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Appendices

APPENDIX I

Authentication of *Spermacoce articularis* L.f.

भारतसरकार
GOVERNMENT OF INDIA
पर्यावरण,वनऔरजलवायुपरिवर्तनमंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
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सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2022/Tech /397 दिनांक/Date: 18th August 2022

पादप प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen given by you for authentication is identified as ***Spermacoce articularis* L.f. - RUBIACEAE.**

अभिनिर्धारित प्रतिरूप को संबंधित कॉलेज/विभाग/संस्थान के पादपालय में परिरक्षण हेतु वापस किया जाता है।/ The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

18/08/2022

डॉ. एम. यु. शरीफ़/DR. M. U. SHARIEF
वैज्ञानिक 'एफ' एवं कार्यालयाध्यक्ष/
SCIENTIST 'F' & HEAD OF OFFICE

सेवा में / To

Ms. GOPIKA S
Ph.D. Research Scholar
Department of Botany
Avinashilingam Institute for Home Science &
Higher Education for Women
COIMBATORE – 641 043

APPENDIX II

Qualitative Phytochemical Analysis

All 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, anthraquinones according to the standard method of Harborne, (1998) as presented in Appendix II

Test for Alkaloids - Dragendorff's test

1 ml of the extract was treated with 1 ml of Dragendroff's reagent. The appearance of an orange precipitate indicated the presence of alkaloids.

Test for Tannins

Braemer's test: 1 ml of the extract was boiled with 2 ml of water, filtered, and then treated 5-6 drops of 5% ferric chloride. The appearance of a dark green, blue, or brown colour indicated the presence of tannins.

Test for Phenols - Ferric chloride test

2 ml of the extract was treated with 2 ml of 5% ferric chloride solution. The formation of deep blue or black indicated the presence of phenols.

Test for Saponins

To 5 ml of the extract, 1 ml of sodium bicarbonate was added, and the mixture was shaken well. The formation of a honeycomb-like structure indicated the presence of saponins.

Test for Proteins - Biuret Test

To qualitatively detect the presence of proteins in the plant extract, the Biuret test was performed. Approximately 1–2 mL of the plant extract was added to a clean test tube, followed by the addition of 1 mL of 10% sodium hydroxide (NaOH) solution to create an alkaline medium. A few drops of 1% copper sulfate ($CuSO_4$) solution

were then added to the mixture. The contents of the test tube were mixed thoroughly and observed for any color change. The appearance of a violet or purple coloration indicated a positive result, confirming the presence of proteins in the plant extract.

Test for Anthocyanin

Sodium Hydroxide test: 1 ml of the extract was treated with 1 ml of 2M NaOH and the formation of blue-green colour indicated the presence of anthocyanins.

Test for Quinones

Hydrochloric acid test: 1 ml of the extract was treated with 1 ml of concentrated HCl. The formation of a yellow colour precipitate indicated the presence of quinones.

Test for Oxalate

Add 2 mL of an extract with 1ml of glacial acetic acid, greenish-black coloration indicated the presence of oxalate.

Test for Flavonoids - Shinoda Test

1 ml of the extract was dissolved in 5 ml of ethanol, treated with 3 drops of Conc. hydrochloric acid and 0.5 g of magnesium turning and observed for the formation of a pink colour, indicating the presence of flavonoids.

Test for Vitamin C - DNPH Test

To 1 ml of extract, a few drops of DNPH (Dinitrophenyl hydrazine) in concentrated sulphuric acid were added. The production of yellow colour indicated the presence of Vitamin C.

Test for Carbohydrates - Molisch's Test for Carbohydrates

To 2 ml of the extract, 2 drops of 5% naphthol in ethyl alcohol were added. Then 1 ml of concentrated sulphuric acid was added along the sides of the tube. The formation of a violet ring at the junction indicated the presence of carbohydrates

Detection of Phytosterols (Kumar *et al*., 2014)

A small quantity of extract dissolved in 5 ml of chloroform was subjected to Salkowski's and Liebermann Burchard's tests for the detection of phytosterols.

Test for Coumarins

1 ml of 10% sodium hydroxide was added to 1 ml of the extract. The formation of a yellow colour indicated the presence of coumarins.

