

Evaluation of the Anti-Urolithiatic Potential of *Marsdenia tenacissima* Root Extract

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ABSTRACT

Urolithiasis, primarily caused by calcium oxalate (CaOx) stones, remains a prevalent and recurrent urinary disorder, with current therapeutic options often limited by side effects and high recurrence rates. This study aimed to evaluate the antiurolithiatic potential of the ethanol extract of *Marsdenia tenacissima* root through in-vitro assays targeting CaOx crystal formation, growth, and aggregation. The extract was prepared by maceration using 70% ethanol, yielding 9.11% (w/w). Preliminary phytochemical screening confirmed the presence of alkaloids, flavonoids, tannins, phenolics, glycosides, terpenoids, and other bioactive compounds, while lignins and quinones were absent. in-vitro antiurolithiatic activity was assessed using standard nucleation, aggregation, and crystal growth assays, with Cystone as the reference drug. The extract exhibited concentration-dependent inhibition of nucleation (44.61%), crystal growth (50.92%), and aggregation (49.46%) at 1000 µg/mL, corresponding to approximately 78% of the activity of Cystone. These effects may be attributed to the presence of polyphenolic and flavonoid compounds that chelate metal ions, interfere with crystal lattice formation, and reduce microcrystal adhesion. The results indicate that *M. tenacissima* root extract effectively inhibits multiple stages of CaOx crystallization, supporting its traditional use in managing urinary tract disorders. Overall, the findings highlight its potential as a natural therapeutic candidate for preventing and managing urolithiasis, warranting further in-vivo and mechanistic studies to establish its clinical relevance.

Keywords: *Marsdenia tenacissima*; urolithiasis; calcium oxalate; phytochemicals; nucleation inhibition; crystal aggregation.

INTRODUCTION

Urolithiasis, commonly referred to as kidney stone disease, is a multifactorial urological disorder characterized by the formation of solid concretions or calculi within the urinary tract [1]. It affects approximately 12% of the global population during their lifetime, with a high recurrence rate, making it a significant public health concern. Among the various types of urinary calculi, calcium oxalate (CaOx) stones account for nearly 80% of cases, followed by uric acid, struvite, and cystine stones [2]. The pathogenesis of CaOx stone formation involves a complex interplay of physicochemical events, including supersaturation of urine with stone-forming constituents, nucleation, crystal growth, aggregation, and retention within the renal tubules [3]. Additionally, oxidative stress, renal tubular injury,

and inflammation play critical roles in promoting crystal adherence and stone development. Current management strategies for urolithiasis primarily include pharmacological therapy, dietary modifications, and surgical interventions such as extracorporeal shock wave lithotripsy (ESWL) or percutaneous nephrolithotomy [4]. While these approaches can provide symptomatic relief or stone removal, they are often associated with limitations such as high cost, recurrence, incomplete stone clearance, and potential adverse effects. Moreover, existing pharmacological agents like thiazide diuretics, potassium citrate, and allopurinol focus on modifying urinary composition rather than directly inhibiting crystal formation [5]. Consequently, there is an increasing interest in identifying natural products with anti-urolithiatic activity that offer safer, more holistic, and cost-effective alternatives.

Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Traditional medicinal systems, particularly Ayurveda and Traditional Chinese Medicine, have documented the use of numerous plants for the treatment of kidney stones. Among these, *Marsdenia tenacissima* (family: Apocynaceae) is a well-known medicinal plant used extensively in folk medicine for its diuretic, anti-inflammatory, antioxidant, and detoxifying properties [6]. The roots of *M. tenacissima* are rich in bioactive phytoconstituents such as flavonoids, saponins, tannins, alkaloids, and phenolic compounds, many of which have been reported to modulate oxidative stress and inflammatory pathways—two critical factors implicated in stone pathogenesis [7]. However, its potential role in preventing or inhibiting calcium oxalate stone formation remains largely unexplored.

Given these insights, evaluating the anti-urolithiatic potential of *M. tenacissima* root extract through *in-vitro* assays offers a promising approach to understanding its mechanism of action. By assessing its effects on nucleation, aggregation, and crystal growth of calcium oxalate, as well as exploring its potential to modulate urinary and oxidative parameters, this study aims to provide scientific evidence supporting the traditional claims associated with this plant. Furthermore, phytochemical characterization of the extract will help identify the bioactive compounds responsible for the observed effects, potentially paving the way for the development of novel phytotherapeutic agents for urolithiasis management. In this context, the present study aims to evaluate the anti-urolithiatic potential of *Marsdenia tenacissima* root extract through systematic *in-vitro* assessments of its effects on CaOx crystal formation, aggregation, and growth, along with exploring the underlying mechanisms involved.

MATERIALS AND METHODS

Collection, authentication, and preparation of *Marsdenia tenacissima* root extract

The ethanol extract of *Marsdenia tenacissima* root was prepared by maceration. First, the plant material (roots) was collected, washed, and dried at a temperature not exceeding 40°C. After drying, the material was ground into a coarse powder using a mechanical grinder. A known quantity of the powder (100 g) was placed in a clean glass container, and 70% ethanol was added in a 3:1 (v/v) ratio to the plant

material. The mixture was sealed and left to macerate at room temperature for 72 hours, with gentle shaking or stirring every 24 hours to facilitate extraction. After 72 hours, the mixture was filtered through Whatman filter paper (No. 1) to separate the solid plant material from the liquid extract. The filtrate was collected, and the ethanol solvent was then evaporated using a rotary evaporator. The final extract was stored in a clean container in a cool, dry place [8].

