



“BIOACTIVITY-GUIDED FRACTIONATION OF *RIVINA HUMILIS* LEAVES AND STEM EXTRACTS FOR ANTI-UROLITHIATIC PHYTOCONSTITUENTS”

Chandra shekar S P ^{a*}, Dr. Roopashree T S ^b, Tejaswi c ^a

^a Department of Pharmacognosy, Government college of Pharmacy, Bangalore-560027, Karnataka.

^b Professor and Head, Department of Pharmacognosy, Government college of Pharmacy, Bangalore-560027, Karnataka, India

ABSTRACT:

Background: Urolithiasis remains a prevalent disorder with recurrence and limitations of current therapies, prompting interest in plant based inhibitors of calcium oxalate crystallization and aggregation. *Rivina humilis* L. (Phytolaccaceae) contains phenolics and flavonoids implicated in antioxidant and nephroprotective actions; preliminary reports also indicate antimicrobial and cytotoxic adjunct bioactivities that support its pharmacological relevance.

Objective: To perform bioactivity guided fractionation of *Rivina humilis* leaves and stems to isolate constituents with antiurolithiatic activity, supported by phytochemical profiling.

Methods: Crude extracts and solvent fractions were prepared via decoction, maceration and soxhlation, followed by guided sub fractionation followed by chromatography; fractions were screened *in vitro* using nucleation and aggregation assays for calcium oxalate inhibition, with absorbance based kinetics adhering to Beer–Lambert principles. Total flavonoid content and total phenolic content (as quercetin and gallic acid equivalents respectively) were quantified to correlate with crystallization inhibition, while phytochemical tests characterized major classes informing target isolation. Leads were advanced for spectroscopic characterization (UV, IR, NMR, MS) through accredited facilities.

Results: Fractions enriched in phenolics/flavonoids demonstrated superior percentage inhibition in nucleation and aggregation assays, aligning with higher TFC and TPC readouts, thereby prioritizing these for isolation and structural elucidation; preliminary evidence suggests contributions from gallic acid related phenolics among active pools. The workflow established a reproducible path from screening to isolation consistent with WHO/academic norms for pharmacognostic evaluations.

Conclusion: Bioactivity guided fractionation of *Rivina humilis* yielded phenolic rich fractions with promising antiurolithiatic potential linked to antioxidant mechanisms, supporting further isolation, spectroscopic identification, and mechanism oriented validation toward phytopharmaceutical development.

Keywords: *Rivina humilis*; Urolithiasis; Calcium oxalate; Nucleation; Crystal aggregation; Antiurolithiatic agents; Phenolic compounds; Flavonoids; Gallic acid; Bioactivity guided fractionation; Phytochemistry; Spectroscopy.

Introduction

Global Burden of Urolithiasis

Urolithiasis, or kidney stone disease, is among the most prevalent urological disorders worldwide, affecting millions across all age groups. Its global incidence and recurrence rates have risen steadily over the past decades, imposing a substantial health and economic burden on individuals and healthcare systems. According to the Global Burden of Disease (GBD) study, more than 115 million new cases occurred globally in 2019, with prevalence ranging

from 1% to 13% depending on geography, diet, and climate. Data from the National Health and Nutrition Examination Survey (NHANES) indicate that prevalence in the United States increased from 3.8% (1976–1980) to 10.1% (2015–2016)^[1,2].

India's Scenario

In India, the prevalence of urolithiasis is approximately 12%, with higher rates in northern regions, reaching up to 15–20% . The incidence jumped dramatically from below 40 per 100,000 in the 1960s to nearly 930 per 100,000 by the 1990s . Factors contributing to India's higher burden include hot climate and dehydration, high intake of oxalate-rich foods, poor water quality, genetic predisposition, and lifestyle changes related to urbanization^[3].

Urolithiasis is defined as the formation of crystalline, non-metallic deposits within the urinary tract . Stone formation occurs through steps such as urinary supersaturation, nucleation, crystal growth, aggregation, and retention. Contributing factors include elevated urinary calcium and oxalate, decreased inhibitors like citrate and magnesium, oxidative stress, and genetic as well as dietary influences ^[4].

Types of Kidney Stones^[5].

Kidney stones are classified by composition :

Calcium stones (75–85%) – mainly calcium oxalate and phosphate; form in acidic or alkaline urine respectively. Uric acid stones (10–15%) – associated with gout and high-protein diets, form in acidic urine.

Struvite stones (5–15%) – infection-related; more common in women. Cystine stones (1–2%) – genetic in origin; have the highest recurrence rate.

Management and Treatment Costs ,Current management includes extracorporeal shock wave lithotripsy (ESWL), ureteroscopic lithotripsy (URS), percutaneous nephrolithotomy (PCNL), and medication-based therapy. In India, costs range from ₹25,000 to ₹1,80,000 depending on procedure type . Despite these treatments, recurrence remains high (around 50% within 5–10 years) , emphasizing the need for affordable, preventive, and non-invasive alternatives^[6].

