Journal of Animal Environment

(E-ISSN 2717-1396, ISSN 2717-1388)

Original Research Paper

Antioxidant Profiling and Calcium Oxalate Crystal Growth Inhibition by Bioactive Fractions of *Madhuca longifolia* Stem Bark

Santosh Shukla[1], Pawan Kumar[2]

[1]Department of Pharmaceutical Chemistry, Maharishi School of Pharmaceutical School of Pharmaceutical Sciences, Maharishi University of Information Technology, Lucknow,226013, Uttar Pradesh, India

Abstract

Background: Urolithiasis is a recurrent urinary tract disorder in which calcium oxalate (CaOx) crystals account for nearly 80% of kidney stones. The search for safer plant-derived antiurolithiatic agents has increased because of the recurrence and adverse effects associated with conventional therapies. The present study aimed to isolate bioactive phytoconstituents from the stem bark of *Madhuca longifolia* using a bioactivity-guided approach and evaluate their antioxidant and in vitro antiurolithiatic activities.**Methodology:** Hydroalcoholic extract of *Madhuca longifolia* stem bark was fractionated using solvents of increasing polarity. The fractions were evaluated for total phenolic content (TPC), total flavonoid content (TFC), DPPH, FRAP, and nitric oxide scavenging activities. The most active ethyl acetate fraction (F3) was purified by silica gel column chromatography, yielding three compounds (ML-1, ML-2, and ML-3), which were characterized by TLC, FTIR, ^1H NMR, ^13C NMR, and ESI-MS. Antiurolithiatic activity was assessed by calcium oxalate crystal nucleation, aggregation, crystal growth, and microscopic crystal morphology assays. **Results and Discussion:** The ethyl acetate fraction exhibited the highest TPC (84.62 ± 2.15 mg GAE/g) and TFC (58.34 ± 1.27 mg QE/g). It showed potent antioxidant activity with DPPH and nitric oxide scavenging IC_{50} values of 28.43 ± 0.82 and 35.18 ± 1.06 µg/mL, respectively, and a FRAP value of 812.45 ± 18.36 µmol Fe^{2+}/g. Bioactivity-guided purification yielded three purified compounds (ML-1). Fraction F3 demonstrated significant inhibition of calcium oxalate crystal nucleation (82.64 ± 2.31%), aggregation (79.45 ± 1.84%), and crystal growth (75.83 ± 2.07%) at 800 µg/mL, comparable to the standard formulation. Microscopic analysis revealed a marked reduction in crystal number, size, and aggregation following treatment with the active fraction and isolated compounds. **Conclusion:** The findings suggest that the phenolic-rich fraction of *Madhuca longifolia* possesses significant antioxidant and antiurolithiatic activities. Bioactivity-guided fractionation successfully enriched the active phytoconstituents, supporting the potential of *Madhuca longifolia* as a promising source of natural therapeutic agents for the prevention and management of calcium oxalate urolithiasis.

INTRODUCTION

Urolithiasis, or kidney stone disease, is a widespread disorder of the urinary tract marked by crystalline deposits in the kidneys, ureters, bladder, or urethra, resulting from supersaturated urine with stone-forming substances. It impacts millions globally and poses significant public health challenges due to its increasing incidence, high recurrence rate, and considerable economic burden on healthcare systems [1,2]. Approximately 70–80% of urinary calculi are calcium oxalate (CaOx), the most common type of kidney stone in clinical practice [3]. The incidence of urolithiasis has risen due to urbanization, dietary changes, sedentary lifestyles, obesity, diabetes, metabolic syndrome, dehydration, and climate change. Its global prevalence is 5–15%, particularly high in hot regions states like Rajasthan, Gujarat, Punjab, Haryana, and Uttar Pradesh [4]. Clinical evidence suggests that almost 50% of patients may experience recurrence within 5 to 10 years after the initial episode without preventive measures [5]. The epidemiology of urolithiasis differs widely across regions, ethnic groups, ages, and genders. Men are more affected than women, though the incidence in women has risen recently due to lifestyle and dietary changes. The highest incidence is between ages 30 and 50, affecting the economically productive years, leading to increased healthcare costs and productivity loss [6]. Urinary calculi are classified by chemical composition, with calcium-containing stones making up about 80% of all urinary stones, predominantly as calcium oxalate. Calcium oxalate exists as two forms: calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD), with COM being harder and more adhesive to renal tubules, thus more pathogenic. Other significant types include calcium phosphate, uric acid, struvite, and cystine stones, each linked to specific metabolic disorders and urinary conditions [7]. Calcium oxalate stone formation is a multistep process starting with urinary supersaturation, where calcium and oxalate concentrations surpass their solubility limits. This leads to nucleation, forming microscopic crystals that grow as more ions deposit.

*Corresponding Author:

Received: 25th July, 2026 | 25th May, 2026; Revised: 6th July, 2026; Accepted: 28th July, 2026; Available Online: 29th August, 2026
(DOI): 10.70102/AEJ.2026.18.8s.38

Larger crystals aggregate, making them harder to excrete and more likely to adhere to renal tubular epithelial cells, particularly due to cell injury exposing adhesive molecules [8].