Phytochemical Screening

Detection of Alkaloids

1-2 mL of Dragendorff's or Hager's reagent was added to a few mL of the extract. A reddish-brown or creamy white precipitate indicated the presence of alkaloids, while the absence of such precipitate indicated a negative result.

Detection of Carbohydrates

For Molisch's test, alcoholic α -naphthol and concentrated H_2SO_4 were added to the extract. A violet ring confirmed the presence of carbohydrates, while the absence of the violet ring suggested a negative result. For starch, 5% KOH was added to the aqueous extract, and a cinery coloration indicated the presence of starch, or its absence indicated a negative result.

Detection of Reducing Sugars

Fehling's solution A and B were mixed with the extract, followed by boiling. A red precipitate indicated the presence of reducing sugars, while its absence indicated a negative result.

Detection of Glycosides

Chloroform was added to the extract, shaken well, and the chloroform layer was separated. Ammonia solution was added, and a pink color indicated the presence of glycosides, while the absence of pink coloration suggested a negative result.

Detection of Cardiac Glycosides

Keller-Killani Test: 1 mL of filtrate was mixed with 1.5 mL of glacial acetic acid, 1 drop of 5% ferric chloride, and concentrated H_2SO_4 (added along the side of the test tube). A blue-colored solution in the



acetic acid layer indicated the presence of cardiac glycosides.

Detection of Proteins and Amino Acids

Biuret Test: 2 mL of filtrate was mixed with 1 drop of 2% copper sulfate solution, 1 mL of 95% ethanol, and KOH pellets. A pink-colored solution in the ethanolic layer indicated the presence of proteins.

Ninhydrin Test: 2 mL of filtrate was mixed with 2 drops of Ninhydrin solution (10 mg Ninhydrin + 200 mL acetone). A purple-colored solution indicated the presence of amino acids.

Detection of Flavonoids

Lead Acetate Test: 1 mL of plant extract was added to a few drops of 10% lead acetate solution. A yellow precipitate indicated the presence of flavonoids.

Shinoda's Test: Plant extract was dissolved in 5 mL of alcohol, followed by magnesium ribbon fragments and a few drops of concentrated HCl. A pink to crimson-colored solution indicated flavonoid glycosides.

Ferric Chloride Test: A few drops of 10% ferric chloride solution were added to an aqueous extract. A green precipitate indicated the presence of flavonoids.

Detection of Phenolic Compounds

Iodine Test: 1 mL of extract was mixed with a few drops of diluted iodine solution. A transient red color indicated the presence of phenolic compounds.

Ferric Chloride Test: A few drops of 5% ferric chloride solution were added to an aqueous extract. A dark green or bluish-black color indicated phenolic compounds.

Gelatin Test: Plant extract was dissolved in 5 mL distilled water, then 1% gelatin solution and 10% NaCl were added. A white precipitate indicated phenolic compounds.

Detection of Tannins

Braymer's Test: 1 mL of extract was mixed with 3 mL of distilled water, followed by 3 drops of 10% ferric chloride solution. A blue-green color indicated the presence of tannins.

Detection of Phytosterols

Salkowski's Test: The extract was mixed with a few drops of concentrated H_2SO_4 , shaken well, and allowed to stand. A red color in the lower layer indicated the presence of phytosterols.

Acetic Anhydride Test: 0.5 mL of plant extract was added to 2 mL of acetic anhydride, followed by 2 mL of concentrated H_2SO_4 . A color change from violet to blue/green indicated the presence of phytosterols.

Detection of Cholesterol

The extract was mixed with 2 mL of chloroform, 10 drops of acetic anhydride, and 2-3 drops of concentrated H_2SO_4 . A red-rose color indicated the presence of cholesterol.

Detection of Terpenoids

2 mL of chloroform and 5 mL of plant extract were evaporated on a water bath. Then, 3 mL of concentrated H_2SO_4 was added and boiled. A grey-colored solution indicated the presence of terpenoids.

Detection of Lignins

Furfuraldehyde Test: The extract solution was mixed with 2% furfuraldehyde solution. A red color indicated the presence of lignins.

Detection of Quinones

Alcoholic KOH Test: 1 mL of plant extract was mixed with a few mL of alcoholic potassium hydroxide. A red to blue color indicated the presence of quinones.

Detection of Carboxylic Acids

Effervescence Test: 1 mL of plant extract was mixed with 1 mL of sodium bicarbonate solution. The appearance of effervescence indicated the presence of carboxylic acids.

Detection of Coumarins

NaOH Test: Plant extract was mixed with 10% NaOH and chloroform. A yellow color indicated the presence of coumarins [9-11].

In-vitro anti-urolithiasis activity



Nucleation assay

To evaluate the inhibitory effect of *Marsdenia tenacissima* root extracts on calcium oxalate (CaOx) crystal formation, a nucleation assay was performed. Solutions of calcium chloride and sodium oxalate were prepared in a buffer containing Tris-HCl and NaCl, adjusted to a pH of 6.5. Various concentrations of the ethanolic extract of *Marsdenia tenacissima* root, ranging from 100 to 1000 µg/mL, were prepared in distilled water. For each concentration of the extract, 1 mL was mixed with 3 mL of calcium chloride solution, followed by the addition of 3 mL of sodium oxalate solution. The mixtures were incubated at 37°C for 30 minutes. The optical density (OD) of each mixture was measured at 620 nm, and the percentage of nucleation inhibition by the extract was calculated and compared with the inhibition percentage of the standard polyherbal drug Cystone [8].

