3

Materials and Methods

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.

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PHASE I

3.1 Collection of Plant Materials and Authentication

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 (Appendix I).

3.2 Preparation of the Plant Extract

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.

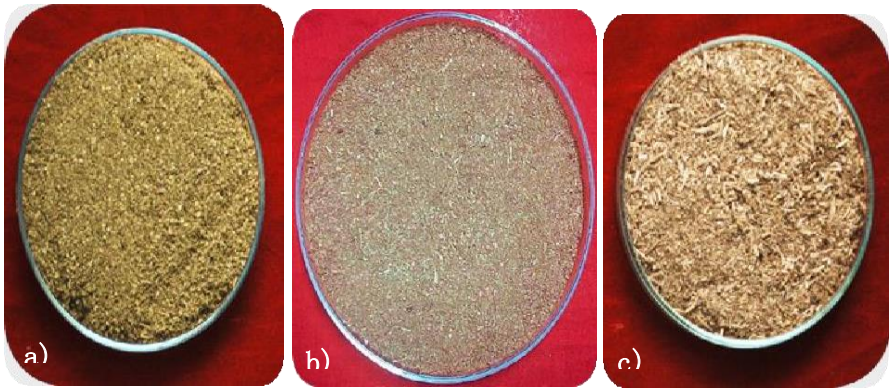

Plate 3.1 Powders of (a) Leaf, (b) Stem, and (c) Root of *Spermacoce articularis* L.f.

3.3 Pharmacognostic Studies

3.3.1 Organoleptic Study (Jackson and Snowdown, 1968)

The organoleptic properties of leaf, stem, and root powders were assessed based on colour variation, odour, and taste.

3.3.2 Fluorescence Analysis (Kokoshi *et al.,* 1958)

A small amount of powdered plant sample was added to separate watch glasses containing different solutions: water, 10% sodium hydroxide (NaOH), 50% sulfuric acid (H_2SO_4), and 1N hydrochloric acid (HCl). Each mixture was properly homogenized and incubated for approximately 30 minutes. The resulting solutions were then observed for their characteristic colour reactions under both visible light and ultraviolet light (UV 365nm).

3.4 Qualitative Phytochemical Analysis

All the extracts of *S. articularis* leaf, stem, and root were analyzed for phytocompounds such as alkaloids, tannins, phenols, saponins, proteins, anthocyanins, quinones, oxalates, flavonoids, vitamin C carbohydrates,

phytosterols, coumarins, terpenoids, volatile oils, resins, carotenoids, glycosides, fixed oils and fats, catechin, reducing sugars, amino acids, starch, acidic compounds, eugenols, cardiac glycosides, and anthroquinones according to the standard method of Harborne, (1998) as presented in Appendix II.

3.5 Quantitative Phytochemical Analysis

Following the qualitative analysis, quantitative assessments were performed for primary metabolites like carbohydrates and proteins, and secondary metabolites including alkaloids, terpenoids, and tannins.

3.5.1 Estimation of Primary Metabolites

3.5.1.1 Estimation of Carbohydrates

The estimation of carbohydrates was done by the method of Hedge and Hofreiter, (1962) in Appendix III-A.

3.5.1.2 Estimation of Protein

The estimation of protein was done by Lowry *et al.* (1951), given in Appendix III-B.

3.5.2 Estimation of Secondary Metabolites

3.5.2.1 Estimation of Total Alkaloid Content

The total alkaloid content was determined using a simple spectrophotometric method (Rakesh and Nair, 2019) in Appendix IV-A.

3.5.2.2 Estimation of Total Terpenoid Content

The estimation of total terpenoid was performed using spectrophotometric method (Troung *et al*., 2021) in Appendix IV-B.

3.5.2.3 Estimation of Total Tannin Content

The quantification of total tannin content was determined using the method of Roghini & Vijayalakshmi (2018) in Appendix IV-C.

3.6 Determination of Antioxidant Activity

3.6.1 Evaluation of Enzymatic Antioxidants

3.6.1.1 Estimation of Catalase

The catalase activity was determined using the method of Chance, (1995) as given in Appendix V-A.

3.6.1.2 Estimation of Peroxidase

The Peroxidase activity was determined by the method of Addy and Goodman (1972) as given in Appendix V-B.

3.6.1.3 Estimation of Polyphenol Oxidase

The polyphenol oxidase activity was assessed based on the method of Wojdylo *et al.* (2007) as given in Appendix V-C.

