SPECIMEN FORMAT FOR THESES OF MONTH

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Abstract with in 300 words:

Kidney stone disease is common with limited treatments and high recurrence. *Spermacoce articularis* is being explored as a safer natural option for its antioxidant and anti-urolithiatic potential. The study aimed to comprehensively evaluate the pharmacognostic, phytochemical, antioxidant, and pharmacological properties of *S. articularis* with a focus on its potential antiurolithiatic activity. Pharmacognostic studies, including organoleptic and fluorescence analysis, revealed distinct characteristics among leaf, stem, and root samples. The leaf and stem extracts exhibited a richer phytochemical profile using methanol, ethanol, acetone, and aqueous solvents than the root extract. The quantitative analysis exhibited a significant amount of both primary (proteins and carbohydrates) and secondary metabolites (alkaloids, tannins, and terpenoids), indicating a rich profile of biologically active constituents. Among all the solvent extracts, the stem methanol and leaf ethanol extracts of *S. articularis* demonstrated the highest antioxidant potential in all enzymatic, non-enzymatic, and radical scavenging assays. The *S. articularis* stem methanol extract (SASM) was found to have the highest dissolution of calcium oxalate crystals through nucleation and aggregation assays, outperforming the leaf ethanol extract. Consequently, further *in vivo* studies were conducted using the SASM at two doses (250 mg/kg and 500 mg/kg) on renal calculi-induced Wistar albino rats, and the results confirmed positive efficacy, with a significant reduction in calcium oxalate crystal deposition and prevention of renal tissue damage, compared to the standard cystone group. To identify the active compounds responsible for the anti-urolithiasis activity, chromatographic methods such as TLC, HPTLC, and GC-MS were employed. TLC and HPTLC analyses confirmed the presence of terpenoids and phenols in the stem methanol extract. GC-MS profiling detected 40 bioactive compounds, and 25 organic compounds from various functional groups were selected for molecular docking. *In silico* analysis showed D-mannitol had high binding affinities to Tamm Horsfall Protein, Calcitonin, and Calcium oxidoreductase. Molecular dynamics simulations suggested D-mannitol may inhibit calcitonin hormone, supporting its potential in kidney stone treatment. The top 10 hit compounds obtained from docking also showed favorable pharmacokinetic properties. Overall, the study highlights that *S. articularis* is a valuable natural resource that warrants further investigation for the development of anti-urolithiasis treatments.

i) Major Objectives

1. To study the pharmacognostic and phytochemical aspects of different extracts of the leaf, stem, and root of *Spermacoce articularis*
2. To evaluate the total antioxidants and free radical scavenging activity of various solvent extracts from the leaf and stem of *S. articularis*

3. To assess the antiurolithiatic potential of the leaf and stem of *S. articularis* using *in vitro* nucleation and aggregation assays
4. To investigate the urolithiasis-inhibiting potential of *S. articularis* stem in the Wistar-Albino rat model
5. To profile the phytochemical constituents of *S. articularis* stem extract using TLC, and HPTLC techniques
6. To identify the chemical composition of S. articularis stem extract using GC-MS profiling
7. To evaluate the binding potential of bioactive compounds from S. articularis stem to urolithiatic receptors using molecular studies.

ii) Hypothesis

The hypothesis set up for the present study is,

- **Null hypothesis:** The *S. articularis* stem extract does not reduce the CaOx crystal growth when compared to Cystone, the standard.
- **Alternative hypothesis:** The *S. articularis* stem extract significantly reduces the CaOx crystal growth when compared to Cystone, the standard.

iii) Methodology

This section displays the materials and methods adopted to prove the *in vitro*, *in vivo* and *in silico* antiurolithiatic efficacy of *Spermacoce articularis* L.f. extract in preventing urinary stone formation.

PHASE I

The whole *Spermacoce articularis* L.f. plant specimen was collected from Attappadi, Kerala, between June and July 2020. The collected plant specimen was submitted to the Botanical Survey of India, Southern Region, Coimbatore, for taxonomical identification and authentication with reference number BSI/SRC/5/23/2022/Tech. The leaf, stem, and root of *S. articularis* L.f. were collected, shade-dried, finely powdered, stored in tightly sealed sterile containers, and labelled. The powdered sample (10 g) was extracted using a Soxhlet apparatus with solvents of increasing polarity (hexane, chloroform, ethanol, methanol) each 100 ml for 4, 8, 16 and 24 hours. The methanol, ethanol, and acetone extracts were evaporated to concentrate using a rotary evaporator and then air-dried. The aqueous extracts were lyophilized and stored for further analysis.