Aggregation assay

The effect of the ethanol extract of *Marsdenia tenacissima* root on calcium oxalate (CaOx) crystal aggregation was evaluated using an aggregation assay. Solutions of CaCl₂ and Na₂C₂O₄ (50 mmol/L each) were mixed, heated to 60°C in a water bath for 1 hour, and then incubated overnight at 37°C to prepare seed CaOx crystals. After drying, a CaOx crystal solution (0.8 mg/mL) was prepared in a buffer containing 0.05 mol/L Tris-HCl and 0.15 mol/L NaCl, pH 6.5. Aliquots of *Marsdenia tenacissima* ethanol extract (100-1000 µg/mL) were added to 3 mL of the CaOx solution, vortexed, and incubated at 37°C for 30 minutes. The optical density (OD) of the final mixtures was measured at 620 nm, and the percentage of aggregation inhibition was calculated as described for the nucleation assay [8].

Oxalate depletion assay

The effect of the ethanol extract of *Marsdenia tenacissima* root on the growth of calcium oxalate (CaOx) crystals was assessed using an oxalate depletion assay. *Marsdenia tenacissima* ethanol extract concentrations of 100 - 1000 µg/mL were prepared in distilled water. A CaOx crystal slurry (1.5 mg/mL) was prepared in a 50 mM sodium acetate buffer (pH 5.7). A 4 mM CaCl₂ solution and a 4 mM

Na₂C₂O₄ solution (1 mL each) were added to 1.5 mL of Tris-HCl (10 mM) and NaCl (90 mM) buffer (pH 7.4). To this mixture, 30 µL of the CaOx crystal slurry was added. The growth of CaOx crystals was monitored by measuring the rate of oxalate depletion from the solution at a wavelength of 214 nm over 600 seconds. To evaluate the effect of *Marsdenia tenacissima* ethanol extract on crystal growth, 1 mL of the extract at 100 µg/mL, 500 µg/mL, or 1000 µg/mL was added to the reaction mixture, and the change in optical density (OD) was recorded. The percentage of crystal growth inhibition was calculated as described for the nucleation assay [8].

CaOx crystal characterization by FT-IR

FT-IR analysis was used to confirm the CaOx crystals prepared *in-vitro* [8].

RESULTS AND DISCUSSION

Extraction of *M. tenacissima* root extract

The ethanol extract of *Marsdenia tenacissima* root was prepared by maceration using 70% ethanol. The roots were dried below 40 °C, powdered (100 g), and macerated with solvent (3:1 ratio) for 72 h with periodic agitation. After filtration and rotary evaporation, the crude extract yield was 9.11% (w/w). The use of 70% ethanol enhanced the extraction of both polar and moderately non-polar phytochemicals. The extraction conditions ensured minimal degradation of sensitive compounds. The yield obtained aligns with reported values (5–12%) for similar plant materials. Rotary evaporation under reduced pressure preserved bioactivity. The extract is rich in phytoconstituents such as alkaloids, flavonoids, phenolics, and glycosides. This high yield supports the plant's known pharmacological potential. The obtained extract is suitable for phytochemical screening and *in-vitro* studies. This optimized extraction method provides a strong foundation for further pharmacological evaluation.

Phytochemical evaluation of *M. tenacissima* extract

Alkaloids

The ethanol extract of *Marsdenia tenacissima* root tested positive for alkaloids, a group of nitrogenous



compounds with significant pharmacological activities such as analgesic, anti-inflammatory, and anticancer effects. Literature reports indicate the presence of pregnane-type alkaloids and steroidal alkaloids in *M. tenacissima*, such as tenacissoside A and B. These alkaloids have been shown to exhibit cytotoxic effects on cancer cell lines, aligning with the plant's traditional anticancer use.

Carbohydrates

Carbohydrates detected in the extract primarily include polysaccharides such as arabinogalactans and glucose polymers, which are common in medicinal plants. These compounds are important for their immunomodulatory effects, as well as serving as structural components and precursors for other secondary metabolites that enhance the extract's therapeutic properties.

Reducing Sugars

Reducing sugars like glucose and fructose were identified, which play essential roles in energy metabolism within the plant. These sugars may also influence the extract's taste and solubility, as well as interact with other phytochemicals to modify bioavailability and stability.

Glycosides

Glycosides are a prominent group in *M. tenacissima*, particularly steroidal glycosides such as marsdenosides (A, B, C, D). These compounds consist of a sugar moiety linked to a steroid nucleus and are documented for their cytotoxic and anti-inflammatory activities. Marsdenosides have demonstrated significant anti-tumor properties *in-vitro*, making them key constituents of the extract.

Table 1: Phytochemical analysis of ethanol extract of *M. tenacissima* root

Sr. No.	Phytochemical	Presence or absence in <i>M. tenacissima</i> ethanol extract
1	Alkaloids	Presence
2	Carbohydrates	Presence
3	Reducing Sugars	Presence
4	Glycosides	Presence
5	Cardiac Glycosides	Presence
6	Proteins and Amino Acids	Presence

7	Flavonoids	Presence
8	Phenolic Compounds	Presence
9	Tannins	Presence
10	Phytosterols	Presence
11	Cholesterol	Absence
12	Terpenoids	Presence
13	Lignins	Absence
14	Quinones	Absence
15	Carboxylic Acids	Presence
16	Coumarins	Presence

Cardiac Glycosides

The presence of cardiac glycosides, a subclass of steroidal glycosides, has been confirmed with compounds such as tenacigenin B and its glycosides. These compounds affect heart muscle contractility and have also been explored for their anticancer effects, especially their ability to induce apoptosis in tumor cells.