3.6.1.4 Estimation of Ascorbate Oxidase

The estimation of ascorbate oxidase activity was based on the method of Vines and Oberbacher, (1965) as given in Appendix V-D.

3.6.2 Evaluation of Non-Enzymatic Antioxidants

3.6.2.1 Estimation of Total Phenolic Content

The total phenolic content was quantified by following the method of Saeed *et al.* (2012) and expressed in milligrams of gallic acid equivalents (GAE) per gram of dried extract, as given in Appendix VI-A.

3.6.2.2. Estimation of Total Flavonoid Content

The total flavonoid content was expressed as milligrams of rutin equivalents per gram and estimated using the method of Saeed *et al.* (2012) as in Appendix VI-B.

3.6.2.3 Estimation of Ascorbic Acid

The ascorbic acid content was determined using the standard method of Roe and Keuther (1943) as given in Appendix VI-C.

3.6.2.4 Estimation of α-Tocopherol

The alpha-tocopherol was estimated using the method of Rosenberg (1992) as in Appendix VI-D.

3.6.3 *In vitro* Radical Scavenging Assays

3.6.3.1 DPPH Radical Scavenging Assay

The DPPH radical scavenging potential of the plant extracts was assessed based on the method described by Senguttuvan *et al.* (2014) as presented in Appendix VII-A.

3.6.3.2 Ferric Reducing Antioxidant Power Assay

The FRAP radical scavenging capacity of the plant extracts was assessed based on the method described by Pallab *et al.* (2013), as presented in Appendix VII-B.

3.6.3.3 ABTS Radical Scavenging Assay

The ABTS radical scavenging potential of the plant extracts was assessed based on the method described by Chintalpani *et al.* (2018) as presented in Appendix VII-C.

PHASE II

3.7 Antiurolithiatic Activity

3.7.1 *In vitro* Antiurolithiatic Activity

3.7.1.1 Effect of Plant Extracts on Calcium Oxalate Crystal Nucleation

The formation of calcium oxalate (CaOx) crystals was assessed using a nucleation assay to evaluate the effect of the stem and leaf extract in Phase II. A buffer solution containing 0.05 M Tris-HCl and 0.15 M sodium chloride (NaCl) was prepared and maintained at pH 6.5. A 5 mM solution of calcium chloride ($CaCl_2$) and a 7.5 mM solution of sodium oxalate ($Na_2C_2O_4$) were also prepared using the buffer. A mixture of 3 ml of the $CaCl_2$ and $Na_2C_2O_4$ solution was combined with various concentrations (100, 200, 300, 400, 500, and 600 µL) of

Spermacoce articularis leaf and stem extracts and kept for incubation at 37 °C for 30 minutes. The optical density (OD) of the solution was measured at 620 nm using UV-Vis spectrometry (Hennequin *et al.,* 1993). The inhibitory percentage of nucleation by plant extract was calculated and compared with cystone as the standard using the formula,

$$\%\ Inhibition = \frac{1 - OD\ Sample}{OD\ Control} X\ 100$$

3.7.1.2 Effect of Plant Extract on Calcium Oxalate Crystal Aggregation

Using a standard procedure with slight modifications, the dissolution of aggregation of CaOx crystals by the plant extract was analyzed. To obtain CaOx monohydrate crystals at 50nM, $CaCl_2$ and $Na_2C_2O_4$ were combined. The solution was then placed in a water bath (60 °C) for 1 hr, followed by cooling to 37 °C. The crystals were collected through centrifugation and subsequently concentrated at 37°C. Calcium oxalate (CaOx) crystals (0.8 mg/ml) were suspended in Tris-buffered saline and incubated at 37°C with plant extract concentrations ranging from 100 to 600 µl (Hess *et al*., 1989). The inhibitory percentage of aggregation (Ir) was estimated by calculating the turbidity of the sample relative to that acquired in the control using the following formula,

$$Ir = \frac{1 - Sample\ turbidity}{Control\ turbidity} X\ 100$$

3.7.1.3 Microscopic Evaluation

A few drops of the mixture from different concentrations were placed on separate slides. The reduction in crystal size and morphology of CaOx crystallization in the presence and absence of *S. articularis* stem methanol and leaf ethanol extracts was examined using a trinocular microscope at 1000x magnification.

3.7.2 *In vivo* Antiurolithiatic Activity

3.7.2.1 Experimental Animals

Thirty healthy adult male Wistar albino rats (*Rattus norvegicus*), weighing 150-200 g, were acquired from the animal house of Biogene Lab, Bangalore.

