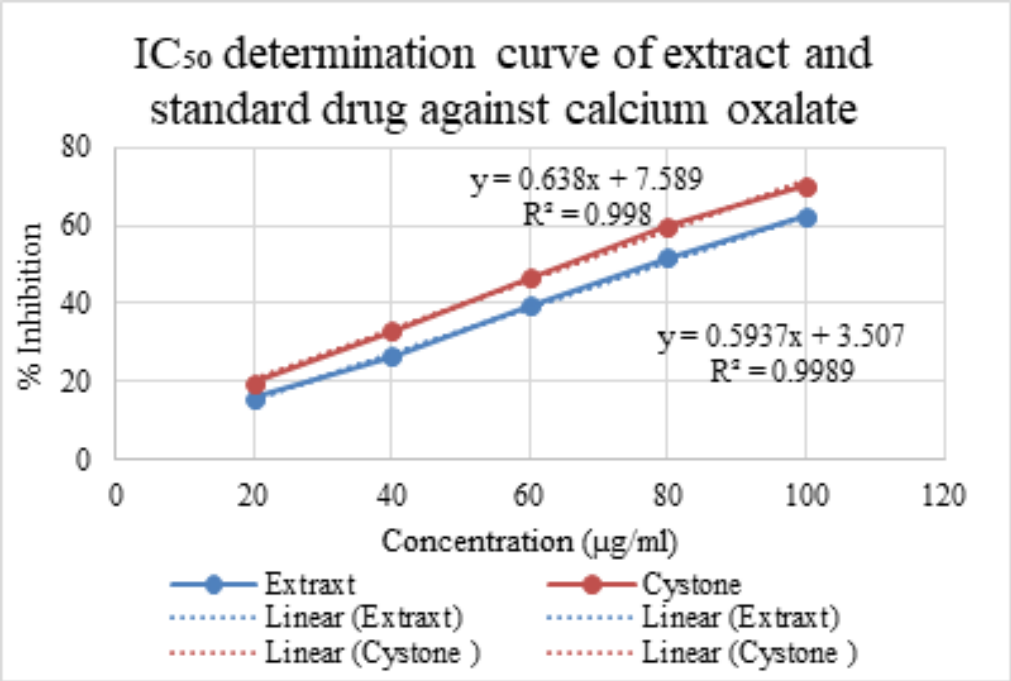


Figure 4: IC_{50} determination curve of extract and standard drug against calcium oxalate

The dose–response curve generated using GraphPad Prism revealed a concentration-dependent inhibition of calcium oxalate, and the IC_{50} value of the plant extract was found to be **78.31 µg/ml**, while the standard drug showed an IC_{50} value of **66.47 µg/ml**, indicating stronger activity of the standard compared to the extract.

Table 3: IC_{50} values of standard and plant extract in spectrophotometric calcium oxalate assay

Concentration (µg/ml)	% Inhibition of	
	Plant Extract	Cistone (Standard)
20	15.76	19.74
40	26.4	33.14
60	39.3	46.48
80	51.69	59.73
100	62.48	70.24
IC 50	**78.31** µg/ml	**66.47** µg/ml

The spectrophotometric estimation of calcium oxalate was performed to evaluate the anti-urolithiatic activity of the plant extract and standard drug. The percentage inhibition of calcium oxalate increased with increasing concentration (20–100 µg/ml) for both plant extract and standard drug, indicating a dose-dependent dissolution of calcium oxalate. The plant extract showed percentage inhibition ranging from 15.76% to 62.48%, while the standard drug showed higher inhibition ranging from 19.74% to 70.24%. The IC_{50} values were discovered to be 78.31 µg/ml for the plant extract and 66.47 µg/ml for the standard drug, indicating stronger calcium oxalate inhibition by the standard drug in contrast to the plant extract. These findings imply that the plant extract possesses significant anti-urolithiatic activity, although slightly lower than the standard drug.

Titrimetric estimation of Calcium oxalate:

An accurate assessment of the risk of stone development can be obtained by analysing the urine chemistry in relation to the minerals that cause stones. The extract's capacity to remove kidney stones is indicated by the percentage of calcium oxalate that dissolves.