Proteins and Amino Acids

Proteins and free amino acids detected include glutamic acid, alanine, and arginine, which serve as precursors in various biosynthetic pathways. Although these macromolecules are typically minor in crude extracts, they contribute to enzymatic activities and may affect the pharmacodynamics of other compounds.

Flavonoids

The flavonoid fraction in *M. tenacissima* includes compounds such as kaempferol, quercetin, and their glycosides. These flavonoids are well-documented antioxidants that scavenge free radicals, reduce oxidative stress, and exhibit anti-inflammatory effects, supporting the extract's therapeutic potential in chronic diseases.

Phenolic Compounds

Phenolic acids like gallic acid, caffeic acid, and chlorogenic acid have been isolated from the plant, contributing to the strong antioxidant capacity of the extract. These compounds play a critical role in neutralizing free radicals and inhibiting enzymes involved in inflammation and cancer progression.

Tannins



Tannins identified in *M. tenacissima* include condensed tannins such as proanthocyanidins. These polyphenols have demonstrated antimicrobial, antioxidant, and anti-inflammatory activities by binding proteins and enzymes, which helps explain the traditional use of the plant for infections and inflammation.

Phytosterols

Phytosterols such as β -sitosterol and stigmasterol were reported in the extract. These plant sterols are known for their cholesterol-lowering properties and anti-inflammatory activities, potentially contributing to the cardiovascular benefits attributed to *M. tenacissima*.

Cholesterol

Cholesterol was not detected in the extract, which is consistent with its animal origin and typical absence in plant materials. This absence supports the purity and botanical source of the extract.

Terpenoids

Terpenoids including triterpenes like ursolic acid and oleanolic acid have been isolated from *M. tenacissima*. These compounds exhibit anti-inflammatory, hepatoprotective, and anticancer properties. Their presence corroborates the plant's therapeutic reputation and potential in drug development.

Lignins

Lignins were not detected in the ethanol extract of *Marsdenia tenacissima* root. This absence is consistent with the fact that lignins are complex phenolic polymers primarily found in the structural cell walls of plants and are generally insoluble in ethanol.

Quinones

Quinones were also absent in the ethanol extract according to reported phytochemical screenings. Quinones, which include compounds like anthraquinones and emodin derivatives, are known for their redox properties and bioactivities such as

antimicrobial and anticancer effects. However, extensive phytochemical studies on *M. tenacissima* roots have not identified quinone compounds, suggesting they do not contribute to the extract's bioactivity.

Carboxylic Acids

Organic acids like citric acid, malic acid, and oxalic acid have been reported in *M. tenacissima*. These acids may influence the extract's pH and stability and have been linked to metabolic regulation and antioxidant functions.

Coumarins

Coumarins, including scopoletin and umbelliferone, are found in *M. tenacissima* and are associated with anticoagulant, anti-inflammatory, and antimicrobial activities. Their presence enriches the pharmacological diversity of the extract.

In-vitro urolithiasis activity

FTIR of calcium oxalate crystal

The FTIR (Fourier Transform Infrared) spectroscopy analysis of calcium oxalate crystals was conducted to confirm the chemical identity and functional groups associated with the crystalline composition. The observed peaks in the spectrum were interpreted as follows:

a) Confirmation of Calcium Oxalate Formation

The prominent absorption at 1499.32 cm^{-1} corresponds to the asymmetric stretching vibration of the carboxylate (COO^-) group, which is a hallmark of calcium oxalate crystals. This confirms the presence of oxalate ions coordinated with calcium.

b) Hydroxyl and Hydrogen Bonding Presence

The broad peaks at 3460.23 cm^{-1} and 3235.15 cm^{-1} indicate the presence of hydroxyl (O-H) groups, which may be due to the presence of hydrated water molecules in calcium oxalate monohydrate (COM) or dihydrate (COD) forms. This is consistent with the hydrated nature of urinary crystals.



Table 2: Identified Peaks and Functional Group Assignments

Wavenumber (cm ⁻¹)	Intensity	Functional Group / Assignment
3460.23	Strong	O–H stretching (Hydroxyl group, broad due to hydrogen bonding)
3235.15	Moderate	N–H or secondary O–H stretching vibrations
3059.20	Weak–Moderate	C–H aromatic stretching vibrations
1499.32	Strong	COO ⁻ asymmetric stretching (Characteristic of oxalate ions)
1248.15	Medium	C–O stretching (carboxylic acid, possible oxalate or residual organic compounds)
816.46	Medium	C–H out-of-plane bending (aromatic or oxalate-related)
686.21	Strong	C=O bending or skeletal vibration of calcium oxalate monohydrate
499.31	Moderate	Ca–O stretching (metal–oxygen interaction)