The rats were housed in plastic cages with filter tops under controlled environmental conditions, such as a temperature (28±2°C), light-dark cycle (12:12 hrs), and humidity (50±10%). All rats were given water ad libitum and fed with commercial rat pellets (SAI Animal Feed Ltd, Bangalore). Before the experimental work, the rats were acclimatized at the animal laboratory for 10 days. The experimental design was cleared ethically for the *in vivo* study by the Institutional Review Board (IRB) of Avinashilingam Institute for Home Science and Higher Education for Women with the study reference (AIW: IAEC.2023:09) (Appendix VIII).

3.7.2.2 Acute Toxicity Assay

The acute oral toxicity study was conducted using a stepwise procedure with a single animal, following the Organization for Economic Co-operation and Development (OECD) Guidelines 425. The *S. articularis* stem methanol (SASM) extract was treated with a dose of 2000 mg/kg, and the mortality rate was observed after 24 hours. The effective dose was determined as one-tenth of the median lethal dose (LD_{50}).

3.7.2.3 Ethylene Glycol-Induced Urolithiasis in Wistar Albino Rats

For 28 days, each rat was administered ethylene glycol at a concentration of 0.75% (0.75 ml of ethylene glycol in 100 ml of tap water) to induce calcium oxalate stones. An experimental urolithiasis model was used to investigate the antiurolithiatic activity in male Wistar albino rats (Pareta *et al.,* 2010). The Wistar albino rats were allocated into groups from Group I to Group V, with each group comprising 6 rats. Group I was designated as the normal control, while all other groups were induced with urolithiasis (stone formation). Group II served as the lithiatic control and received a vehicle of 1% Tween 80. Groups III to V were administered a 0.75% ethylene glycol solution orally daily for the first 14 days. From days 15 to 28, each group was treated with the following: standard cystone (100 mg/kg), SASM low dose (PEG+L.D 250 mg/kg), and SASM high dose (PEG+H.D 500 mg/kg), respectively. Groups IV and V served as the curative treatment groups.

3.7.2.4 Collection and Analysis of Urine, Blood, and Serum

Rats were housed in metabolic cages with water available freely after the completion of treatment. The pH of the 24-hour urine sample was measured using a digital pH meter shortly after collection. Before storing the collected urine at 4°C, a drop of con. hydrochloric acid (HCl) was added. It was then analyzed for Blood Urea Nitrogen (BUN), creatinine, uric acid, oxalate, calcium, citrate, magnesium, phosphorus using commercial kits (Molybdate U.V., OCPC, Calmagite, Erba diagnostics Mannheim Gmbh methods). The hematological parameters like RBC (Appendix IX-A), WBC (Appendix IX-B), and Hb percentage (Appendix IX-C) were estimated (Dacie and Lewis, 1975). On day 28, serum samples were obtained through retro-orbital blood collection from the rat's eyes using 5% isoflurane. The rats were euthanized using a cervical dislocation procedure for histopathological examination. The serum separation was achieved by centrifuging blood at 10,000 rpm for 10 minutes at cooled conditions, and the samples were labelled. The serum was stored in a refrigerator before being analysed for BUN, creatinine and uric acid levels (Singh *et al.,* 2022) given in Appendix IX-D, E, and F.

3.7.2.5 Measurement of Body and Kidney Weight

The body weight was measured for each group of rats at the beginning and end of the treatment, and the changes were recorded. All the rats were sacrificed, and at the end of the study, the actual weight of the dissected kidney was measured.

3.7.2.6 Determination of Antioxidant Enzymes and Lipid Peroxidation

3.7.2.6.1 Preparation of Tissue Homogenate

The liver was isolated from rats sacrificed after treatment with SASM extract, rinsed with 0.9% saline water, and stored for 12 hours for *in vivo* antioxidant studies. A 10% homogenate of the separated liver was achieved using a motor-driven Teflon-coated homogenizer and 0.1 M Tris-HCl buffer (pH 7.4) and centrifuged at 10,000 rpm for 10 minutes at 5˚C. The collected supernatant was used for further *in vivo* experiments. Antioxidant enzymes, viz.

Superoxide dismutase (SOD) (Kakkar *et al.,* 1984), Catalase (CAT) (Sinha, 1972), Glutathione peroxidize (GPX) (Rotruck *et al.,* 1973), Reduced glutathione (GSH) (Ellman, 1959), and Lipid peroxidation (LPO) (Ohkawa *et al.,* 1979), and protein content of liver tissues of all the tested rats were determined as given in Appendix X (A-F).