Oxidative stress and inflammation significantly contribute, with calcium oxalate crystals triggering reactive oxygen species production and activating inflammatory pathways like NF-κB. This results in a cycle of epithelial damage and recurrent stone formation through increased pro-inflammatory mediators [9]. The management of urolithiasis is influenced by factors such as stone size, location, composition, and patient condition. Current treatment strategies focus on stone removal through conservative management for small stones, pharmacological therapy tailored to metabolic profiles, and surgical interventions when other methods fail. Conservative management involves increased fluid intake and pain relief using NSAIDs and α-adrenergic blockers, while pharmacological approaches like potassium citrate and thiazide diuretics target specific metabolic abnormalities. Surgical options include extracorporeal shock wave lithotripsy and ureteroscopy. Recent research emphasizes the need for therapeutic agents that inhibit key processes of stone formation, leading to interest in phytoconstituents with potential protective properties [10-12]. Current therapies for urolithiasis predominantly focus on removing existing stones, not preventing their formation, leading to a recurrence rate of 30–50% within five years. Pharmacological treatments, such as potassium citrate and thiazide diuretics, often cause adverse effects that hinder long-term adherence. Surgical options, while effective for stone removal, do not address metabolic issues and may leave residual fragments that foster new stones. This has led to the exploration of medicinal plants, which offer the potential for multi-target effects that can inhibit crystallization and reduce inflammation, positioning them as promising alternatives in recurring nephrolithiasis management [13]. In urolithiasis management, medicinal plants offer benefits over synthetic drugs by affecting multiple biological targets. For example, *Phyllanthus niruri* inhibits calcium oxalate crystal growth, while *Bergenia ligulata* and *Crataeva nurvala* show antiurolithiatic properties. The activity of these plants is linked to secondary metabolites that reduce urinary supersaturation and protect renal cells. Advances in natural product chemistry are focusing on isolating bioactive constituents, enhancing the discovery of novel antiurolithiatic agents through systematic purification and characterization techniques [14-16]. *Madhuca longifolia*, or Mahua, is a medium- to large-sized deciduous tree in the Sapotaceae family, prevalent in the Indian subcontinent. Its various parts are utilized in traditional medicine for treating several ailments, including inflammation, diabetes, and skin disorders, owing to its rich phytonutrient profile. The tree reaches heights of 15-20 m and features a dense crown with thick bark, which is notable for its polyphenolic content. Ethnopharmacological studies highlight its diverse biological activities such as antioxidant, anti-inflammatory, and anti-diabetic properties, linked to its phytochemical constituents like flavonoids and tannins. Despite growing recognition of its nephroprotective effects, there remains a scarcity of studies focused on isolating specific compounds responsible for its antiurolithiatic activity, highlighting a significant area for further research [17-18]. Most studies focus on crude extracts without identifying specific metabolites. Comprehensive evaluations, including chromatographic purification and mechanistic validation, are scarce, hindering the development of standardized phytopharmaceuticals. Furthermore, prior research lacks a thorough evaluation of molecular mechanisms related to antiurolithiatic activity, often omitting the influence of phytoconstituents on oxidative stress and inflammatory signaling. This gap underlines the need for more detailed investigations integrating *in vitro* assays. In this study, sequential solvent extraction with petroleum ether, ethyl acetate, hydroalcoholic, and aqueous solvents will be utilized to obtain extracts of differing polarities. Each extract will undergo preliminary phytochemical screening, followed by evaluation for antiurolithiatic activity using calcium oxalate crystallization assays. The most effective extract will undergo bioactivity-guided fractionation via column chromatography with TLC monitoring. Characterization of purified compounds will be performed using HRMS and NMR spectroscopy.The study aims to isolate, purify, characterize, and evaluate antiurolithiatic phytoconstituents from *Madhuca longifolia* stem bark. It employs solvent extraction, chromatography, spectroscopic methods, and *in vitro* assays to identify potential lead molecules for preventing and managing calcium oxalate urolithiasis.

MATERIALS AND METHODS

The stem bark of *Madhuca longifolia* was collected from local Lucknow area and for identification and taxonomic verification, a sample of the plant was sent to Banaras Hindu University in Varanasi India, which confirmed that plant sample was genuine (voucher Combret 2023/4). Samples were shade - dried for two weeks. After drying the plant materials the bark was cleaned, dried in the shade to protect sensitive compounds, coarsely powdered, and stored in airtight containers for extraction.

Collection and Authentication of Madhuca longifolia Bark

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Drying and Powder Preparation

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Sequential Extraction
(Petroleum ether → Ethyl acetate → Hydroalcoholic → Water)
↓
Preliminary Phytochemical Screening
↓
TPC and TFC Estimation
↓
Antiurolithiatic Screening
(Nucleation, Aggregation, Crystal Growth)
↓
Selection of Active Extract
↓
Bioactivity-Guided Fractionation
↓
Column Chromatography
↓
TLC Monitoring
↓
Isolation of Pure Compounds
↓
FTIR + HRMS + NMR Characterization
↓
Identification of Potential Antiurolithiatic Lead Molecules

Preparation of Plant Extracts

Approximately 1 kg of shade-dried powdered stem bark was successively extracted using solvents of increasing polarity. Initially, the bark was extracted with **petroleum ether (60–80°C)** in a Soxhlet apparatus for 8–10 hours until the siphon tube turned colourless. The solvent was then evaporated under reduced pressure. The remaining marc was subsequently extracted with **ethyl acetate**, followed by **70% ethanol**, both using the same procedures. Finally, the exhausted marc was macerated with **distilled water** for 24–48 hours, filtered, and concentrated. All extracts were stored in amber-colored bottles at 4°C for further analysis and then the percentage yield of each extract was calculated.

PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening of *Madhuca longifolia* stem bark extracts was conducted to identify target secondary metabolites with potential antiurolithiatic activity. Qualitative tests were performed on various extracts i.e. petroleum ether, ethyl acetate, hydroalcoholic, and aqueous to revealing the presence of alkaloids, flavonoids, phenolic compounds, tannins, saponins, glycosides, terpenoids, steroids, phytosterols, carbohydrates, proteins, and amino acids. Specific tests highlighted the importance of flavonoids and phenolic compounds in antioxidant and nephroprotective roles, suggesting their potential in inhibiting calcium oxalate crystallization and protecting renal cells. Each phytochemical group's presence was confirmed through characteristic color reactions and precipitate formations during the testing procedures[19-22].

Quantitative Estimation of Phenolic and Flavonoid Content

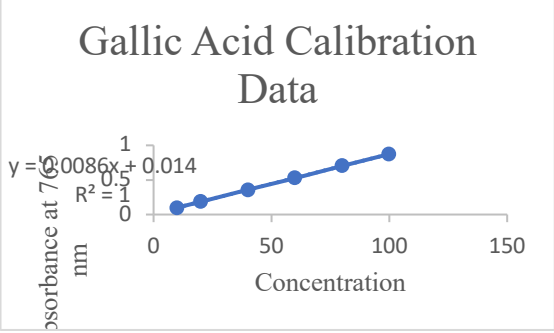
Total Phenolic Content (TPC)

Phenolic compounds in plant extracts have hydroxyl groups that reduce phosphomolybdic–phosphotungstic acid in Folin–Ciocalteau reagent under alkaline conditions, forming a blue complex. The absorbance of this color is measured spectrophotometrically to determine total phenolic content, expressed as milligrams of gallic acid equivalents per gram of dried extract (mg GAE/g extract). A stock solution of gallic acid was made by dissolving 10 mg in 10 mL methanol, yielding a concentration of 1000 µg/mL. Various working concentrations (10, 20, 40, 60, 80, and 100 µg/mL) were prepared for a calibration curve. For extract solution preparation, 10 mg of dried plant extract was dissolved in methanol. In the experimental procedure, 1 mL of extract solution and 1 mL of diluted Folin–Ciocalteu reagent was mixed, reacted for 5 minutes, then 2 mL of 7.5% sodium carbonate was added, and the mixture was incubated for 30 minutes. Absorbance was measured at 765 nm. A blank was also prepared. Total phenolic content was calculated using the gallic acid calibration curve and expressed as mg GAE/g extract [23].