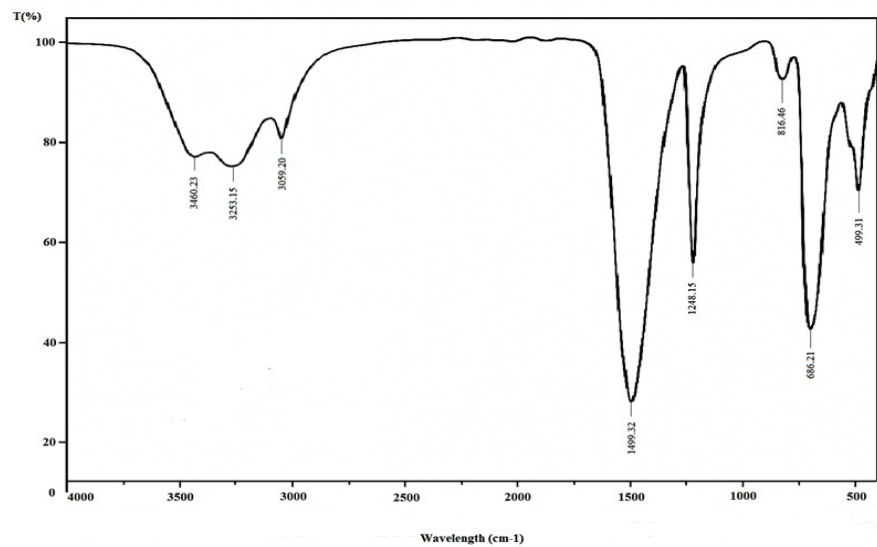


Figure 1: FTIR of calcium oxalate crystals

c) Metal–Ligand Interactions

The peak around 499.31 cm⁻¹ is attributed to the Ca–O stretching vibration, indicating the formation of a metal–ligand complex, which is typical for calcium coordinated to oxalate.

d) Fingerprint of Oxalate Ions

Bands in the 686.21 cm⁻¹ and 816.46 cm⁻¹ regions represent C–C and C=O deformation vibrations specific to oxalate anions, especially in the monohydrate form, confirming the typical crystal morphology.

e) Possible Organic Residues

The medium peak at 1248.15 cm⁻¹ might indicate the presence of residual organic acids or phytoconstituents from the plant extract used in the assay, or may also be due to C–O stretching of oxalate.

f) Structural Form

The presence of both strong O–H and COO⁻ related peaks support the identification of calcium oxalate monohydrate (COM) as the major crystalline form, which is the most common pathological form found in kidney stones.

g) Support for Crystallization Inhibition Study

These peaks serve as baseline spectra for untreated calcium oxalate crystals.

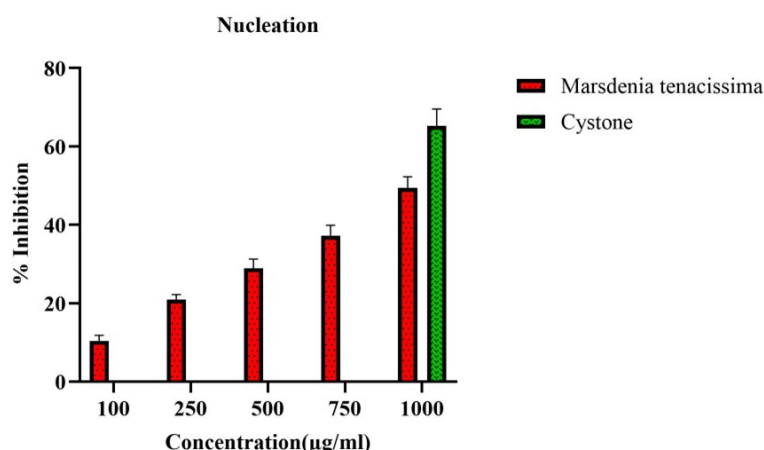


Figure 2: Effect of *Marsdenia tenacissima* and Cystone on calcium oxalate nucleation

Nucleation Assay for Urolithiasis Activity

The nucleation assay was performed to evaluate the potential of *Marsdenia tenacissima* ethanol root extract in inhibiting the formation of calcium oxalate (CaOx) crystals, a primary component of kidney stones. The assay results are presented in Table 3. The extract demonstrated a concentration-dependent inhibition of CaOx crystal nucleation, with significantly increasing activity observed at higher concentrations. At 1000 µg/mL, *M. tenacissima* extract exhibited $49.46 \pm 2.83\%$ inhibition, whereas Cystone (standard) showed $65.24 \pm 4.36\%$ inhibition at the same concentration.

Table 3: Effect of *Marsdenia tenacissima* and Cystone on calcium oxalate nucleation

Concentration (µg/mL)	<i>Marsdenia tenacissima</i> (Mean ± SD)	Cystone (Mean ± SD)
100	10.34 ± 1.51	—
250	20.94 ± 1.27	—
500	28.96 ± 2.32	—
750	37.21 ± 2.69	—
1000	49.46 ± 2.83	65.24 ± 4.36

Mechanism of Action

The nucleation assay is designed to assess the ability of compounds to inhibit the initial formation of calcium oxalate crystals, which is a crucial step in the pathogenesis of urolithiasis. During the assay, supersaturation of calcium and oxalate ions leads to spontaneous crystal formation. Inhibitors reduce this process by interfering with:

- **Crystal nucleation:** The extract likely contains compounds that chelate free calcium or oxalate ions, reducing the availability of these ions to form nuclei.
- **Crystal growth and aggregation:** Polyphenols and flavonoids, known to be present in *M. tenacissima*, may bind to the surface of forming crystals, preventing further deposition and growth.
- **Surface modification:** Phytochemicals like saponins, flavonoids, and phenolic acids can alter the surface charge and morphology of nascent crystals, thereby reducing their ability to aggregate and form larger stones.