3.7.2.7 Histopathological Analysis of Kidneys

On day 29, rats were anesthetized and sacrificed through abdominal incision, and their kidneys were removed. A cold solution of normal saline was used to rinse the extraneous tissue of isolated kidneys to remove fat deposits, then it was fixed in a neutral formalin (pH 7.4) solution for 10%. One of the two isolated kidneys was immersed in paraffin films using a traditional method and then sliced into thin sections (5 µm) using a rotary vertical microtome. Changes in the histopathological analysis and crystal deposition in the sections were stained with a haematoxylin and eosin solution (Singh *et al.,* 2022). Haemotological parameters like haemoglobin content, total erythrocyte count, and leukocyte count were analysed in the blood given in Appendix IX (A-C).

3.8 *In vitro* Cytotoxicity

3.8.1 Brine Shrimp Lethality Assay

The cytotoxicity of SASM was assessed using *Artemia salina* (nauplii) hatched in saline solution. Different volumes (100, 250, 500, 1000, and 1500 µg/ml) of SASM were diluted in DH_2O to prepare a 1 mg/ml stock. A total of 30 shrimps were added to the solution (25 mL), and the movement and mortality of the shrimps were monitored at different intervals (1,2,4,6, and 24h) using a magnifying lens. The same was followed for negative blank and positive control potassium dichromate ($K_2Cr_2O_7$) (1mg/ml). After 24 hrs, the mortality rate of the shrimps was calculated through regression probit analysis using SPSS statistical software to measure the toxicity of the extract (Olowa and Nuneza, 2013).

$$Mortality\ (\%) = \frac{No.of\ Dead\ nauplii}{No.of\ Dead\ nauplii + No.of\ Live\ nauplii} X\ 100$$

3.8.2 Cytotoxic Properties of Test Drugs Against Kidney HEK 293 Cell Line

The cytotoxic properties of test drugs against the Kidney HEK 293 cell line were determined using the MTT method by Melendez *et al.* (2022), given in Appendix XI.

PHASE III

3.9 Chromatographic Techniques

3.9.1 Thin Layer Chromatography

The cell- free supernatant was extracted using methanol, and the organic phase was carefully transferred to a beaker. It was then placed on a water bath set at a temperature range of 55-60°C, allowing the solvent to evaporate until dryness. The prepared SASM crude extract and the standards linalool (terpenoids) and gallic acid (phenols) were subsequently compared to thin-layer chromatography (TLC) analysis. This involved applying the extract to TLC plates using capillary tubes, followed by development in a TLC chamber with toluene, ethyl acetate, and formic acid as a mobile phase in the ratio 5:5:0:5. After development, the TLC plates were air-dried and then visualized under UV light at 254nm and 366nm. Some plates were prepared for further analysis with spraying reagents, while others were briefly heated in a hot air oven for 10 minutes to induce color development. The TLC plate analysis revealed the presence of distinct, active-coloured spots, which were designated as spots a-e. The active bands on the silica plates, derived from selected isolates, were recovered and desorbed in methanol until dryness was reached.

Visualization of Bands

The TLC plates were dried and treated with anisaldehyde sulphuric acid (H_2SO_4) reagent to detect the presence of compounds. The movement of the compounds on the TLC plates was expressed in terms of their Rf values, which were calculated following a method similar to that described by Ahamed *et al.* (2017).

$$Retention\ time\ (Rf) = \frac{Distance\ travelled\ by\ the\ solute}{Distance\ travelled\ by\ the\ solvent}$$

3.9.2 High-Performance Thin Layer Chromatography Profiling Activation of pre-coated TLC plates and Application of sample

The HPTLC (CAMAG) Linomat 5 instrument was utilized for the analysis of secondary metabolites in *S. articularis* stem extracts. The silica gel (60 F^{254}) coated TLC plates (Merck) used were of size 20.0x10.0 cm. The instrument settings included a 100 µl syringe, with 20 tracks in total (5 tracks for each sample), with the respective standards oleanolic acid for terpenoids, and gallic acid for phenols. The application parameters were set as follows: the application position Y was 8 mm, the application volume was 5 µl, and the band length was 8 mm. The automatic applicator sprayed the sample and nitrogen gas from the syringe onto the TLC plates in the form of bands. This band-wise application of samples resulted in better separation of the compounds on the TLC plates (Preethi *et al.,* 2014).