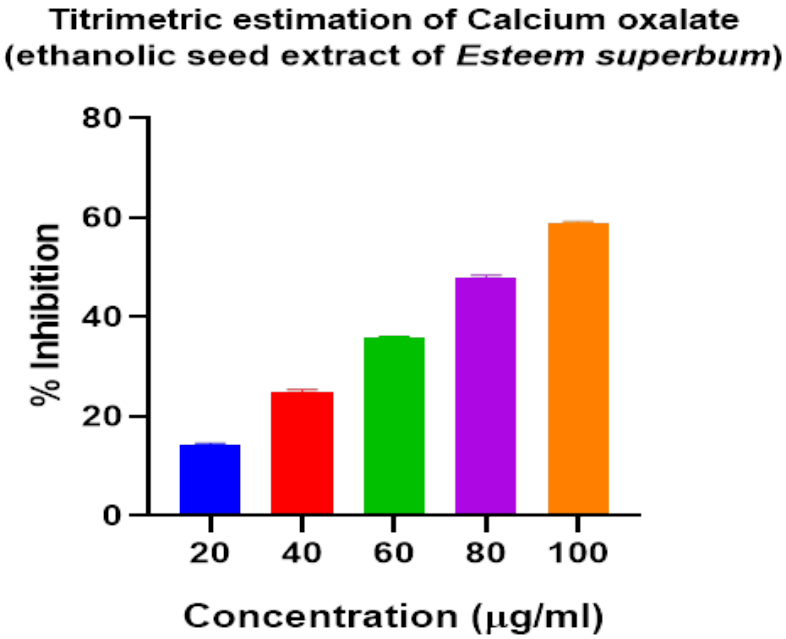


Fig 5: Titrimetric estimation of Calcium Oxalate with Plant Extract

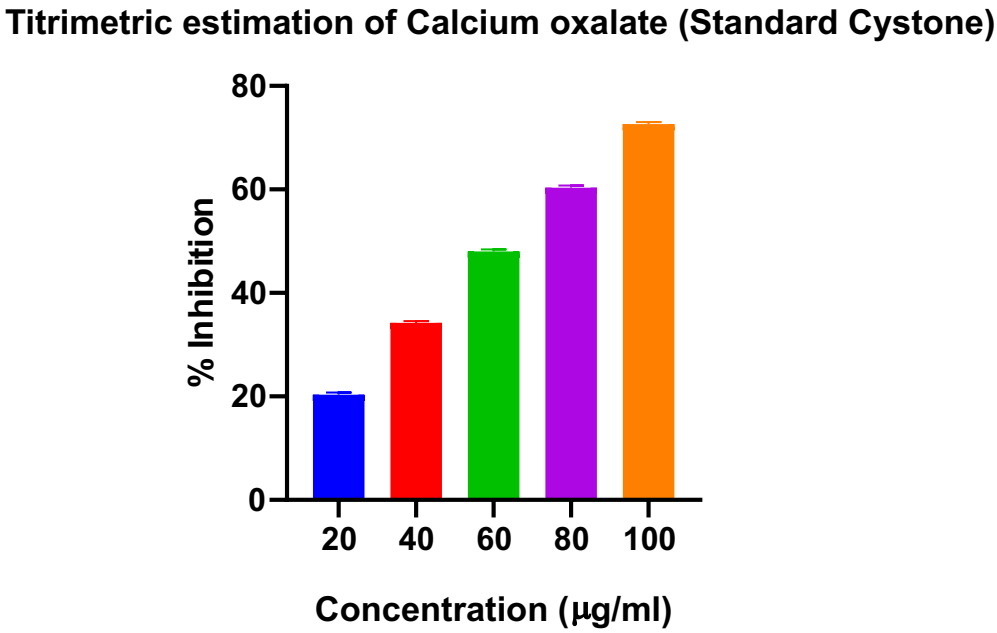


Figure 6: Titrimetric estimation of Calcium Oxalate with Standard Drug Cystone

The titrimetric estimation of calcium oxalate showed a concentration-dependent increase in percentage inhibition by the plant extract. The proportion of calcium oxalate inhibition progressively increased as the concentration rose from 20 to 100 µg/ml, indicating enhanced dissolution and reduced crystal formation. This suggests that the plant extract possesses significant anti-urolithiatic activity and is effective in inhibiting calcium oxalate formation in a dose-dependent manner.