Test for Terpenoids - Knoller's Test

To a small quantity of the extract taken in a dry test tube, add a few tin granules and 1 mL of thionyl chloride and shake well. The appearance of pink colour indicated the

presence of triterpenoids.

Test for Volatile oils

NaOH test 2.0 ml of the extract was shaken with 0.1 ml of dilute sodium hydroxide and 1 ml of dilute HCl. The formation of a white precipitate indicated the presence of volatile oils.

Tests for Resins

1 ml of extract was treated with a few drops of acetic anhydride solution followed by one ml of conc. H_2SO_4. Variation in colour from orange to yellow indicated the presence of resin.

Test for Carotenoids - Carr-Price reaction (Jagessar RC, 2017)

Add 10mL extract evaporated to dryness and 2-3 drops of a saturated solution of antimony trichloride in chloroform A blue-green color eventually changed to red.

Test for Glycosides

To 2 ml of extract with dilute HCl and 2 ml Sodium nitropruside in pyridine, sodium hydroxide solution were added. The formation of pink to blood red color indicated the presence of cardiac glycosides.

Test for Fixed oils and fats - Saponification Test

To the extract, 4 ml of 2 % sodium carbonate solution was added and shaken vigorously. After boiling, a clean soapy solution was formed, which was allowed to

cool, and to this, a few drops of Conc.HCl was added. Fatty particles separate and float up, indicating the presence of oil and fat.

Test for Catechin

The matchstick was dipped in the extract and dried. Then it was moist with Conc. HCl and warm near flame. The appearance of red or pink colour in the match stick indicated the presence of catechin.

Test for Reducing Sugar

The residue was re-dissolved in water in the water bath. To 2 mL of the solution, in the test tube, was added, 1ml each of Fehling's solutions A and B. The mixture was shaken and heated in a water bath for 10 minutes. The colour obtained was recorded. A brick-red precipitate indicated reducing sugar.

Test for Amino Acids - Ninhydrin Test (Bhandary *et al.*, 2012)

The test solution, when boiled with a 0.2% solution of Ninhydrin, would result in the formation of a purple colour, suggesting the presence of free amino acids.

Test for Starch

By using Iodine as a reagent, the appearance of dark blue colour which disappeared on heating and reappeared on cooling indicated the presence of starch in 1 ml of the sample.

Test for Acidic Compounds

To 2 ml of ethanol extract, 1 ml sodium bicarbonate solution was added. The effervescence produced indicated the presence of acidic compounds.

Test for Eugenols

Eugenol, a phenolic compound primarily found in clove oil, can be identified through several qualitative chemical tests. One of the most common methods is the Ferric Chloride Test, where the presence of the phenolic hydroxyl group in eugenol produced a characteristic violet or blue coloration upon reaction with ferric chloride, indicating a positive result.

Test for Cardiac Glycosides

One of the most common is the Keller-Killiani test, which identifies the deoxysugar component of cardiac glycosides. In this test, the presence of a reddish-brown ring at the junction of two layers and a blue-green coloration in the upper layer indicated a positive result.

Test for Anthraquinone

In 2 ml plant extract, 3 ml of benzene and 5 ml of 10% ammonia were added. The appearance of pink, violet or red colouration in the ammonia layer indicated the presence of anthraquinones.

APPENDIX III

Estimation of Primary Metabolites

A) Estimation of Total Carbohydrate (Hedge and Hofreiter, 1962)

Principle

Carbohydrate is first hydrolysed into simple sugars using dilute hydrochloric acid. In a hot acidic medium, glucose is dehydrated to hydroxymethylfurfural. This compound forms with anthrone a green-coloured product with an absorption maximum of 630 nm. The estimation of carbohydrates was done

Reagents

1. Glucose stock standard: 100 mg of glucose was dissolved in 100 ml of water in a standard flask
2. Working standard: 10 ml of the stock was diluted to 100 ml. 1.0 ml of this solution contains 10 mg of glucose.
3. Anthrone reagent: 0.2% anthrone was dissolved in ice-cold concentrated sulphuric acid prepared fresh before use.

4. 2.5 ml HCl.