Procedure

Partial purification of the crude extract was carried out using the High-Performance Thin Layer Chromatography (HPTLC) technique. The crude extract was dissolved in methanol and applied to TLC plates (20x10 cm) using a syringe. The mobile phase for terpenoids consisted of toluene, ethyl acetate, and formic acid in a 4.5:0.5:0.1 ratio with 10% ethanolic sulphuric acid as derivatization reagent. The mobile phase for phenols consisted of toluene, ethyl acetate, formic acid, and methanol in a 3:3:0.8:0.2 ratio with 10% ethanolic sulfuric acid, followed by Folin-Ciocalteau reagent for derivatization. After running the plates with this mobile phase, they were dried, and the positions of the spots were visualized under UV light. The Rf values were calculated for each spot, and the spots were labelled as spots a-g.

Detection of bands

Detection of bands was carried out under UV light, and separated bands of compounds were seen at 254 nm and at 366 nm.

3.9.3 Gas Chromatography-Mass Spectrometry Analysis

GC-MS is an analytical method used by Tyagi and Agarwal, (2017) to identify the various bioactive substances present within medicinal plants, and it is given in Appendix XII.

PHASE IV

3.10. *In silico* Analysis

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.

3.10.1 Molecular Docking using GLIDE

Molecular docking was performed using the Schrodinger suite (academic license, Version 2023-1) to predict the binding affinities of the ligand-receptor. The ligands' 2D structures were obtained using the PubChem repository. Protein structures (2D) were obtained from RCSB PDB. Protein preparation starts by removing water molecules beyond 5Å from heteroatoms, eliminating hydrogens, co-solvents, and existing lead components, followed by selecting respective force fields (OPLS4) and root mean square deviation (RMSD) charges (0.30 Å). Using the receptor grid generation tool, a grid was generated around the active sites with X, Y, and Z coordinates of 10Å. The SDF format of the ligand chemical structures of the 31 filtered compounds was downloaded from PubChem and processed with the Ligprep module of the Schrodinger suite. The ligands were neutralized and desalted, and electron ionization was performed. Molecular docking was carried out using GLIDE (Grid-based Ligand Docking with Energetics). GLIDE identifies the potential possible ligand locations within the active site region of the receptor. The protein grid file was uploaded as a zip document along with the ligand output file. Docking was performed using Xtra Precision (XP) mode, and high-scoring outputs were analyzed with interaction diagrams to determine the amino acid residues involved (Friesner *et al.,* 2006; Yuriev *et al.,* 2011).

3.10.2 Molecular Dynamics Simulation

The previously docked protein-ligand complex structure (PLCS) was assessed using the Desmond module (Version 7.3) of Schrodinger, to provide

receptor-ligand interactions insights under specified thermodynamic conditions (temperature, volume, density, and pressure) for the duration of 100ns. The simulation protocol involved the TIP3P water model solvation complexes, the addition of ions for neutralization, the removal of overlapping water molecules, and the creation of an orthorhombic water boundary box (10Å) (Mark & Nilsson, 2001). Energy minimization was executed at a 1000 KJ/M/nm tolerance. Equilibration of the system was achieved using NVT and NPT ensembles for 100 ps. The OPLSE4 force field was employed to develop an MDS system for a simulation and performed unrestricted production runs for a 100ns simulation time. The various parameters of MDS were analyzed, such as protein-root mean square deviation (P-RMSD), root mean square fluctuation (RMSF), PL contacts, secondary structure elements (SSE), and torsion tree to evaluate stability, compactness, structural fluctuations, and PL interactions in the solvated system**.**

3.10.3 Pharmacokinetic Analysis and Drug-Likeness Prediction

Determining ADMET properties, such as absorption, distribution, metabolism, excretion, and toxicity, of a drug candidate early in the drug development process can help identify potential issues and optimize development. *In silico* online prediction tool Swiss ADME was utilized to calculate the ADMET properties of the phytoconstituents quantified through GC-MS, which were first converted into Simplified Molecular Input Line Entry System (SMILES) format using the PubChem database and analyzed. The evaluation of ADMET factors encompassed various parameters, including physicochemical properties, QED score, gastrointestinal absorption, blood-brain barrier permeability, plasma protein binding (fraction unbound), volume of distribution, CYP3A4 substrate and inhibition potential, clearance, rat oral acute toxicity, and adherence to acute toxicity rules.

3.12 Statistical Analysis

Data were presented as the arithmetic mean±SEM of three separate experiments. Statistical significance was determined using a one-way ANOVA followed by Dunnett's post-hoc test, with significance set at $p<0.05$.