Nucleation assay

The calcium oxalate particles crystallization within the urinary tract is attributed by Urine supersaturation. Clusters of stone-forming salts begin to form during this nucleation phase as There are further constituents. When it comes to the nucleation of calcium oxalate salts, the Cystone standard solution exhibits greater inhibitory action than the extract. The crystal growth observed in the Extract were less compared to that of Cystone.

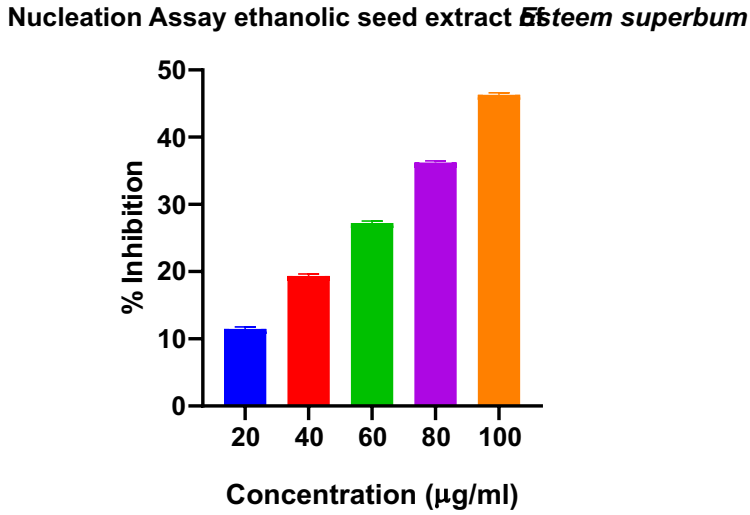


Fig 7: Nucleation assay of Plant Extract

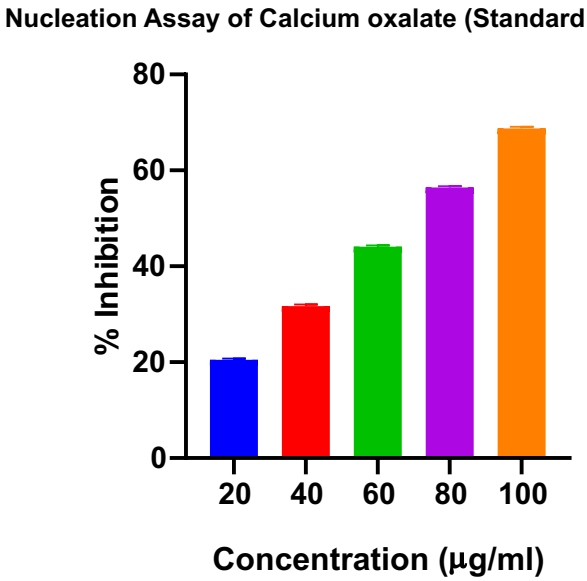


Fig 8: Nucleation assay of Standard

DISCUSSION

The current investigation assessed the anti-urolithiatic potential of ethanolic extracts of *Ensete superbum* seeds using in-vitro calcium oxalate crystal dissolution and inhibition models. The findings indicate that the plant extracts possess notable inhibitory effects on calcium oxalate crystal formation and promote dissolution of preformed crystals, suggesting their potential role in the management of urolithiasis. The gradual reduction in undissolved calcium oxalate with increasing concentration of extracts demonstrates a dose-dependent activity, which may be ascribed to the existence of phytoconstituents like flavonoids, phenolics, and saponins known for antioxidant and crystal inhibitory properties.

The anti-urolithiatic activity observed in this study may be due to the ability of plant phytochemicals to chelate calcium ions, reduce crystal aggregation, and prevent nucleation and growth of calcium oxalate crystals. Antioxidant compounds present in the seeds may also reduce oxidative stress in renal tissues, thereby minimizing stone formation.

However, the study has certain limitations. The investigation was limited to *in-vitro* experimental models and did not include *in-vivo* or clinical evaluation. The exact mechanism of action and identification of specific active compounds responsible for the activity were not determined. Further studies involving isolation of active constituents, molecular docking, and *in-vivo* validation are recommended to confirm the therapeutic potential of *Ensete superbum* seeds.

Overall, the study suggests that *Ensete superbum* seeds could be considered a promising natural source for the development of anti-urolithiatic agents, and further

pharmacological and clinical investigations are necessary to support its safe and effective use.

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