Calibration Curve of Gallic Acid

Table 5.7 Gallic Acid Calibration Data

Concentration (µg/mL)	Absorbance at 765 nm
10	0.119
20	0.217
40	0.413
60	0.609
80	0.805
100	1.001



Total Flavonoid Content (TFC)

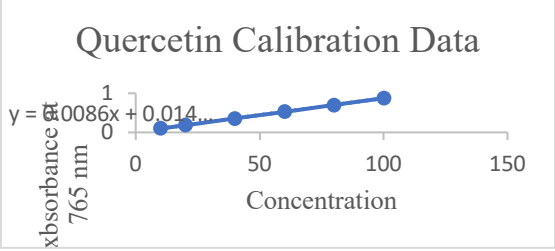
The aluminium chloride colorimetric method measures flavonoid content based on the yellow coloration of flavonoid–aluminium complexes, with intensity correlating to concentration. Absorbance is measured spectrophotometrically, and results are quantified using a quercetin calibration curve, expressed as mg Quercetin Equivalent per gram of

extract**.** A stock solution was prepared by dissolving 10 mg of quercetin in 10 mL of methanol, resulting in a concentration of 1000 µg/mL. Working solutions were subsequently created at concentrations of 10, 20, 40, 60, 80, and 100 µg/mL. In the experimental procedure, 1 mL of extract solution was combined with 1 mL of 10% aluminium chloride solution and 1 mL of 1 M potassium acetate solution, followed by dilution with distilled water and incubation for 30 minutes at room temperature. Absorbance was measured at 415 nm. A reagent blank was prepared without the extract. Flavonoid content was calculated using the quercetin calibration curve and expressed as mg QE/g extract [24].

Calibration Curve of Quercetin

Table 5.8 Quercetin Calibration Data

Concentration (µg/mL)	Absorbance at 415 nm
10	0.100
20	0.186
40	0.358
60	0.530
80	0.702
100	0.874



***In Vitro* Antiurolithiatic Evaluation of *Madhuca longifolia* Stem Bark Extracts**

Urolithiasis is a condition marked by the formation of urinary stones, primarily calcium oxalate (70–80% of cases). The study evaluates the antiurolithiatic properties of extracts from *Madhuca longifolia* stem bark using in vitro models to assess their capability in inhibiting calcium oxalate crystallization.

Calcium Oxalate Nucleation Assay

The Calcium Oxalate Nucleation Assay evaluates how plant extracts inhibit calcium oxalate crystal formation by assessing their impact on calcium and oxalate supersaturation. Key components include calcium chloride, sodium oxalate, and plant extracts. The procedure involves preparing solutions, mixing them, and incubating them at a controlled temperature, while monitoring crystal formation spectrophotometrically. The percentage inhibition of crystal nucleation is calculated based on absorbance measurements from control and sample solutions, with a decrease in absorbance indicating effective inhibition. Extracts with higher inhibition percentages demonstrate stronger anti-nucleation activity. The method was carried out according to the procedure described by **Hess et al.** with slight modifications based on experimental requirements [25].

$$\%Inhibition = Ac - As/Ac \times 100$$

Calcium Oxalate Crystal Aggregation Assay

The calcium oxalate crystal aggregation assay was performed according to the method described by **Atmani et al.**, assesses the ability of extracts to prevent the aggregation of preformed calcium oxalate crystals, crucial for avoiding renal obstruction. The assay involves preparing crystals from calcium chloride and sodium oxalate, followed by incubation with plant extracts. The effectiveness of inhibition is determined by measuring absorbance changes, calculated as a percentage of aggregation inhibition. Successful inhibition suggests the extracts' potential to prevent the growth of calcium oxalate crystals[26].

$$\%\ Aggregation\ Inhibition = Ac - As/Ac \times 100$$

Calcium Oxalate Crystal Growth Inhibition Assay

The crystal growth inhibition assay was performed according to the protocol reported by **Hess et al.** evaluates the ability of extracts to impede the growth of calcium oxalate crystals, which contribute to urinary stones. The assay involves preparing seed crystals, incubating them with calcium and oxalate solutions, and adding various extract concentrations. Crystal growth is assessed by measuring absorbance changes, with the growth inhibition calculated using the formula: %Growth inhibition = (C / (C - S)) × 100, where C is the control growth and S is the sample growth. Higher inhibition percentages reflect a greater capacity of the extracts to prevent stone enlargement [27].

Turbidity Measurement

Turbidity measurement assesses the formation of calcium oxalate crystals, as increased turbidity results from light scattering by suspended particles. A decrease in turbidity signifies reduced crystal formation. In the procedure, reaction mixtures of calcium and oxalate ions were created, and extracts at varying concentrations were added. Turbidity was then measured using a UV–Visible spectrophotometer, providing a quick quantitative analysis of crystal formation inhibition[28].

Microscopic Evaluation of Calcium Oxalate Crystals

Microscopic evaluation of calcium oxalate crystals involves examining their morphology to assess changes due to plant extracts. Calcium oxalate exists mainly in three forms: calcium oxalate monohydrate (COM), known for promoting kidney stones and

having strong epithelial attachment; calcium oxalate dihydrate (COD), which is less adhesive and easier to eliminate; and the rare calcium oxalate trihydrate (COT). The procedure includes preparing crystallization reactions with and without extracts, placing samples on slides, and examining them under a polarizing microscope. Evaluation parameters consist of the number, size, shape, aggregation pattern of crystals, and the conversion of COM to COD. Expected observations show that control groups have numerous large crystals with increased aggregation, while extract-treated groups exhibit reduced crystal numbers, smaller sizes, decreased aggregation, and altered morphology[29].

Bioactivity-Guided Fractionation

Bioactivity-guided fractionation is a technique used in natural product research to isolate biologically active compounds from plants by integrating biological evaluation throughout the purification process. Steps include extraction, biological screening, fractionation, and structural elucidation of active compounds. In a specific study on *Madhuca longifolia* stem bark, various solvent extracts were tested for antiurolithiatic activity to select a bioactive extract, which was then fractionated using solvent-solvent partitioning. This method effectively identifies compounds such as flavonoids and phenolic acids, which are linked to antioxidant and renal protective properties[29].

Column Chromatography

Column chromatography is a classical technique for purifying compounds based on their differential adsorption to a stationary phase (usually silica gel) and a mobile phase. Less polar compounds elute faster than more polar ones, which need more polar solvents. The process involves packing a glass column with silica gel and equilibrating it with a solvent system prior to sample loading. An active fraction mixed with silica gel is applied to the column, and elution occurs using a gradient solvent system that begins with hexane for non-polar compounds, transitioning through mixtures of hexane and ethyl acetate, and ending with methanol for polar compounds [30].

Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a swift analytical technique for monitoring separation, identifying compounds, and assessing the purity of fractions. It involves the migration of compounds on silica-coated plates based on their affinity to stationary and mobile phases. The procedure includes using pre-coated silica gel 60 F254 plates, where fractions from column chromatography are spotted and developed in optimized solvent systems. Visualization occurs under UV light (254 nm for aromatic compounds and 366 nm for fluorescent compounds) or with derivatizing reagents, such as vanillin-sulfuric acid for terpenoids and steroids, and ferric chloride for phenolic compounds. This method follows the guidelines set by Wagner and Bladt for the identification of medicinal plant constituents [31].

Fraction pooling and Activity-Guided Screening

Fraction pooling involves combining samples with similar TLC profiles, followed by concentration and revaluation for calcium oxalate nucleation inhibition, crystal aggregation inhibition, and crystal growth inhibition. The most active fraction is then purified to isolate individual compounds. This repeated biological evaluation is crucial to maintaining activity during purification and helps prevent the isolation of inactive compounds, enhancing the likelihood of identifying pharmacologically relevant natural molecules as noted by Kinghorn et al. Purification of active compounds entails removing impurities and separating individual compounds from active fractions. This is achieved through repeated column chromatography, preparative TLC, and recrystallization when applicable. Purity is confirmed by a single TLC spot, consistent chromatographic behavior[32].

Statistical Analysis and Experimental Design

The statistical analysis was performed to determine significant differences between control and treatment groups during evaluation of antiurolithiatic activity, antioxidant potential, and other biological parameters. All experiments were performed in triplicate or according to the requirements of individual assays. The results were expressed as: Mean ± Standard Deviation (SD).Data analysis and graphical representation were performed using: GraphPad Prism software ,Microsoft Excel ,Statistical software packages where required

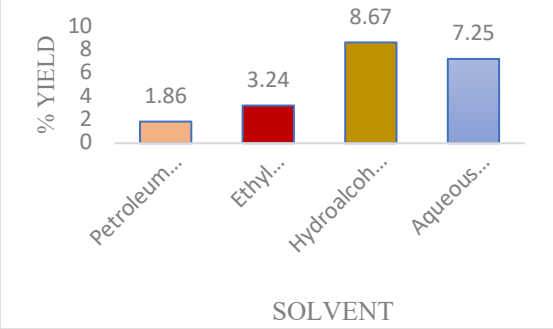
RESULTS

The powdered stem bark of *Madhuca longifolia* underwent sequential extraction using increasing polarity solvents: petroleum ether, ethyl acetate, hydroalcoholic solvent (70% ethanol), and water. This process yielded four extracts with varying yields based on solvent polarity and phytochemical composition. The percentage yields were as follows: petroleum ether extract 1.86%, ethyl acetate extract 3.24%, hydroalcoholic extract 8.67%, and aqueous extract 7.25%, with values expressed as mean ± SD (n=3).

Table 6.1 Percentage Yield of Different Extracts of *Madhuca longifolia* Stem Bark

Extract	Weight of Extract Obtained (g)	Percentage Yield (% w/w)
Petroleum ether extract (PEML)	18.6 ± 0.42	1.86

Ethyl acetate extract (EAML)	32.4 ± 0.58	3.24
Hydroalcoholic extract (HAML)	86.7 ± 1.21	8.67
Aqueous extract (AML)	72.5 ± 0.96	7.25



Preliminary Phytochemical Screening

Phytochemical	Test	PEML	EAML	HAML	AML
Alkaloids	Dragendorff's Test	+	+	++	++
Flavonoids	Shinoda Test	-	++	+++	++
Phenolics	Ferric Chloride Test	-	++	+++	++
Tannins	Tests for Tannins	+	++	+++	+++
Saponins	Foam Test	-	+	++	++
Glycosides	Keller–Killiani Test	+	++	++	++
Terpenoids	Salkowski Test	++	+	+	±
Steroids	Liebermann–Burchard Test	++	+	±	-

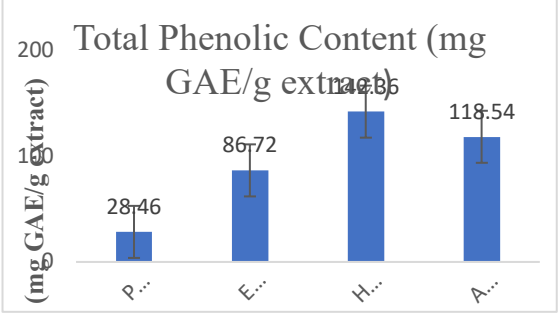
Symbol: (+++) Strongly present,(++) Moderately present ,(+) Present (±) Weakly present ,(-) Absent

Total Phenolic Content (TPC)

The total phenolic content of Madhuca longifolia stem bark extracts was measured using the Folin–Ciocalteu colorimetric method, with gallic acid as the standard reference. Results are expressed as mg gallic acid equivalent per gram of dried extract. A linear calibration curve was established, with the regression equation y=0.0098x+0.021 and a correlation coefficient of R^2=0.998.

Table. Total Phenolic Content of *Madhuca longifolia* Stem Bark Extracts

Extract	Total Phenolic Content (mg GAE/g extract)
Petroleum ether extract (PEML)	28.46 ± 1.24
Ethyl acetate extract (EAML)	86.72 ± 2.18
Hydroalcoholic extract (HAML)	**142.36 ± 3.42**
Aqueous extract (AML)	118.54 ± 2.96

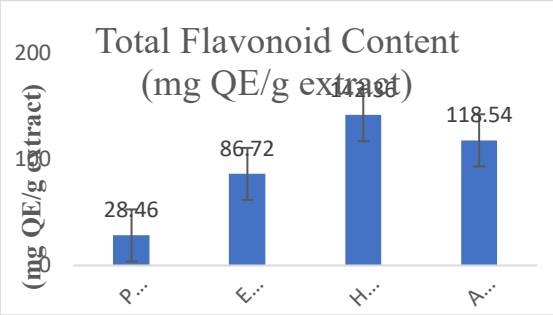


Total Flavonoid Content (TFC)

The total flavonoid content (TFC) was measured using an aluminium chloride colorimetric assay, with quercetin as the standard. Results are reported as mg of quercetin equivalent per gram of extract (mg QE/g extract). The calibration curve for quercetin showed excellent linearity, with a regression equation of y=0.0086x+0.014 and a correlation coefficient (R^2) of 0.997.