- Pharmacognostic Studies
  - Organoleptic Study (Jackson and Snowdown, 1968)

- Fluorescence Analysis (Kokoshi et al., 1958)

- Qualitative Phytochemical Analysis (Harborne, 1998)
- Quantitative Phytochemical Analysis
    - Estimation of Primary Metabolites
        - Estimation of Carbohydrates (Hedge and Hofreiter, 1962)
        - Estimation of Protein (Lowry et al. 1951)
    - Estimation of Secondary Metabolites
        - Estimation of Total Alkaloid Content (Rakesh and Nair, 2019)
        - Estimation of Total Terpenoid Content (Troung et al., 2021)
        - Estimation of Total Tannin Content (Roghini & Vijayalakshmi, 2018)
    - Determination of Antioxidant Activity
        - Evaluation of Enzymatic Antioxidants
            - Estimation of Catalase (Chance, 1995)
            - Estimation of Peroxidase (Addy and Goodman, 1972)
            - Estimation of Polyphenol Oxidase (Wojdylo et al. 2007)
            - Estimation of Ascorbate Oxidase (Vines and Oberbacher, 1965)
        - Evaluation of Non-Enzymatic Antioxidants
            - Estimation of Total Phenolic Content (Saeed et al. 2012)
            - Estimation of Total Flavonoid Content (Saeed et al. 2012)
            - Estimation of Ascorbic Acid (Roe and Keuther, 1943)
            - Estimation of α-Tocopherol (Rosenberg 1992)
        - *In vitro* Radical Scavenging Assays
    - DPPH Radical Scavenging Assay (Senguttuvan et al. 2014)
    - Ferric Reducing Antioxidant Power Assay (Pallab et al. 2013)
    - ABTS Radical Scavenging Assay (Chintalpani et al. 2018)

PHASE II

This study investigated the antiurolithiatic potential of *S. articularis* extracts, evaluating their ability to inhibit calcium oxalate crystal formation, nucleation, and aggregation, providing insights into their therapeutic potential for managing kidney stones. The *in vitro* antiurolithiatic activity of *S. articularis* extracts was evaluated using methanol stem extract (SASM) and ethanol leaf extract at concentrations of 100-600 µg/ml. The stem methanol extract showed high inhibitory activity in *in vitro* and was chosen for further *in vivo* studies. For the *in vivo* studies, Wistar albino rats were divided

into five groups: Group 1 (Control), Group II (stone-induced), Group III (Cystone treated), Group IV (SASM 250 mg/kg), Group V (SASM 500 mg/kg).

- *In vitro* Antiurolithiatic Activity (Hennequin et al., 1993)
  - Effect of Different Concentrations of Plant Extracts on Calcium Oxalate Crystal Nucleation
  - Effect of Different Concentrations of Plant Extracts on Calcium Oxalate Crystal Aggregation
  - Microscopic Evaluation
- *In vivo* Antiurolithiatic Activity
  - Acute Toxicity
  - Ethylene Glycol-Induced Urolithiasis in Wistar Albino Rats (Pareta et al., 2010)
  - Collection and Analysis of Urine, Blood, and Serum (Singh et al., 2022)
  - Measurement of Body and Kidney Weight
  - Determination of Antioxidant Enzymes and Lipid Peroxidation
  - Preparation of Tissue Homogenate
  - Histopathological Analysis of Kidneys (Singh et al., 2022)
- *In vitro* Cytotoxicity
  - Brine Shrimp Lethality Assay (Olowa and Nuneza, 2013)
  - Cytotoxic Properties of SASM Extract Against Kidney HEK 293 Cell Line (Melendez et al. 2022)

PHASE III

TLC was used to separate and identify terpenoids and phenolics in the SASM extract with visualization at 254nm and 366nm based on their UV absorbance. The analysis showed Rf values ranging from 0.24 to 0.98, indicating effective separation of bioactive compounds in the extract. The mobile phase comprising toluene, ethyl acetate, and formic acid (TEAF) proved optimal for the separation. The chromatogram of SASM extracts confirms the presence of terpenoids and phenolics.

- Chromatographic Techniques
  - Thin Layer Chromatography (Ahamed et al. 2017).
  - High-Performance Thin Layer Chromatography Profiling (Preethi et al., 2014)
  - Gas Chromatography-Mass Spectrometry Analysis (Tyagi and Agarwal, 2017)

PHASE IV

An *in-silico* approach was employed to predict the potential compounds present in the SASM extract, as identified through GC-MS, which were docked against the selected three targets: Tamm-Horsfall protein (THP), calcium oxidase enzyme, and calcitonin hormone. Furthermore, molecular dynamic simulations were performed on the top-hit compound to elucidate its binding dynamics and stability.