Role of Plant-Based Medicine in Urolithiasis. Traditional Use and Scientific Validation

Traditional medical systems such as Ayurveda describe *Mutrashmari* (urinary stones) as a severe urinary ailment, prescribing herbal formulations for treatment and recurrence prevention . Increasing scientific attention has confirmed that plant derived polyphenols such as quercetin, catechins, and curcumin exhibit antioxidant, anti-inflammatory, and crystal inhibitory properties . These compounds prevent calcium oxalate crystallization and aggregation, thereby reducing stone formation. Plants like *Camellia sinensis* (green tea), *Punica granatum* (pomegranate), Cranberry, and Hibiscus have shown significant crystal inhibition *in vitro* and antioxidant properties beneficial to renal tissues ^[7].

Importance of Herbal Research

Plant-based therapies offer affordability, low toxicity, and accessibility, especially in developing countries. They act on multiple biological targets and are better accepted among traditional medicine-practicing populations. Ethnobotanical findings can serve as leads for discovering novel antiurolithiatic agents, making herbal research an essential complement to modern pharmaceuticals^[8].

Botanical and Ethnomedicinal Overview

Rivina humilis L. (family Petiveriaceae), known as pigeonberry or bloodberry, is a perennial herb distributed across tropical regions including India . Ethnobotanical literature documents its use for kidney and urinary ailments, diuresis, and inflammation relief . The plant contains phenolics, flavonoids, saponins, and triterpenoids compounds frequently associated with antioxidant and antiurolithiatic activities^[9].

Scientific Rationale

The strong ethnomedicinal background of *Rivina humilis*, presence of polyphenolic constituents, and absence of systematic validation support its selection for detailed study. This research aims to scientifically evaluate its antiurolithiatic potential through bioactivity-guided fractionation and *in vitro* assays^[10].

Research Models

In vitro Studies^[11,12].

Common experimental approaches include:

Nucleation assay: assesses inhibition of calcium oxalate crystal formation in supersaturated solutions at 620 nm absorbance .

Aggregation assay: measures inhibition of crystal clustering and stability.

These models are rapid and reproducible, providing insight into inhibition mechanisms.

Bioactivity-Guided Fractionation^[13].

This method integrates chromatographic separation with biological testing to isolate active compounds responsible for measurable effects . Steps include extract screening, sequential fractionation, activity testing, and structural elucidation using advanced spectroscopic techniques. Such targeted approaches enhance discovery efficiency and enable extract standardization.

Reference Standard – Cystone

Cystone, a polyherbal formulation, is widely used as a reference standard in kidney stone research owing to its demonstrated clinical effectiveness . It contains extracts of *Didymocarpus pedicellata*, *Saxifraga ligulata*, *Rubia cordifolia*, and others, working synergistically to promote stone expulsion and normalize urinary chemistry. Global regulatory acceptance, safety, and herb-based composition make it an appropriate comparative control for evaluating new plant extracts^[14].

Need for the Present Study

Despite traditional claims, scientific validation of *Rivina humilis* for nephrolithiasis remains limited. The present work focuses on: Evaluating its antiurolithiatic activity using *in vitro* models.

Isolating active polyphenolic fractions through bioactivity-guided fractionation.

Establishing phytochemical and pharmacognostical correlation for potential development into a safe, affordable, and effective antiurolithiatic remedy.

Methodology

An experimental, *in-vitro*, bioactivity-guided fractionation study was conducted on *Rivina humilis* L. leaves and stems to identify antiurolithiatic phytoconstituents using calcium oxalate nucleation and aggregation assays, with orthogonal support from TFC/TPC assays and chromatographic/spectroscopic characterization.

1.1 Plant material and authentication

Fresh leaves and stems of *Rivina humilis* were collected in the month of December from local areas of Ramamurthy Nagar, KR Puram, Bengaluru, India, and authenticated by a Research Officer (Botany), Central Ayurveda Research Institute, Bengaluru (voucher-based authentication).

Pharmacognostic evaluation

1.2 Macroscopy : Color, odor, size, and texture were recorded under uniform daylight using visual comparison, ruler/caliper, and a 10× lens for surface features^[15].

1.3 Microscopy : Freehand transverse sections were bleached with sodium hypochlorite, stained with phloroglucinol–HCl, mounted in glycerin, and observed at 10×/45×; diagnostic powder features were examined after sodium hypochlorite clearing and phloroglucinol–HCl staining^[15].

1.4 Proximate constants : Moisture (LOD, 100–105 °C), total/acid-insoluble/water-soluble ash (muffle furnace 450–600 °C), and alcohol/water-soluble extractives (24 h maceration) were determined in triplicate as per WHO procedures^[15].

1.5 Extraction

Successive extractions of leaf and stem powders were performed by:

Maceration: Petroleum ether → chloroform → ethyl acetate → methanol → water (48 h per solvent; gentle evaporation).

Soxhlation: 40 g powder in cellulose thimble; 150 mL cycles with petroleum ether, chloroform, ethyl acetate, methanol to exhaustion; rotary evaporation at

≤40 °C; storage at 4 °C.

Decoction: 40 g powder boiled in 400 mL water (100 °C, 2 h), filtered, oven-dried ; yields were calculated against starting mass.