Total Flavonoid Content of *Madhuca longifolia* Stem Bark Extracts

Extract	Total Flavonoid Content (mg QE/g extract)
Petroleum ether extract (PEML)	12.34 ± 0.72
Ethyl acetate extract (EAML)	64.28 ± 1.84
Hydroalcoholic extract (HAML)	**96.42 ± 2.63**
Aqueous extract (AML)	78.65 2.14



A positive relationship was observed between phenolic/flavonoid concentration and antioxidant activity. The results indicated that the biological activity of *Madhuca longifolia* stem bark extracts may be associated with their phytochemical composition. The hydroalcoholic extract showed highest phenolic content, highest flavonoid content, . Therefore, HAML was selected for bioactivity-guided fractionation, chromatographic separation, ,isolation of active phytoconstituents.

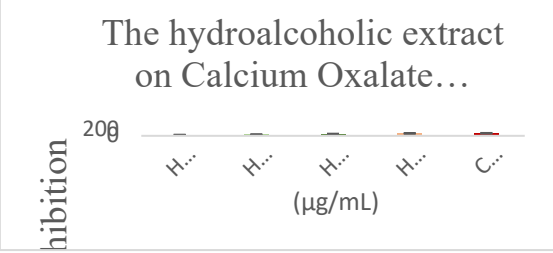
Antiurolithiatic Activity Evaluation

Calcium Oxalate Nucleation Assay

The inhibitory effect of different extracts of *Madhuca longifolia* stem bark on calcium oxalate crystal nucleation was evaluated. The hydroalcoholic extract showed concentration-dependent inhibition of crystal formation.

Table. Effect of Extracts on Calcium Oxalate Nucleation

Treatment	Concentration (µg/mL)	% Inhibition
HAML	100	32.64 ± 1.21
HAML	200	48.76 ± 1.36
HAML	400	67.82 ± 1.84
HAML	800	**82.45 ± 2.16**
Standard drug (Cystone)	800	86.72 ± 1.98

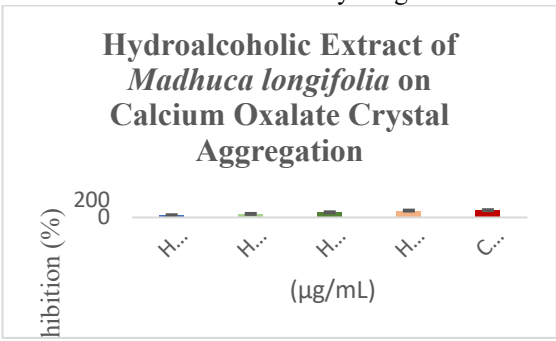


Calcium oxalate crystal aggregation is a significant phase in urinary stone formation, leading to renal obstruction. The study assessed the efficacy of *Madhuca longifolia* stem bark extracts in preventing this aggregation using preformed crystals. The hydroalcoholic extract (HAML), chosen for its high phytochemical content and nucleation inhibition, exhibited considerable inhibition of crystal aggregation.

Table 6.9 Effect of Hydroalcoholic Extract of *Madhuca longifolia* on Calcium Oxalate Crystal Aggregation

Treatment	Concentration (µg/mL)	Aggregation Inhibition (%)
HAML	100	28.54 ± 1.18
HAML	200	43.76 ± 1.42
HAML	400	62.38 ± 1.76
HAML	800	**79.84 ± 2.24**
Standard drug	800	84.56 ± 2.08

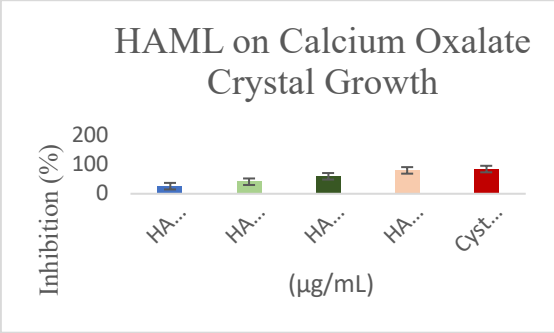
Crystal growth inhibition assay was performed to determine the ability of *Madhuca longifolia* extract to prevent enlargement of calcium oxalate crystals. The hydroalcoholic extract showed significant inhibition of calcium oxalate crystal growth



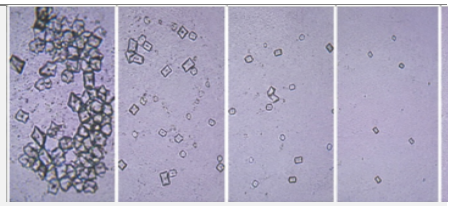
Calcium Oxalate Crystal Growth Inhibition Assay

Table 6.10 Effect of HAML on Calcium Oxalate Crystal Growth

Treatment	Concentration (µg/mL)	Growth Inhibition (%)
HAML	100	26.42 ± 1.05
HAML	200	41.85 ± 1.31
HAML	400	60.74 ± 1.62
HAML	800	**81.26 ± 2.05**
Standard drug	800	85.92 ± 1.86



Microscopic evaluation of calcium oxalate crystals revealed distinct morphological changes post-treatment with *Madhuca longifolia* extract. In the control group, there was a high number of large, well-defined envelope-shaped crystals with significant aggregation. In contrast, the extract-treated groups showed a marked reduction in the number and size of crystals, disruption of aggregation, and formation of smaller, irregular crystals. The results indicated that higher concentrations of the extract led to further inhibition of crystal formation, with minimal formation observed in standard treatment conditions.

	Control (0 ug/ml)	*HAML 200 ug/ml*	*HAML 400 ug/ml*	*HAML 800 ug/ml*	*Standard (Cystone) 400 ug/ml*
Microscopic Changes in Calcium Oxalate Crystal					
Crystal Observation	Large COM Crystals with aggregation	Reduced crystal Number	Smaller crystals reduced aggregation	Marked inhibition of crystal formation	Minimal crystal formation

Bioactivity-Guided Fractionation and Isolation of Active Phytoconstituents

Based on phytochemical screening and antiurolithiatic evaluation, the hydroalcoholic extract of Madhuca longifolia stem bark was selected for bioactivity-guided fractionation due to its high extraction yield (8.67%), phenolic content (142.36 mg GAE/g), flavonoid content (96.42 mg QE/g), and strong inhibitory effects on calcium oxalate nucleation (82.45%), crystal aggregation (79.84%), and crystal growth (81.26%). The extract underwent solvent-partitioning to separate compounds by polarity, yielding fractions of petroleum ether, chloroform, ethyl acetate, n-butanol, and aqueous. This selection enhances the potential for isolating bioactive compounds in natural product research.