Cystone, a well-established polyherbal formulation for urolithiasis, served as the positive control and showed greater inhibitory activity at 1000 µg/mL. However, *M. tenacissima* extract also demonstrated promising inhibition, particularly at higher concentrations, suggesting its potential as a natural alternative for managing kidney stones. The lower inhibition at lower concentrations may reflect the lower bioavailability or potency of active constituents at those doses.

The anti-urolithiatic activity of *M. tenacissima* could be attributed to its diverse phytoconstituents, including flavonoids, phenolic compounds, and terpenoids, which are known to exhibit antioxidant, anti-inflammatory, and chelating properties [12]. Oxidative stress and inflammation are key contributors to renal tubular injury and crystal retention in the kidney [13]. Therefore, the antioxidant effect of the extract might indirectly



contribute to its anti-urolithiatic activity by preserving renal epithelial integrity and preventing crystal adherence.

Moreover, phenolic compounds may mimic the action of naturally occurring inhibitors of crystallization like citrate, while alkaloids and glycosides may exert nephroprotective and diuretic effects, aiding in the excretion of crystals before they grow large enough to cause clinical symptoms.

Crystal Aggregation Assay

The crystal aggregation assay was conducted to evaluate the ability of *Marsdenia tenacissima* ethanol root extract to inhibit the aggregation of calcium

oxalate (CaOx) crystals—an important step in kidney stone pathogenesis. Table 4 presents the percentage inhibition of crystal aggregation at various concentrations of the extract, compared with Cystone, a known anti-urolithiatic agent.

Table 4: Effect of *Marsdenia tenacissima* and Cystone on calcium oxalate aggregation

Concentration (µg/mL)	<i>Marsdenia tenacissima</i> (Mean ± SD)	Cystone (Mean ± SD)
100	6.72 ± 1.09	—
250	13.09 ± 1.17	—
500	23.34 ± 2.74	—
50	31.63 ± 2.18	—
1000	44.61 ± 2.29	62.14 ± 4.05

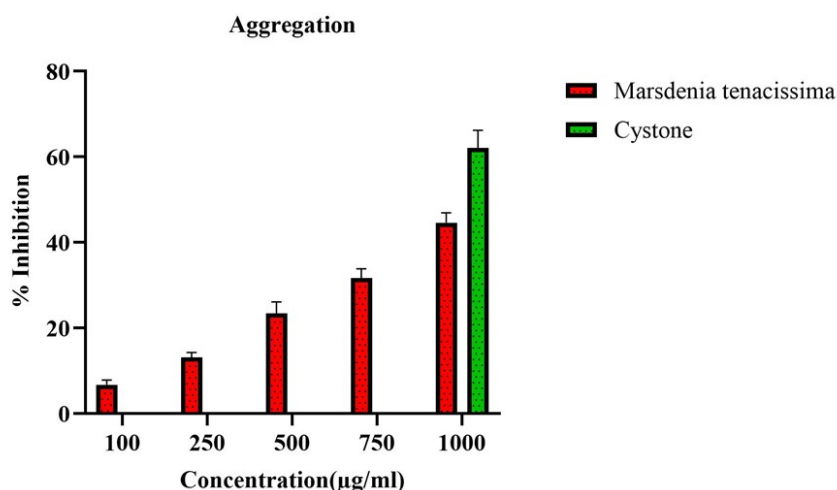


Figure 3: Effect of *Marsdenia tenacissima* and Cystone on calcium oxalate aggregation

Mechanism

The crystal aggregation assay determines the potential of a substance to prevent individual calcium oxalate crystals from clustering together into larger stones, which can obstruct urinary pathways. In this assay *Marsdenia tenacissima* extract demonstrated a concentration-dependent inhibition of crystal

aggregation. At the highest concentration (1000 µg/mL), the extract showed 44.61 ± 2.29% inhibition, whereas Cystone exhibited 62.14 ± 4.05% inhibition, serving as a benchmark. This result suggests that although Cystone was more potent, *M. tenacissima* extract exhibited substantial inhibitory activity, particularly at higher doses.