Preliminary phytochemical tests

Standard color reactions were run on all extracts: carbohydrates (Molisch, Benedict, Fehling), proteins/amino acids (Biuret, Millon, Ninhydrin), fixed oils (filter paper), alkaloids (Dragendorff's, Wagner, Mayer, Hager), glycosides (pre/post-hydrolysis Fehling), flavonoids (alkali, lead acetate, Shinoda), tannins/polyphenols (FeCl₃, gelatin, lead acetate), saponins (foam), steroids/triterpenoids (Liebermann–Burchard)^[15].

Antiurolithiatic assays

Reagents: 5 mM CaCl₂, 7.5 mM Na₂C₂O₄ prepared in Tris–NaCl buffer (pH 6.5); Cystone stock (10 mg/mL; water; sonication; 0.45 μm filtration). Working solutions: Extracts/fractions/cystone at 200–1000 μg/mL from 10 mg/mL stocks via serial dilution.

1.6 Nucleation assay: 1 mL CaCl₂ mixed with 1 mL sample, then 1 mL Na₂C₂O₄; incubated at 37 °C for 30 min; absorbance at 620 nm; percent inhibition = $(C-S)/C \times 100$ the assays were performed in triplicates^[16].

1.7 Aggregation assay: Synthetic COM crystals prepared and suspended at 0.8 mg/mL in Tris–NaCl (pH 6.5), incubated with sample at 37 °C; turbidity measured at 620 nm; inhibition computed as above; Cystone as reference; triplicates throughout^[17].

Total flavonoid content (TFC)

AlCl₃ colorimetry at 415 nm with quercetin standards (10–80 μg/mL); extracts at defined dilutions; TFC expressed as quercetin equivalents using calibration linearity; triplicate determinations^[18].

$T = C \times V/M$

Total phenolic content (TPC)

Folin–Ciocalteu assay at 725 nm with gallic acid standards (10–50 µg/mL), 20% Na₂CO₃, 40-min room-temperature development; TPC expressed as gallic acid equivalents via $T=C \times V/M$; triplicates^[19].

Bioactivity-guided fractionation

Methanolic extract underwent sequential liquid–liquid partitioning: n-butanol (upper), ethyl acetate (upper), dichloromethane (lower); repeated extractions to color exhaustion; fractions concentrated by gentle heating aligned to solvent boiling points.; yields recorded; all fractions screened in nucleation and aggregation assays (200–1000 µg/mL) ^[20].

Column chromatography and isolation

Active n-butanol fraction (3 g) was dry-loaded on silica (1:1 w/w) over a toluene-conditioned silica column; eluted with toluene:ethyl acetate:formic acid

(50:40:10) under gravity; fractions pooled by TLC similarity; dried and re-assayed to confirm activity before spectroscopic workup^[22].

HPTLC fingerprinting

System: CAMAG platform; silica gel 60 F254 plates; application by Linomat V; chamber saturation 20 min; development to 90 mm; densitometry at 254 nm (D2/W lamps); WinCATS v1.4.6 ^[21].

Conditions: Mobile phase toluene:ethyl acetate:formic acid (5:4:1); standards quercetin, gallic acid (1 mg/mL; 2 µL); samples 5 µL; band width 8 mm;

Rf-based comparison

Spectroscopic characterization^[23,24,25]

UV–Vis: Methanolic solutions (50 µg/mL); 200–400 nm scans; λ_{max} recorded using quartz cuvettes; methanol blank.

FT-IR: ATR mode on PerkinElmer; background, sample scans, baseline/ATR correction; peak assignment for functional groups.

NMR: ¹H/¹³C in deuterated solvent (5 mm tube); lock, tune, shim; standard parameters with proton-decoupled ¹³C; referencing to TMS/residual solvent; spectra exported.

LC-MS/MS: Reversed-phase LC with ESI; polarity as appropriate; runs with blanks, calibration, and QCs; chromatograms and mass spectra evaluated for selectivity and signal quality.

Data analysis and quality control

All assays were performed in triplicate, with Cystone as positive control where applicable; absorbance measured on UV-Vis spectrophotometer at specified wavelengths; yields and inhibition percentages calculated using standard equations; HPTLC and spectroscopy conducted with documented instrument settings and external standards.

Results

Macroscopy and microscopy

Leaves were opposite, ovate, 5.1–7.2 cm × 2.8–4.5 cm, glabrous with reticulate venation and obtuse apex; stems were cylindrical, solid, 4–10 mm girth, glabrous to sparsely pubescent in young regions, with prominent nodes and elongated internodes.

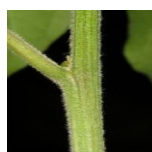
Transverse sections showed typical dicot features in leaf midrib and stem, and powder microscopy revealed anisocytic stomata, prism and cluster calcium oxalate crystals, lignified fibres, tracheids, sclereids, starch grains, and parenchyma, supporting species identification and raw material quality.