Procedure

Prepared the standards by taking 0.2 - 1.0 ml of the working standards. After 1.0 ml of water serves as a blank make up the volume to 1.0 in all the tubes with distilled water. Add 0.1 ml of the sample extract in all the samples. Then 4.0 ml of anthrone reagent in all the test tubes. Heated for eight minutes in a boiling water bath, cooled rapidly, and read the green color at 630 nm.

Calculation

A standard graph was drawn by taking the concentration of glucose on the X axis and the spectrophotometer reading on the Y-axis from the graph the concentration of glucose in the sample was calculated.

B) Estimation of Protein (Lowry *et al.,* 1951)

Principle

The blue color developed by phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids, tyrosine, and tryptophan present in the protein, plus the color developed by the biuret reaction of the protein with the alkaline cupric tartrate

Reagents

1. Two percent sodium carbonate in 0.1 N sodium hydroxide (Reagent A).
2. 0.5 percent copper sulfate ($CuSO_4.5H_2O$) in one percent potassium sodium tartrate (Reagent B).
3. Alkaline Copper Solution: Mixed 50 ml of A and 1 ml of B before use (Reagent C).
4. Folin – Ciocalteau Reagent (Reagent D)
5. Protein Solution (Stock Standard): Accurately weighed 50 mg of bovine serum albumin (Fraction V) and dissolved in distilled water and the volume was made up to 50 ml in a standard flask.
6. Working Standard: About 10 ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. One milliliter of this solution contains 200 mg of protein.

Extraction of Protein from the Sample

Extraction is usually carried out with phosphate buffer used for the enzyme assay. About 50 mg of each sample was weighed and ground well with a pestle and mortar in 10 ml of the buffer and centrifuged. The supernatant of each sample was used for protein estimation.

Estimation of Protein

About 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard were pipetted into a series of test tubes, and 0.1 ml and 0.2 ml of each extract in two test tubes,

respectively. The volume was made up to 1 ml in all the test tubes. A tube with 1 ml of water served as the blank. About 5 ml of reagent C was added to each tube, including the blank, mixed well, and allowed to stand for 10 minutes. Then 0.5 ml of reagent D was added. Mixed well and incubated at room temperature in the dark for 30 minutes. The blue colour developed was read at 660 nm. A standard graph was drawn, and the amount of protein present in the sample was calculated.

Calculation

The amount of protein in the stem and leaf sample was expressed in mg/g or 100 g sample.

APPENDIX IV

Estimation of Secondary Metabolites

A) Estimation of Total Alkaloid Content (Rakhesh and Nair, 2019)

Principle

Alkaloids are naturally occurring nitrogen-containing pharmacologically active organic compounds present in the plant kingdom. The determination of total alkaloids was performed by a simple Spectrophotometric method using the reaction of the sample with Bromocresol green solution which forms the yellow color complex.

Reagents

1. Bromocresol green solution (BCG)
2. 2 N hydrochloric acid (HCl)
3. Phosphate Buffer solution (pH 4.7)
4. Stock Standard Solution: 100 mg Atropine was dissolved in 100 ml distilled water.
5. Working Standard solution: 10 ml of the stock solution was made up to 100 ml by adding distilled water.

Procedure

A quantity of 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard solution and 0.1 ml of the sample extract was taken in test tubes. About 5 ml of phosphate buffer (pH 4.7) was added. Then 5 ml of Bromocresol green solution was added. This mixture was added with 1, 2, 3, and 4 ml of chloroform. The absorbance of the complex in chloroform was read at 470 nm using a spectrophotometer against the blank prepared as above. Concentration was calculated using Atropine as standard and expressed as mg of AE/g of extract.

B) Estimation of Total Terpenoid Content (Truong *et al.,* 2021)

Principle

Plant terpenoids are utilized in traditional herbal medicines because of their aromatic properties. In this reaction, reddish-brown precipitation has been formed,

which is fully soluble in methanol but partially soluble in chloroform. Estimation of terpenoids has been done spectrophotometrically at 538 nm.

Reagents

1. Chloroform
2. Concentrated Sulphuric acid (H_2SO_4)
3. Stock Standard Solution: 100 mg linalool was dissolved in 100 ml distilled water. Working Standard solution: 10 ml of the stock solution was made up to 100 ml by adding distilled water.