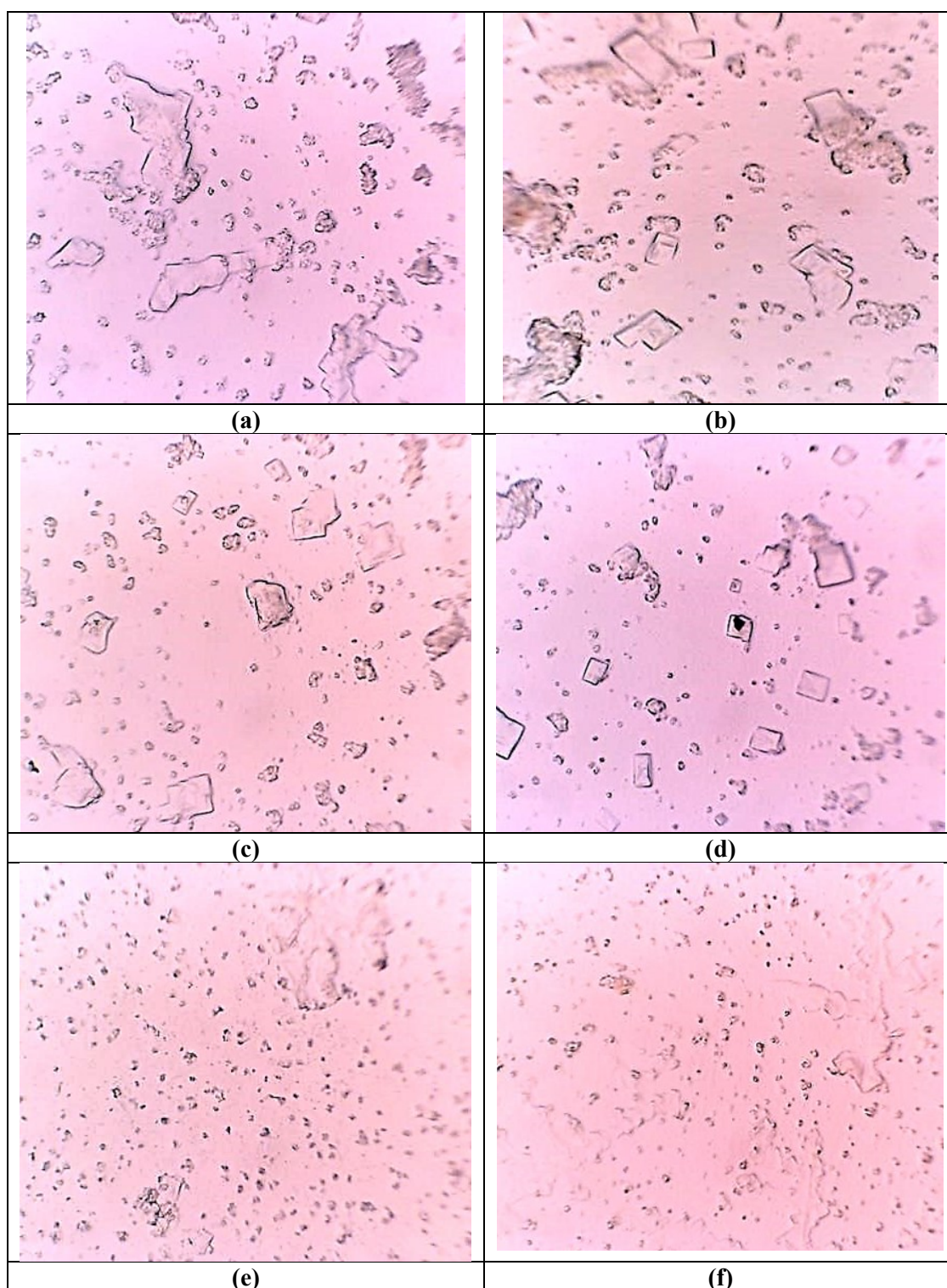


Figure 4: Calcium oxalate crystals in presence of *Marsdenia tenacissima* and cysteine. (a) 100 µg/ml *M. tenacissima*; (b) 250 µg/ml *M. tenacissima*; (c) 500 µg/ml *M. tenacissima*; (d) 750 µg/ml *M. tenacissima*; (e) 1000 µg/ml *M. tenacissima*; (f) 1000 µg/ml Cysteine

The observed anti-aggregation activity can be attributed to several phytochemicals in the extract:

- **Flavonoids and phenolics** can bind to the crystal surface and inhibit inter-crystalline adhesion, effectively reducing aggregate size.
- **Saponins and terpenoids** are known to disrupt the aggregation process by modifying the crystal surface or altering ionic interactions.

Antioxidants present in the extract may help preserve the integrity of renal epithelial cells, preventing them from serving as nucleation sites.

The ability to prevent crystal aggregation is crucial because larger aggregates are more likely to be retained in the kidney and cause obstruction. Preventing aggregation thus not only reduces the size of the formed crystals but also promotes their easier excretion through urine. The results imply that *Marsdenia tenacissima* may serve as a natural therapeutic agent for urolithiasis, by mitigating the progression from microscopic crystals to clinically significant stones.

Crystal Growth Assay

The crystal growth assay was employed to evaluate the inhibitory potential of the ethanol extract of *Marsdenia tenacissima* roots on the progressive enlargement of calcium oxalate crystals, which plays a critical role in the pathogenesis of urolithiasis. In this assay, the percent inhibition of calcium oxalate crystal growth was measured at various concentrations of the extract (100–1000 µg/mL) and compared with a standard antiurolithiatic drug, Cystone, at 1000 µg/mL.

Table 5: Effect of *Marsdenia tenacissima* and Cystone on calcium oxalate growth

Concentration (µg/mL)	<i>Marsdenia tenacissima</i> (Mean ± SD)	Cystone (Mean ± SD)
100	17.61 ± 0.96	–
250	26.39 ± 1.27	–
500	34.01 ± 1.68	–
750	41.27 ± 2.03	–
1000	50.92 ± 2.13	65.43 ± 3.94

The data clearly demonstrate that *Marsdenia tenacissima* extract inhibits calcium oxalate crystal growth in a dose-dependent manner. At the lowest concentration tested (100 µg/mL), the extract showed a moderate inhibitory activity of 17.61%, which increased progressively with concentration, reaching 50.92% at 1000 µg/mL. This concentration-dependent trend indicates the extract's growing efficacy in limiting the enlargement of crystals as its availability increases.

When compared to the standard reference drug, Cystone, which exhibited 65.43% inhibition at 1000 µg/mL, the plant extract showed substantial activity, achieving approximately 78% of the standard drug's effectiveness at the same concentration. This suggests that the ethanol extract of *M. tenacissima* possesses a significant antiurolithiatic effect, particularly in inhibiting the growth phase of stone formation.