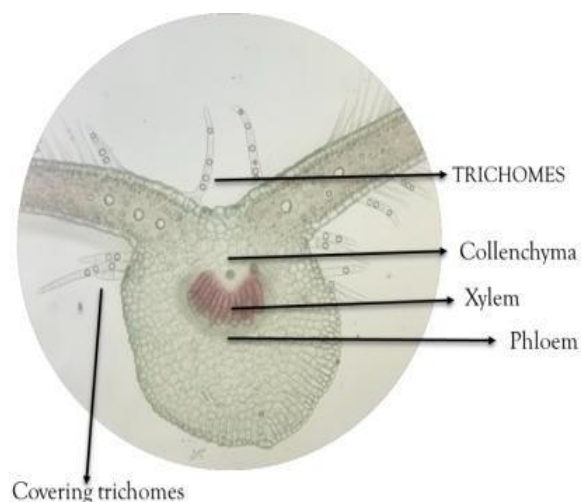
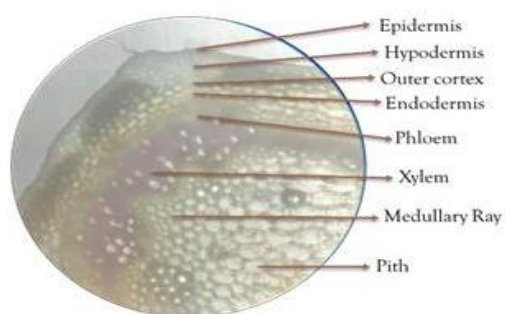
Rivina humilis twig



Rivina humilis leaf



Rivina humilis stem

Rivina humilis leaf transverse section*Rivina humilis* stem transverse section

Proximate analysis

Leaves and stems met good pharmacognostic constants; leaves had slightly higher total ash and extractives than stems, indicating richer inorganic and polar constituents.

Table 1: proximate analysis of *Rivina humilis* leaves and stem

Sl. No	PARAMETERS	<i>Rivina humilis</i> leaves	<i>Rivina humilis</i> stem
1.	Moisture content	7.12±0.416	6.85±0.001
2.	Total ash	11.34±1.178	9.87±0.236
3.	Acid insoluble ash	2.45±0.235	1.98±0.001
4.	Water soluble ash	6.78±0.235	5.92±0.624
5.	Water soluble extractive	18.56±0.653	15.43±0.719
6.	Alcohol soluble extractive	12.34±0.653	10.76±0.377

Figure 1 : proximate analysis of *Rivina humilis* leaves and stem

Extraction yields

Maceration

Decoction

Soxhlation

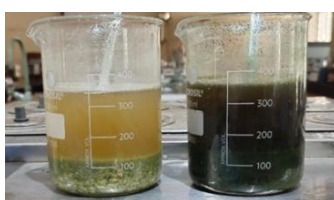


Table 2: Extraction yield by soxhlation

Sl. No	SOLVENTS USED FOR SOXHLATION	% YIELD	NATURE OF THE EXTRACT	
			COLOUR	CONSISTENCY
Rivina humilis leaves				
1.	Petroleum ether	2.5%	Green	Semisolid
2.	Chloroform	2%	Green	Semisolid
3.	Ethyl acetate	2.6%	Green	Semisolid
4.	Methanol	10.12%	Green	Semisolid
5.	Aqueous	8.17%	Pale brown	Powder
Rivina humilis stem				
6.	Petroleum ether	1.65%	Green	Semisolid
7.	Chloroform	0.73%	Green	Semisolid
8.	Ethyl acetate	0.78%	Brownish green	Semisolid
9.	Methanol	10.75%	Brown	Semisolid granular
10.	Aqueous	7%	Brown	Powder/flakes

Table 3: Extraction yield by maceration

Sl. No	SOLVENTS USED FOR MACERATION	% YIELD	NATURE OF THE EXTRACT	
			COLOUR	CONSISTENCY
Rivina humilis leaves				
1.	Petroleum ether	1.52%	Green	Semisolid
2.	Chloroform	1.04%	Dark green	Semisolid
3.	Ethyl acetate	1.96%	Dark Green	Semisolid
4.	Methanol	9.08%	Dark Green	Semisolid
5.	Aqueous	12.44%	Brown	Semisolid
Rivina humilis stem				
6.	Petroleum ether	0.2%	Green	Semisolid
7.	Chloroform	3.2%	Green	Semisolid
8.	Ethyl acetate	0.4%	Green	Semisolid
9.	Methanol	5.76%	Dark olive green	Semisolid
10.	Aqueous	7.4%	Brown	Semisolid

Table 4: Extraction yield by decoction

Sl. No	SOLVENT USED FOR DECOCTION	% YIELD	NATURE OF THE EXTRACT	
			COLOUR	CONSISTENCY
Rivina humilis leaves				
1.	Distilled water	20%	Dark Brown	Powder
Rivina humilis stem				
2.	Distilled water	11.35%	Pale brown	Powder

Methanol maximized Soxhlet yields for leaves, while aqueous often dominated in maceration/decoction, with chloroform consistently lowest; visual attributes matched solvent polarity.