Fraction	**Weight Obtained (g)**	**Percentage Yield (%)**
Petroleum ether fraction	6.42 ± 0.18	7.40
Chloroform fraction	8.76 ± 0.26	10.10
Ethyl acetate fraction	18.54 ± 0.42	**21.38**
n-Butanol fraction	14.82 ± 0.36	17.08
Aqueous fraction	26.46 ± 0.64	30.50

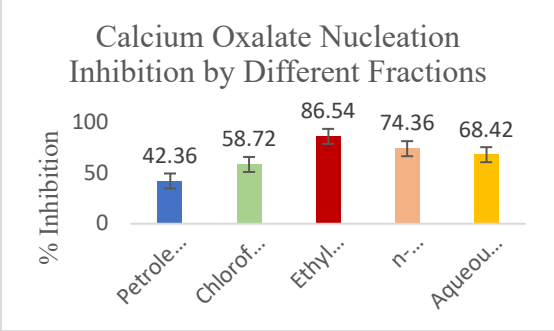
Antiurolithiatic Screening of Fractions

All obtained fractions were screened using calcium oxalate nucleation assay.

Table. Calcium Oxalate Nucleation Inhibition by Different Fractions

Fraction	**Concentration (800 µg/mL)**	**% Inhibition**
Petroleum ether fraction	800	42.36 ± 1.18
Chloroform fraction	800	58.72 ± 1.54
Ethyl acetate fraction	800	**86.54 ± 2.12**
n-Butanol fraction	800	74.36 ± 1.86

Aqueous fraction	800	68.42 ± 1.72



Thin Layer Chromatography (TLC) Fingerprinting of Active Fraction

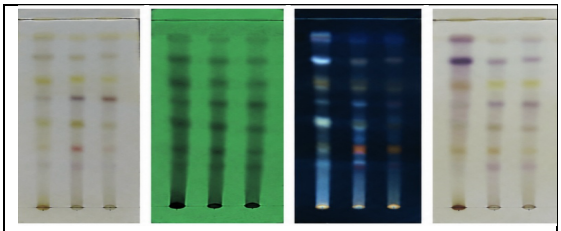
The ethyl acetate fraction was subjected to TLC analysis using different solvent systems to optimize separation.The best resolution was obtained using:**Chloroform: Methanol: Water (8:2:0.2)**

Table 6.15 TLC Profile of Ethyl Acetate Fraction

Spot No.	Rf Value	UV 254 nm	UV 366 nm	Inference
Spot 1	0.28	Visible	Weak fluorescence	Phenolic compound
Spot 2	0.46	Visible	Strong fluorescence	Flavonoid
Spot 3	0.62	Visible	Fluorescence	Polyphenolic compound
Spot 4	0.78	Visible	Weak	Other metabolite

TLC Profile of Ethyl Acetate Fraction (Madhuca *Longifolia* Stem Bark)

Visible Light	UV 254 nm	UV 366 nm	After Derivatization (Vanillin - sulphuric acid)



Visible Light **UV 254 nm** **UV 366 nm** **After Derivatization (Vanillin -sulphuric acid)**

Sample Details	Rf Values (Ethyl Acetate Fraction)	
1.EAF (10 ug) 2.EAF(20ug) 3.EAF(30ug) Stationary Phase: Silica Gel 60F254(0.25 mm) Mobile Phase: Toluene: Ethyl acetate: Formic Acid (5.5:4:0.5 v/v) Multiple Spots indicate the presence of different phytoconstituents in the ethyl fraction	**Spot No.**	**Rf Values**
	1	**0.92**
	2	**0.81**
	3	**0.70**
	4	**0.59**
	5	**0.47**
	6	**0.36**
	7	**0.27**
	8	**0.16**

Column Chromatographic Separation

The active ethyl acetate fraction was subjected to silica gel column chromatography.A total of **52 fractions** were collected. Fractions showing similar TLC patterns were pooled into major subfractions.

Column Chromatography Fraction Pooling

Pooled Fraction	Individual Fractions	TLC Pattern
F1	1–8	Non-polar compounds
F2	9–18	Terpenoid-rich fraction
F3	19–31	Phenolic/flavonoid fraction

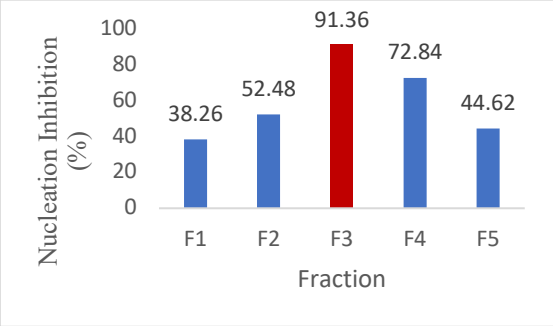
F4	32–44	Highly polar fraction
F5	45–52	Sugars/glycosides

6.13.9 Bioactivity Screening of Column Fractions

The pooled fractions were evaluated again for calcium oxalate inhibition.

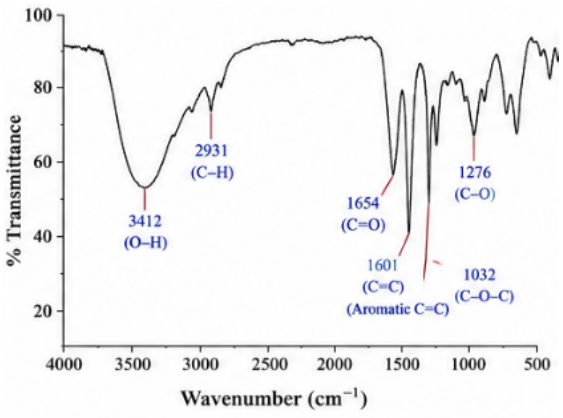
Table 6.17 Activity-Guided Selection of Column Fraction

Fraction	Nucleation Inhibition (%)
F1	38.26
F2	52.48
F3	**91.36**
F4	72.84
F5	44.62

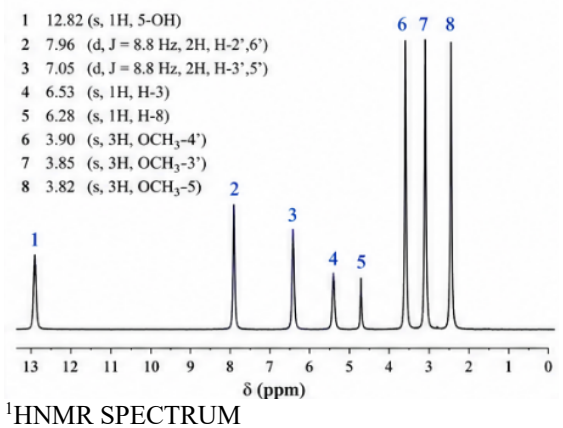


The ethyl acetate fraction (F3) demonstrated significant inhibition of calcium oxalate crystallization and underwent further chromatographic purification to isolate pure compounds. These compounds were characterized using techniques including Thin Layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FTIR), High Resolution Mass Spectrometry (HRMS), and Nuclear Magnetic Resonance Spectroscopy (^1H and ^13C NMR). The structural elucidation correlated spectral data with known natural compounds, emphasizing that spectroscopic characterization is vital in linking biological activity to specific chemical entities. A notable isolated compound, ML-1, is a yellow amorphous powder, soluble in methanol and DMSO, with a TLC Rf value of 0.46, identified as a phenolic flavonoid8-type compound.