- *In silico* Analysis

- Molecular Docking using GLIDE (Friesner et al., 2006; Yuriev et al., 2011).
- Molecular Dynamics Simulation
- Pharmacokinetic Analysis and Drug-Likeness Prediction

iv) Findings

PHASE I

Spermacoce articularis L.f., used in traditional medicine for centuries, is used to treat urinary tract infections and kidney stones, due to its pharmacological properties. The current study of 'Antiurolithiatic Potential of *Spermacoce articularis* L.f. through *In Vivo* and *In Silico* Analysis aims to demonstrate the effectiveness of *S. articularis* to prevent the formation of calcium oxalate crystals, mitigate oxidative stress, and address urinary risk factors related to kidney stones. This investigation could provide a scientific basis for using *S. articularis* in kidney stone treatment, offering new therapeutic approaches for urolithiasis. The study comprises four phases; the first phase includes a pharmacognostic study, like organoleptic and fluorescence analysis. The results confirmed the purity and quality of the *S. articularis* powder samples. Qualitative analysis of leaf, stem, and root extracts of *S. articularis* using different solvents exhibited a significantly higher number of phytochemicals in the extracts of stem and leaf, when compared to the root. The quantification of secondary metabolites exhibited the higher concentrations of alkaloids, terpenoids, and tannins in stem methanol and leaf ethanol extracts of *S. articularis*. Indicating its potential as a natural antioxidant, the enzymatic and non-enzymatic ability was reported to be higher in the stem methanol and leaf ethanol extract, when compared to other solvents. Radical scavenging assay revealed significant activity, with stem methanol extracts exhibiting the highest FRAP and ABTS values (13.84±0.085 µg/ml and 20.08±0.144 % µg/ml) and leaf ethanol extracts exhibiting the highest DPPH activity (23.55±1.270 % µg/ml). The results revealed that stem and leaf extracts of *S. articularis* are rich in bioactive compounds with substantial antioxidant properties.

PHASE II

The results of the second phase indicated that S. articularis stem methanol and leaf ethanol extracts demonstrated in vitro anti-crystallization properties by effectively inhibiting CaOx crystal formation by reducing the size and number of the crystals, with increasing concentrations (100-500 µg/ml). The growth and aggregation of CaOx crystals were significantly reduced by stem methanol extracts, when compared to leaf ethanol extracts. Thus, *in vivo* antiurolithiatic experiments were carried out using *S. articularis* stem methanol (SASM) extracts at two different dosages, 250 mg/kg and 500 mg/kg, in the Wistar albino rat model. Substantial decreases in the kidney weight and CaOx crystal precipitation were noted in the SASM-treated group than the disease control group. In particular, the SASM extract at 500mg/kg exhibited high antioxidant potential and lowered lipid peroxidation, supporting renal health. Besides, the extract decreased the levels of urinary biomarkers like urea, uric acid, and creatinine. The traces of cellular necrosis and tissue inflammation were absent in the kidney sections of the SASM-treated animal groups. The brine shrimp lethality assay demonstrated that SASM extract is relatively safe, exhibiting low toxicity observed at both lower and higher concentrations, with an LC50 value of 180.00 µg/ml. The results indicated that SASM, due to its minimal cytotoxic effects, may be considered for further development as a therapeutic agent.

PHASE III

The Phase III revealed the presence of terpenoids and phenols in the SASM extract in both TLC and HPTLC analysis. The presence of these phytoconstituents, terpenoids (16.96 µg/ml) and phenols (13.46 µg/ml) in SASM at 3mg/ml is detected by HPTLC analysis. A total of 40 bioactive compounds were detected using GC-MS analysis, of which 25 organic compounds with various functional groups were differentiated based on the retention time and peak area. Catechol, D-mannitol, vanillin, gamma-tocopherol, eugenol, and vitamin E were identified as major phytocompounds present in the SASM extract.

PHASE IV

The fourth phase highlights the molecular docking studies of 25 major compounds from GC-MS analysis against three key proteins, like Tamm-Horsfall protein (THP), Calcitonin, and calcium Oxidoreductase. Among them, D-mannitol demonstrated the highest binding affinity against all three proteins. The docked complex of D-mannitol and calcitonin exhibited the highest binding affinity of -8.41 kcal/M, and was further validated for its stability.
MD simulations suggest that D-mannitol could potentially inhibit calcitonin hormone, which plays a

crucial role in regulating kidney function. The bioactive compounds demonstrated favourable pharmacokinetic properties, closely adhering to Lipinski's rule, indicating their drug like properties. Based on these findings, *Spermacoce articularis* holds potential natural resource for developing antiurolithiasis treatment, warranting further studies.

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