Soxhlet yields and extract properties

Leaves: Petroleum ether 2.5% (green, semisolid); Chloroform 2.0% (green, semisolid); Ethyl acetate 2.6% (green, semisolid); Methanol 10.12% (green, semisolid); Aqueous 8.17% (pale brown, powder)

Stems: Petroleum ether 1.65% (green, semisolid); Chloroform 0.73% (green, semisolid); Ethyl acetate 0.78% (brownish green, semisolid); Methanol 10.75% (brown, semisolid granular); Aqueous 7.00% (brown, powder)

Maceration yields and extract properties

Leaves: Petroleum ether 1.52%; Chloroform 1.04%; Ethyl acetate 1.96%; Methanol 9.08%; Aqueous 12.44%

Stems: Petroleum ether 0.20%; Chloroform 3.20%; Ethyl acetate 0.40%; Methanol 5.76%; Aqueous 7.40%

Decoction yields
Leaves: Water 20.00% (dark brown, powder) Stems: Water 11.35% (pale brown, powder)

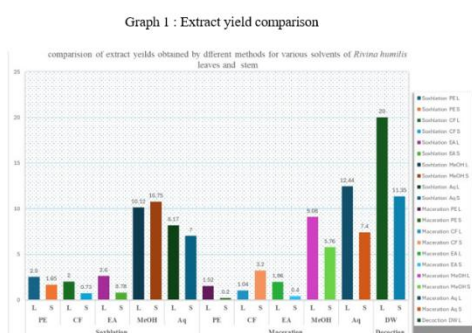
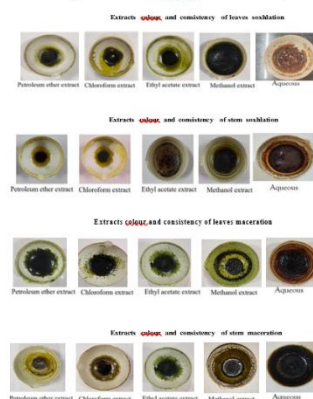


Figure 2 : Extract colour and consistency



Preliminary phytochemistry

Methanolic and ethyl acetate extracts contained broad-spectrum secondary metabolites, including alkaloids, glycosides, flavonoids, tannins, saponins, and polyphenols; petroleum ether captured oils/fats, steroids, and triterpenoids as expected.

Phytochemical screening leaves

Positive classes by solvent: methanol/aqueous/ethyl acetate positive for carbohydrates, proteins/amino acids, alkaloids, glycosides, flavonoids, tannins, saponins, polyphenols; petroleum ether positive for fixed oils/fats, steroids/triterpenoids

Phytochemical screening stems

Patterns mirrored leaves with slightly reduced breadth in some classes for stems

Antiuro lithiatic activity Nucleation assay:

Leaf methanolic extracts showed strong, dose-dependent inhibition up to ~66% at 1000 µg/mL, approaching the Cystone standard trend; aqueous and ethyl acetate were moderate; non-polar extracts were weak.

Nucleation assay inhibition (%) at 200–1000 µg/mL, mean ± SD (n=3)

RHL MeOH Soxhlet: 34.23% ± 0.36 (200 µg/mL) to 66.36% ± 0.59 (1000 µg/mL)

RHL EA Soxhlet: 27.51% ± 0.34 to 31.12% ± 0.57

RHL Aq Soxhlet: 19.80% ± 0.60 to 28.26% ± 0.46

RHS MeOH Soxhlet: 23.59% ± 0.90 to 31.46% ± 0.17

Cystone: 40.06% ± 0.20 to 76.56% ± 0.20

Graph 2 : Nucleation assay results



Antiuro lithiatic activity aggregation assay:

Leaf methanolic extracts again led inhibition, reaching ~55% at 1000 µg/mL; ethyl acetate and chloroform showed moderate activity; aqueous extracts were modest; non-polars were minimal.

Aggregation assay inhibition (%) at 200–1000 µg/mL, mean ± SD

(n=3) RHL MeOH Soxhlet: 37.73% ± 0.11 to 55.34% ± 0.36

RHL EA Soxhlet: 18.48% ± 0.17 to 24.60% ± 0.35

RHS MeOH Soxhlet: 25.92% ± 0.23 to 31.97% ± 0.40

Graph 3 : Aggregation assay results



Total flavonoid content (TFC)

TFC peaked for leaf methanol Soxhlet (55.40 ± 1.03 mg quercetin equivalents/g), with high values for leaf methanol maceration and ethyl acetate extracts; stems were lower overall.