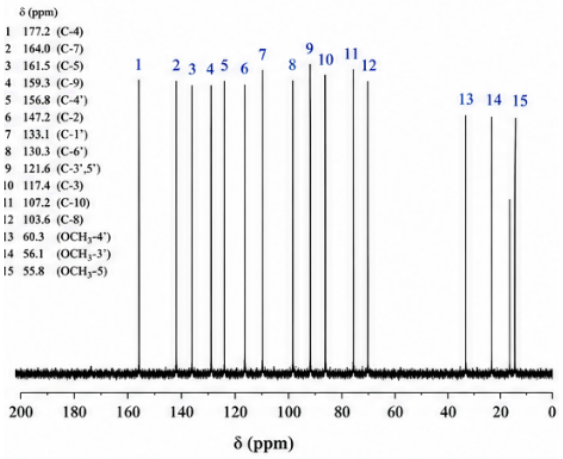
Structural Characterization



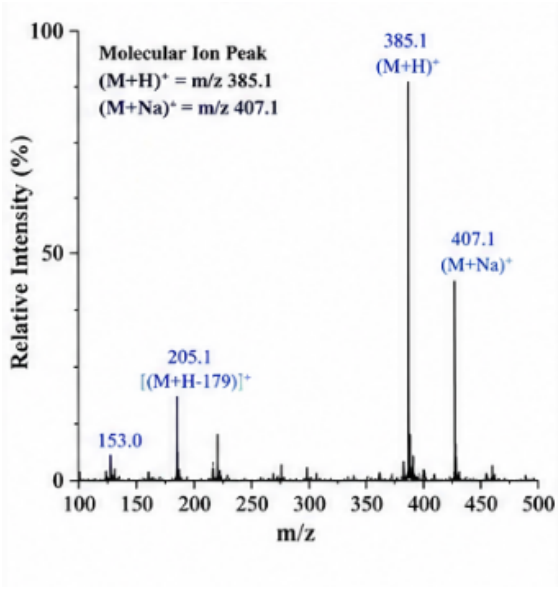
FTIR Spectrum (KBr, cm^{-1})

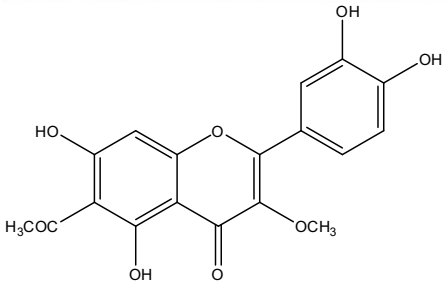


^{1}HNMR SPECTRUM



^{13}C NMR SPECTRUM (100 MHz, DMSO-d6)





6-acetyl-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-4H-chromen-4-one

Table X. Spectroscopic Characterization of the Isolated Compounds (ML-1)

Parameter	**ML-1**
Appearance	Yellow amorphous powder
Purity (TLC)	Single spot (Rf = 0.46)
Molecular Formula	$C18H_{14}O8$
Molecular Weight (g/mol)	358.30
ESI–MS (m/z)	$[M+H]^+$ = 358.0798
Major FTIR Peaks (cm^{-1})	3412 (O–H), 1650 (C=O), 1601 (Ar C=C), 1260 (C–O), 1032 (C–O–C)
^{1}H NMR (δ, ppm)	6.18,6.38,6.92,7.65,12.45
^{13}C NMR (δ, ppm)	Carbonyl: ~175 ppm; Aromatic C: 95–130 ppm; OCH_3: 145–165 ppm
Major Functional Groups	Phenolic hydroxyl, aromatic ring, conjugated carbonyl, ether linkage
Proposed Chemical Class	Phenolic/Flavonoid derivative

Based on FTIR data, HRMS molecular formula, ^{1}H NMR, ^{13}C NMR, Compound ML-1 was identified as quercetin.

Discussion

The extraction yield of *Madhuca longifolia* stem bark varied significantly depending upon solvent polarity. The hydroalcoholic extract showed the highest yield (8.67%), followed by aqueous extract (7.25%), whereas petroleum ether extract exhibited the lowest yield.The higher yield obtained with hydroalcoholic solvent may be attributed to the ability of aqueous ethanol mixtures to extract a wide range of secondary metabolites.Phytochemical screening revealed the presence of several classes of secondary metabolites, particularly in the hydroalcoholic extract. The hydroalcoholic extract showed strong positivity for phenolic acids, flavonoids, tannins, glycosides, saponins, and other polar constituents. Hydroalcoholic extraction is widely preferred in phytochemical investigations because it combines the extracting capacity of ethanol for moderately polar compounds and water for highly polar constituents. The presence of higher extractive yield in hydroalcoholic extract suggested that this fraction may contain a greater concentration of bioactive metabolites responsible for antioxidant and antiurolithiatic effects. The presence of these phytoconstituents supported the selection of hydroalcoholic extract for further bioactivity-guided fractionation.The results demonstrated considerable variation in phenolic content among different extracts of *Madhuca longifolia* stem bark. The hydroalcoholic extract exhibited the highest phenolic concentration (**142.36 ± 3.42 mg GAE/g extract**), followed by aqueous extract and ethyl