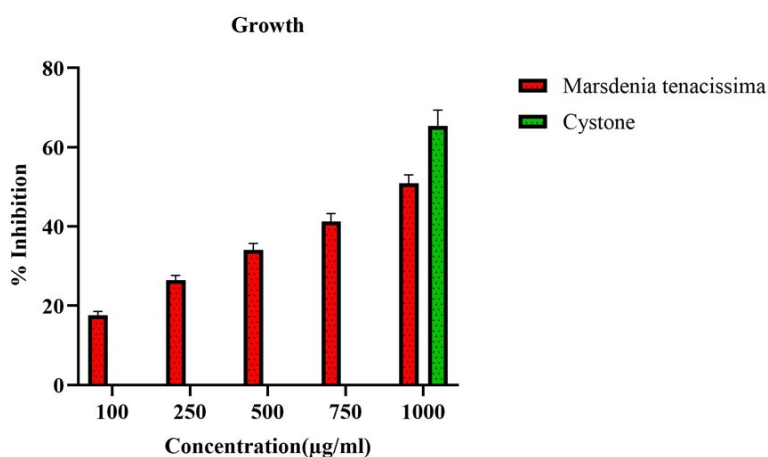


Figure 5: Effect of *Marsdenia tenacissima* and Cystone on calcium oxalate growth

Mechanistic Insights into Crystal Growth Inhibition

The crystal growth phase is critically important in the overall development of kidney stones. Even after

nucleation has occurred, the aggregation and enlargement of crystals into clinically relevant calculi depend largely on the availability of free ions (calcium, oxalate) and their deposition on preformed



crystals. Inhibiting this phase can halt stone progression, reduce the burden of existing microcrystals, and facilitate their excretion via urine.

The observed inhibitory activity of *M. tenacissima* may be attributed to several possible mechanisms:

Calcium Ion Chelation

Many phytochemicals, particularly flavonoids and phenolic acids, have the ability to bind divalent metal ions like calcium [14]. This chelation reduces the free calcium concentration in the surrounding medium, thereby limiting the supersaturation necessary for crystal growth. Flavonoids such as luteolin and apigenin, previously reported in *Marsdenia* species, could play such roles.

Surface Binding and Morphological Disruption

Certain compounds may adsorb onto the surface of calcium oxalate crystals, altering their structure and preventing further ion deposition. This mechanism also interferes with the lattice formation, leading to irregular crystal morphology and reduced crystal maturation [15].

Antioxidant Action

Oxidative stress can contribute to renal epithelial damage, leading to nucleation sites for crystal retention and further growth. Antioxidants present in the extract, including flavonoids, tannins, and phenolics, may reduce oxidative damage, thereby limiting crystal attachment and secondary growth.

Hydrophilic Barrier Formation

Saponins and other surface-active molecules might contribute to the formation of a protective colloidal barrier around crystals, inhibiting coalescence and crystal–cell interactions. This steric hindrance could prevent growth by blocking access to the crystal surface.

Role of Phytoconstituents

The phytochemical profile of *Marsdenia tenacissima* ethanol extract revealed the presence of several classes of bioactive compounds, including:

- **Flavonoids** (e.g., luteolin, kaempferol): Possess strong metal-chelating and antioxidant properties [16].
- **Phenolic compounds**: Involved in free radical scavenging and calcium-binding.
- **Tannins**: Known to interact with proteins and crystal surfaces, thereby preventing crystal aggregation and growth.
- **Saponins**: Surface-active agents that may reduce crystal–cell adhesion and act as dispersing agents.
- **Terpenoids and sterols**: May modulate inflammation and oxidative stress in the urinary tract environment.

The presence of these compounds supports the extract's ability to interfere with multiple stages of stone formation, particularly crystal enlargement. In addition, the cumulative or synergistic effects of these phytoconstituents may enhance the therapeutic potential of the extract beyond the action of a single compound.

While *Marsdenia tenacissima* extract did not surpass Cystone, it demonstrated promising antiurolithiatic activity with over 50% inhibition at the highest concentration. Given that Cystone is a polyherbal formulation standardized over time, the performance of a crude ethanol extract of a single plant achieving comparable efficacy is significant. This finding highlights *M. tenacissima* as a valuable candidate for further phytopharmacological exploration, fractionation, and formulation development.

CONCLUSION

The ethanol extract of *Marsdenia tenacissima* root showed a high extraction yield and a diverse phytochemical composition, confirming the presence of several bioactive constituents. The extract demonstrated strong inhibition of calcium oxalate crystal nucleation, growth, and aggregation in a concentration-dependent manner, suggesting its multiphase protective role against kidney stone formation. These effects, comparable to about 78% of the activity of Cystone, indicate promising antiurolithiatic potential. The observed actions are likely mediated by flavonoids, tannins, and phenolics that interfere with crystal lattice formation, promote metal chelation, and prevent crystal adhesion.



Additionally, their antioxidant and membrane-protective effects may help reduce renal epithelial injury and crystal retention. The absence of lignins and quinones points toward other active phytochemical groups as the primary contributors to the bioactivity. Collectively, the findings validate the traditional use of *M. tenacissima* in treating urinary and renal ailments and support its potential development as a safe, plant-based therapeutic agent for managing urolithiasis.

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AUTHOR CONTRIBUTIONS

All authors contributed significantly to the conception and design of the study, formulation development, data acquisition, analysis, and interpretation.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this work.

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