TFC (mg QE/g), triplicates with mean $\pm$ SD

RHL MeOH Soxhlet: 55.40 ± 1.03 ; RHL MeOH Maceration: 36.62 ± 0.25 ; RHL EA Soxhlet: 35.16 ± 3.73 ; RHS EA Maceration: 31.09 ± 1.93

Total phenolic content (TPC)

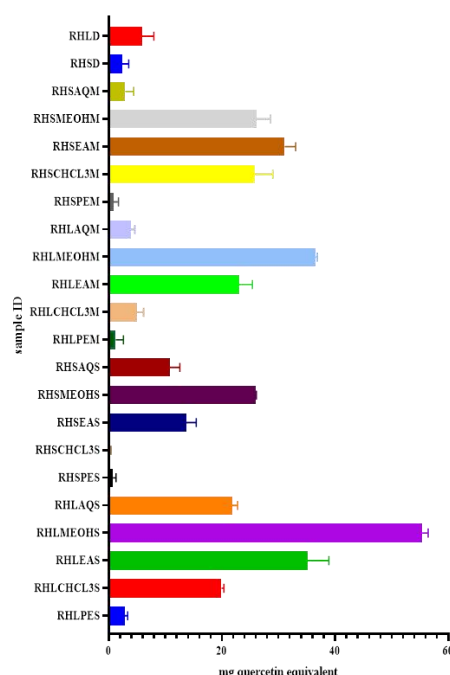
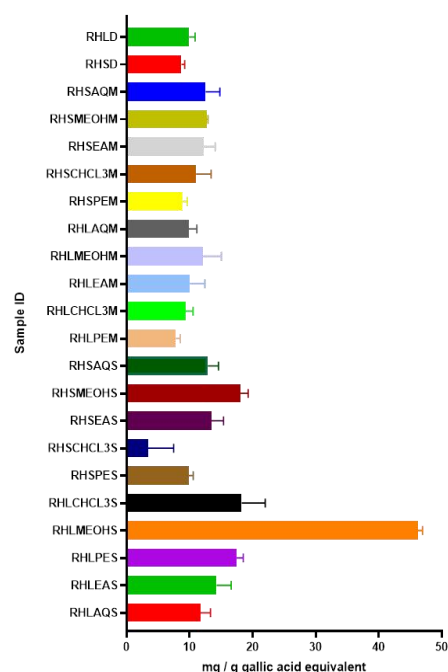
TPC was highest in leaf methanol Soxhlet (46.18 ± 0.77 mg gallic acid equivalents/g), with methanol generally outperforming other solvents; leaf extracts exceeded stems overall.

TPC (mg GAE/g), triplicates with mean $\pm$ SD

High: RHL MeOH Soxhlet 46.18 ± 0.77 ; RHS MeOH Soxhlet 18.08 ± 1.21 ; RHL PE Soxhlet 17.46 ± 1.05

Moderate: RHL EA Soxhlet 14.29 ± 2.30 ; RHS EA Soxhlet 13.43 ± 1.95 ; RHS Aq Soxhlet 12.89 ± 1.69

Low: RHL DECO 9.91 ± 0.97 ; RHS CHCl₃ Soxhlet 3.43 ± 4.05

Graph 3 : Total flavonoid contents**Graph 4 : Total phenolic content****Bioactivity-guided fractionation and isolation**

Liquid-liquid partitioning of leaf methanol extract identified n-butanol fraction as most active; column chromatography of this fraction yielded five subfractions, with F- 4 (TEF 50:40:10) as the major, bioactive pool (0.98 g).

Column fractions

(solvent system) F 1

TEF 80:10:10, 0.01 g;

F 2 TEF 70:20:10, 0.02 g;

F 3 TEF 60:30:10, 0.013 g;

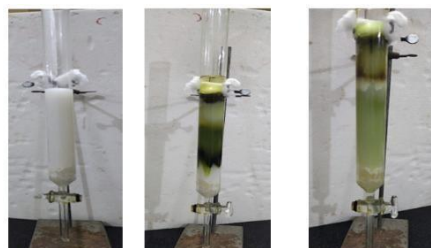
F 4 TEF 50:40:10, 0.98 g;

F 5 TEF 40:50:10, 0.001 g

Antiurothiatic activity of F 4 vs. Cystone (nucleation/aggregation representative inhibition %) Out of all 5 column fractions f-4 showed best inhibition which is described below,

F 4: 28.37% (200 μ g/mL), 42.31% (400), 57.21% (600), 61.82% (800), 69.84% (1000)

Cystone: 44.02% (200), 53.14% (400), 68.21% (600), 71.41% (800), 79.07% (1000)

Figure 3: Liquid-liquid partition**Figure 4: Column chromatography**

HPTLC fingerprinting

HPTLC profiles showed polyphenolic bands for standards and active fractions; tracks indicated alignment of active fraction with polyphenol standards, supporting enrichment of phenolic constituents.

HPTLC tracks: standards (gallic acid and quercetin) and F 4 (fourth fraction)

Figure 5: TLC plate Q-STD-
Quercetin G-STD-Gallic
acid, S – F-4 sample

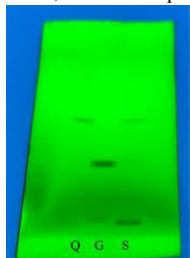
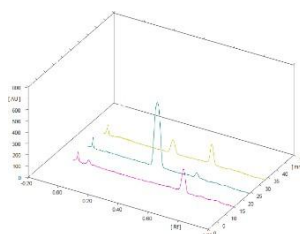


Figure 6: HPTLC chromatogram



Spectroscopic identification

The UV–visible spectra of the isolated sample (red) and gallic acid standard (blue), recorded using distilled water as blank, show strong absorbance around 270–280 nm. The close match in peak position and spectral shape indicates the isolated compound possesses a UV profile similar to gallic acid, confirming the presence of phenolic constituents.