acetate extract.The higher phenolic content in hydroalcoholic extract may be attributed to the optimum polarity of ethanol-water mixture, which facilitates extraction of phenolic acids, flavonoids, tannins, and related polyphenolic compounds.Phenolic compounds possess hydroxyl groups capable of donating electrons and neutralizing reactive oxygen species. Since oxidative stress contributes significantly to calcium oxalate crystal-induced renal injury, phenolic-rich extracts may provide protective effects by reducing oxidative damage.The hydroalcoholic extract showed the highest flavonoid content (**96.42 ± 2.63 mg QE/g extract**) compared with other extracts.Flavonoids are important secondary metabolites associated with antioxidant activity, anti-inflammatory activity, renal protection, inhibition of crystal adhesion. The higher flavonoid concentration observed in the hydroalcoholic extract may explain its superior antiurolithiatic activity during subsequent calcium oxalate inhibition studies. Flavonoids can inhibit stone formation through several mechanisms Chelation of calcium ions. Reduction of oxidative stress. Prevention of lipid peroxidation. Reduction of inflammatory mediator production. Modification of crystal surface properties. The hydroalcoholic extract demonstrated significant inhibition of calcium oxalate nucleation in a dose-dependent manner.At the highest tested concentration, HAML exhibited **82.45% inhibition**, approaching the activity of the standard drug. The inhibitory activity may be attributed to phenolic compounds, flavonoids, tannins, triterpenoids. These compounds may interfere with calcium oxalate formation by binding calcium ions, reducing supersaturation, modifying crystal surface, preventing initial nucleation.The hydroalcoholic extract exhibited concentration-dependent inhibition of calcium oxalate crystal aggregation.At 800 µg/mL concentration, HAML showed **79.84% inhibition**, indicating significant prevention of crystal aggregation. The extract showed strong inhibition of calcium oxalate crystal growth in a concentration-dependent manner. The maximum inhibition observed at 800 µg/mL suggests that phytoconstituents present in HAML interfere with deposition of calcium and oxalate ions on existing crystal nuclei. Microscopic analysis confirmed the inhibitory effect observed in spectrophotometric assays. The untreated control exhibited typical calcium oxalate monohydrate (COM) crystals, which are considered highly adhesive and associated with renal stone formation. Following treatment with HAML, crystals showed: altered morphology, reduced size, decreased aggregation. These changes indicate that phytoconstituents present in *Madhuca longifolia* interfere with crystal nucleation and maturation.Phenolic compounds may adsorb on crystal surfaces and modify crystal growth kinetics. Based on the combined results highest extractive yield (8.67%)., maximum TPC (142.36 ± 3.42 mg GAE/g extract)., maximum TFC(96.42 ± 2.63 mg QE/g extract), highest calcium oxalate inhibition (82.45%)., the hydroalcoholic extract of *Madhuca longifolia* stem bark was selected as the **bioactive extract** for further chromatographic fractionation and isolation of active phytoconstituents.The hydroalcoholic extract was subjected to successive solvent partitioning to separate compounds according to polarity.The obtained fractions were:Petroleum ether fraction (PEF) ,Chloroform fraction (CF) ,Ethyl acetate fraction (EAF) ,n-Butanol fraction (BF) ,Aqueous fraction (AF).Among all fractions, the aqueous fraction showed the highest yield; however, the ethyl acetate fraction demonstrated superior biological activity.These compounds are frequently associated with antioxidant and antiurolithiatic effects.Therefore, the ethyl acetate fraction (EAF) was selected for further chromatographic separation.This observation supports the principle that biological activity does not always correlate with extraction yield but depends on the presence and concentration of active metabolites. The ethyl acetate fraction showed maximum inhibition of calcium oxalate nucleation (**86.54 ± 2.12%**) and was therefore considered the most active fraction.The enhanced activity of EAF may be attributed to enrichment of phenolic and flavonoid compounds, which may inhibit crystal formation through: calcium ion binding, crystal surface modification, antioxidant action, inhibition of oxidative renal injury.The TLC profile demonstrated the presence of multiple chemically distinct constituents in the active ethyl acetate fraction.Fluorescent spots under UV 366 nm indicated the presence of aromatic compounds, particularly flavonoids and phenolic derivatives.The presence of multiple metabolites justified further purification through column chromatography.Fraction F3 showed maximum antiurolithiatic activity and was selected for purification of individual compounds.The high activity of F3 suggested enrichment of active metabolites responsible for calcium oxalate inhibition.Based on TLC behaviour and phytochemical reactions, F3 was suspected to contain flavonoid/phenolic compounds.Further purification resulted in isolation of pure compounds designated as **Compound ML-1.**The FTIR spectrum indicated the presence of hydroxyl, aromatic, and carbonyl functional groups, suggesting that ML-1 belongs to a polyphenolic class of compounds.The broad absorption around 3420 cm^{-1} indicated hydrogen-bonded hydroxyl groups, which are commonly observed in flavonoid structures.The carbonyl absorption around 1652 cm^{-1} suggested the presence of conjugated ketone functionality characteristic of flavonoids.The accurate molecular mass obtained from HRMS supported the presence of a flavonoid-type

compound containing multiple oxygenated functional groups.The molecular formula suggested a highly oxygenated aromatic structure.The presence of aromatic proton signals and a characteristic downfield hydroxyl proton indicated a flavonoid skeleton.The singlet signals corresponding to A-ring protons suggested oxygenated flavonoid structure.

Conclusion

The present study successfully demonstrated that the stem bark of *Madhuca longifolia* is a promising source of bioactive phytoconstituents with significant antioxidant and in vitro antiurolithiatic potential. Bioactivity-guided fractionation proved to be an effective strategy for enriching the biologically active constituents from the hydroalcoholic extract. Among the solvent fractions, the ethyl acetate fraction (F3) exhibited the highest total phenolic content (84.62 ± 2.15 mg GAE/g) and total flavonoid content (58.34 ± 1.27 mg QE/g), indicating a high concentration of antioxidant phytochemicals.

Bioactivity-guided purification of Fraction F3 resulted in the isolation of three purified compounds, designated ML-1, ML-2, and ML-3, which were characterized using TLC, FTIR, ^{1}H NMR, ^{13}C NMR, and ESI-MS.

The active fraction exhibited remarkable in vitro antiurolithiatic activity by significantly inhibiting calcium oxalate crystal nucleation (82.64 ± 2.31%), aggregation (79.45 ± 1.84%), and crystal growth (75.83 ± 2.07%) at 800 µg/mL, with activity comparable to the standard formulation. Microscopic examination further confirmed a substantial reduction in crystal number, size, and aggregation, demonstrating the ability of the active phytoconstituents to interfere with the early stages of calcium oxalate stone formation , the findings provide scientific evidence supporting the traditional medicinal use of *Madhuca longifolia* for the management of urolithiasis. The phenolic-rich fraction and its isolated compounds possess considerable potential as natural antiurolithiatic agents owing to their combined antioxidant and calcium oxalate crystal inhibitory activities. Further studies involving complete structural elucidation, mechanistic investigations, in vivo pharmacological evaluation, toxicity assessment, and clinical validation are warranted to establish their therapeutic efficacy and facilitate the development of safe and effective phytopharmaceuticals for the prevention and treatment of calcium oxalate urolithiasis.

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