Procedure

To 1 ml of the extract add 2 ml of chloroform. The sample mixture was then vortexed thoroughly before incubating for 3 min. Subsequently, 200 µL of concentrated sulfuric acid (H_2SO_4) was added to the mixture. Again, it is left for incubation at room temperature for 1.5–2 hr in the dark. A reddish-brown precipitate was formed in the mixture during incubation. The supernatant was carefully decanted without disturbing the precipitation. 3 ml of absolute methanol was added and vortexed until complete dissolving of the precipitation in methanol. Absorbance was read at 538 nm using a UV/Visible spectrophotometer. The same procedures were repeated for standard solutions. The Total terpenoid content was expressed as linalool equivalent in mg of linalool/g of extract.

C) Estimation of Total Tannin Content (Roghini and Vijayalakshmi, 2018)

Principle

Spectrophotometric estimation of tannin is based on measuring the blue color formed by the reduction of phosphotungstic molybdic acid by tannin-like compounds in an alkaline solution. The intensity was measured in a spectrophotometer at 700 nm.

Reagents

1. Folin Ciocalteu reagent: One part of commercially available Folin-Ciocalteau's reagent was mixed with two parts of distilled water before use.
2. Sodium carbonate (35 %)

3. Stock Standard Solution: 100 mg tannic acid was dissolved in 100 ml distilled water.
4. Working Standard solution: 10 ml of the stock solution was made up to 100 ml by adding distilled water.

Procedure

To 0.1 ml of sample extract 0.9 ml distilled water was added. To this mixture, 0.5 ml of Folin's reagent followed by 5 ml of 35 % sodium carbonate was added and kept at room temperature for 5 min. The experiment was repeated in triplicates. The same procedure was repeated for a standard solution and read against a blank. 1 ml of water served as blank without the sample. The blue color formed was read at 700 nm using a UV/Visible spectrophotometer. The content of tannin was calculated using a standard graph and the result was expressed as mg TAE/g of fraction.

APPENDIX V

Estimation of Enzymatic Antioxidant Assays

A) Estimation of Catalase (Chance, 1995)

Principle

The UV absorption of Hydrogen peroxide can be measured at 240nm, whose absorbance decreases when degraded by the enzyme catalase. From the decrease in absorbance, the enzyme activity can be calculated

Reagents

1. Phosphate buffer: 0.067 M (pH 7.0)
2. Hydrogen peroxide (2mM) in phosphate buffer

Procedure

H_2O_2-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of 40µl of the extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a spectrophotometer. The enzyme solution containing H_2O_2-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

B) Estimation of Peroxidase (Addy and Goodman, 1972)

Principle

In the presence of the hydrogen donor pyrogallol or dianisidine, peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of pyrogallol or dianisidine to a colored product called purpurogalli can be followed spectrophotometrically at 430nm.

Reagents

1. Pyrogallol: 0.05 M in 0.1M phosphate buffer (pH 6.5)
2. H_2O_2: 1% in 0.1M phosphate buffer, pH 6.5

Procedure

To 3.0ml of pyrogallol solution, 0.1ml of the extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5ml of H2O2 was added and mixed. The change in absorbance was recorded every 30

seconds up to 3 minutes in a spectrophotometer. One unit of peroxidase is defined as the change in absorbance/minute at 430 nm.

C) Estimation of Polyphenol Oxidase (Wojdylo, 2013)

Principle

Phenol oxidases are copper-containing proteins that catalyze the aerobic oxidation of phenolic substrates to quinines, which are auto-oxidized to dark brown pigments known as melanin. These can be estimated spectrophotometrically at 495nm.

Reagents

1. Phosphate buffer (0.1M, pH 6.5)
2. Catechol solution (0.01M)

Procedure

The reaction mixture contained 3.0 ml of phosphate buffer, 1.0 ml of 0.01 M catechol in phosphate buffer, and 2.0 ml of the plant extract. Changes in absorbance were recorded in a spectrophotometer at 495 nm for 3 minutes at an interval of 1 minute. The enzyme activity was expressed as changes in absorbance in minutes/g tissue.

D) Estimation of Ascorbate Oxidase (Vines and Oberbacher, 1965)

Principle

Ascorbic acid+1/2 O_2 dehydro ascorbic acid+ O_2 The disappearance of ascorbic acid is measured at 245 nm by a spectrophotometer.