IR showed broad phenolic O–H ($\sim 3270\text{ cm}^{-1}$), conjugated acid C=O ($\sim 1698\text{ cm}^{-1}$), aromatic C=C ($1614, 1540\text{ cm}^{-1}$), and phenolic C–O bands ($1306, 1244\text{ cm}^{-1}$), consistent with gallic acid.

High-resolution MS in negative ESI revealed base peak at $m/z \sim 169$ ($M-H^-$) and diagnostic decarboxylation fragment at $m/z \sim 125$, confirming gallic acid

framework.

NMR and LC data supported identity of the isolated principal component as gallic acid-kind of compound, correlating with HPTLC standard behaviour and IR/MS assignments.

UV–Vis λ_{max} and scan

FT IR spectrum with key bands

MS spectrum with m/z 169 base

peak ^1H and ^{13}C NMR spectra

Figure 7: UV- spectra

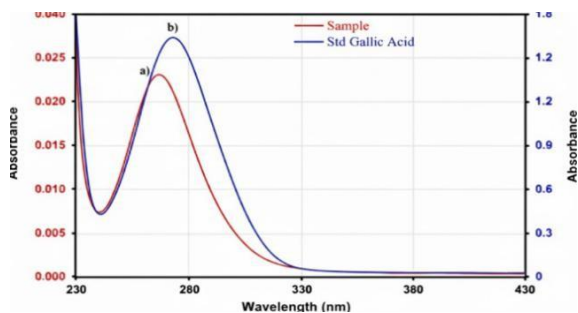


Figure 8: IR- spectra

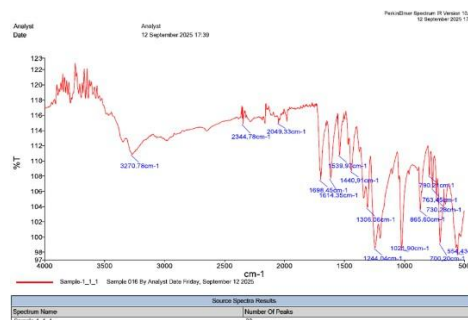
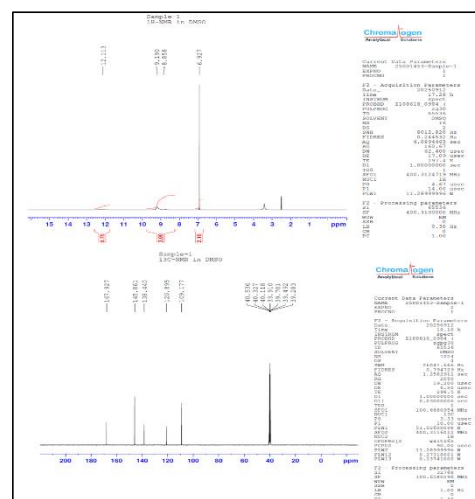
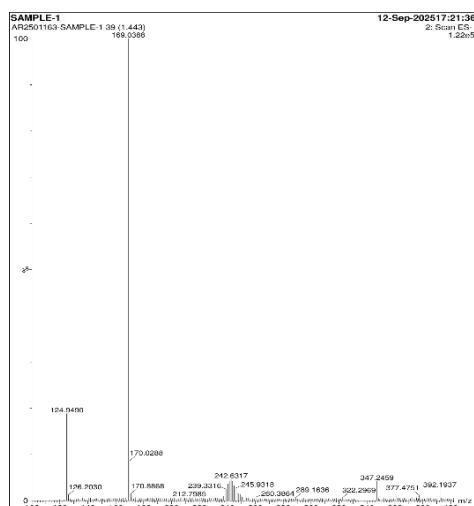


Figure 9: Mass spectra

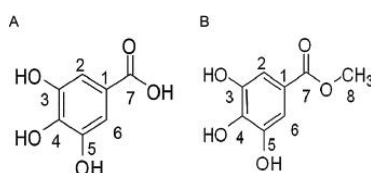
Figure 10: NMR- spectra



Key integrated findings

Activity tracked with phenolic/flavonoid content across solvents and methods, with leaf methanol extracts showing highest nucleation and aggregation inhibition in a dose-dependent manner.

n Butanol fractionation concentrated activity: subsequent isolation and spectra identified gallic acid as a principal bioactive correlating with antiurolithiatic effects *in vitro*.



Discussion

This study followed a simple, careful path from plant to active compound so that every step could be trusted and repeated. Leaves and stems of *Rivina humilis* were collected around Rammurthynagar, Krishnarajapura (Bengaluru) and the identity was confirmed by a recognized institute. Clear photos of the live plant, plus basic quality checks (moisture, ash, and solvent extractives), showed that the raw material was clean and stable before testing. Then documented what the plant looked like outside and under the microscope. Macroscopy covered easy-to-see features like leaf color, size, shape, margins, venation, and stem traits (girth, nodes, internodes, surface). Microscopy and powder microscopy added fine details epidermis, palisade and spongy cells, vascular bundles, anisocytic stomata, different trichomes, lignified fibres, sclereids, starch grains, and calcium oxalate crystals. These features acted like ID proof, so later chemical and biological results were not wasted on the wrong plant.

Extraction used three practical methods, Soxhlet (hot, continuous) helped recover heat-stable compounds efficiently. Maceration (cold) protected heat-sensitive compounds. Decoction (water-based, traditional) matched how people might use the plant and recovered water-loving compounds. The solvents moved from non-polar to polar (petroleum ether, chloroform, ethyl acetate, methanol, water) so “like dissolved like.” Recording yields and how extracts looked or felt helped compare methods and batches. A clear pattern emerged: methanol Soxhlet leaf extracts gave higher yields and richer secondary metabolites.

Simple chemical tests then mapped the main groups present: carbohydrates, proteins and amino acids, alkaloids, glycosides, flavonoids, tannins, saponins, steroids, triterpenoids, oils/fats, and polyphenols. Next, the study measured total phenolic content (TPC, in mg gallic acid equivalents per gram) and total flavonoid content (TFC, in mg quercetin equivalents per gram) in triplicate for reliability. These tests used standard curves and absorbance

readings (Beer–Lambert principle) so the numbers were not just relative; they could be compared across samples and runs. As expected, methanol and ethyl acetate extracts carried more phenolics and flavonoids, and petroleum ether extracts carried more lipophilic groups like oils and triterpenoids.

To see if the chemistry mattered for kidney stones, two simple *in-vitro* tests were used. The nucleation test checked whether extracts could slow the very first appearance of calcium oxalate crystals. The aggregation test checked whether extracts could slow the clumping of small crystals into bigger ones. Both were read at 620 nm across 200–1000 µg/mL, with triplicate readings and proper controls. A known product (Cystone) acted as a positive control so the setup could be trusted. The pattern was clear: polar extracts, especially methanolic leaf extracts, reduced nucleation and aggregation in a dose-dependent way, which matched their higher TPC/TFC. In plain terms, extracts richer in phenolics and flavonoids did a better job at slowing crystal birth and crystal clumping.

To focus the activity further, the methanolic extract was split by liquid–liquid partitioning into n-butanol, ethyl acetate, and dichloromethane fractions. This sorted compounds by polarity and often concentrates the effect. When these fractions were retested in the same two assays, the n-butanol fraction

repeatedly performed best, suggesting that the active compounds were medium-to-high polarity phenolics and related molecules. High-performance thin-layer chromatography (HPTLC) created simple “chemical barcodes” of the active extracts and fractions, which helped compare batches and guide isolation. Using this map, the n-butanol fraction was taken through column chromatography, and each subfraction was tested again so only the parts that kept the activity moved forward.

When a lead compound was isolated, it was checked with standard spectroscopy. UV–Vis showed typical absorption features; IR showed the functional groups; NMR (1D/2D) showed how atoms were connected; MS gave the molecular weight and fragments. Together, these pointed to a phenolic structure like gallic acid, which made sense given the earlier results: higher phenolics, stronger inhibition, and the n-butanol fraction winning in both assays. While “similar to gallic acid” is not the final word, it strongly supports the idea that phenolic chemistry sat behind the antiurolithiatic effects.

Quality and fairness were built in throughout: triplicates, blanks and controls, fixed timing and wavelengths, transparent math for percentage inhibition, and awareness of possible interference from color or turbidity. *In-vitro* tests do not capture the whole urinary system, its flow, pH shifts, proteins, or long-term tissue changes. Crude extracts are complex. The study addressed these by moving toward fractions and isolates and by planning next steps: using small-scale flow systems to mimic moving urine, checking if the compounds bind calcium or oxalate

A practical standardization path was also set up. HPTLC fingerprints and simple markers (for example, gallic acid or co-migrating phenolics) can act as batch IDs and quality checks. Validated analytical methods (linearity, precision, accuracy, robustness) can turn these into routine tests for later development. On the product side, simple formulation steps solid dispersions, complexation, or nano-systems could improve solubility, stability, and absorption of phenolic actives. Early pharmacokinetic and interaction checks can then guide safe dosing and watch for herb–drug interactions. Overall, the study kept to good habits in pharmacognosy and pharmaceutical science: confirm the plant first, measure carefully, connect chemistry to function, simplify mixtures by fractionation, confirm identity with more than one tool, and plan for translation with standardization and safety in mind.

Conclusion

Rivina humilis was identified, carefully processed, and extracted by conventional methods, then evaluated in lab models of calcium oxalate stone formation; the methanolic leaf extract showed the strongest, dose-dependent suppression of nucleation and aggregation, in line with its higher phenolic and flavonoid levels, and fractionation focused this effect in the n-butanol fraction where an isolated lead with a gallic acid–like phenolic profile tied chemistry to biology, together indicating that phenolic-rich, n-butanol enriched material is the most promising path forward, with immediate priorities being full structure confirmation, practical marker-based standardization and stepwise *in vitro* safety–efficacy study.

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