Reagents

1. 0.1 M Phosphate buffer (pH 6.5)
2. Ascorbic acid

Procedure

In a test tube, 3 ml of ascorbic acid (8. 8 mg in 300ml phosphate buffer) mixed with 0.1 ml of the extract was taken. The absorbance at 265 nm was measured after every 30 seconds for 5 minutes. The ascorbate oxidase activity is expressed in terms of units/ g. One enzyme unit is equivalent to 0.01OD changes per minute.

APPENDIX VI

Evaluation of Non-Enzymatic Antioxidants

A) Estimation of Total Phenolic Content (Saeed et al., 2012)

Principle

Folin-Ciocalteu reagent contains polymeric ions due to the combination of phosphomolybdic acid and phosphotungstic heteropolyacid. The reagent consists of an integrated polymeric series with many octahedral molybdenum oxyacid units that surround the central tetrahedral phosphate. In this structure, molybdenum can be easily substituted by tungsten. Folic - Ciocalteu reagent oxidizes phenolates, and heteropoly acid is reduced in its balanced state from +6 to a mixture of +6 and +5 valencies, due to which the blue-colored molybdenum tungsten complex has been formed, and the maximum absorbance was measured at 765 nm.

Reagents

1. Folin-Ciocalteu's reagent.
2. Sodium carbonate (Na_2CO_3)
3. Stock Standard Solution: 100 mg of Gallic acid was dissolved in 100 mL of distilled water.
4. Working Standard solution: 10 ml of the stock solution was made up to 100 ml by adding distilled water.

Procedure

Different concentrations (0.2, 0.4, 0.6, 0.8, 1 mg/ml) of extracts and gallic acid were taken and 1 ml of Folin-Ciocalteu's reagent was added to the test tubes. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to the mixture followed by the addition of 13 ml of distilled water and mixed thoroughly. The estimation of the phenolic compound was carried out in triplicates. The mixture was kept in the dark for 90 min after which the absorbance was read at 750nm.The Total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per g of dried sample.

B) Estimation of Total Flavonoid Content (Saeed *et al.,* 2012)

Principle

Formation of acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavanols in addition to aluminum chloride. Aluminum chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B ring of flavonoids. For building the calibration curve, rutin was used as a standard material. Various concentrations of rutin solution were used to make a standard calibration curve.

Reagents

1. Aluminium chloride ($AlCl_3$) - 0.3 M
2. Sodium nitrite ($NaNO_2$) - 0.5 M
3. Methanol - 30%
4. Sodium hydroxide (NaOH) - 1 M
5. Stock Standard Solution: 100 mg rutin was dissolved in 100 ml distilled water.
6. Working Standard solution: 10 ml of the stock solution was made up to 100 ml by adding distilled water

Procedure

In a 10 ml test tube, different concentrations (0.2, 0.4, 0.6, 0.8, 1 mg/ml) of extracts of rutin were taken and made up to 1 ml with distilled water. Add 3.4 ml of methanol to the test tubes. Followed by 0.15 ml of $NaNO_2$ and 0.15 ml of $AlCl_3.6H_2O$. After 5 min, 1 ml of NaOH was added. The estimation of the flavonoid compound was carried out in triplicates. The solution was mixed well and the absorbance was measured against a blank at 506 nm. The standard curve for total flavonoids was made using rutin as a standard solution (0 to 100 mg/l). The total flavonoids were expressed as milligrams of rutin equivalents per gram.

C) Estimation of Ascorbic Acid (Roe and Keuther, 1943)

Principle

Ascorbate is converted into dehydroascorbate on treatment with activated charcoal, which reacts with 2,4-dinitrophenyl hydrazine to form osazones. These osazones produce an orange-colored solution when dissolved in sulphuric acid, whose absorbance can be measured spectrophotometrically at 540nm.

Reagents

1.TCA (4%)

2. 2,4-dinitrophenyl hydrazine reagent (2%) in 9N H_2SO_4 Methodology

3. Thiourea (10%)

4. Sulphuric acid (85%)

5. Ascorbic acid (100mg of Ascorbic acid in 100ml of 4% TCA)

Procedure

0.2 to 1 ml of ascorbic acid solution and 0.1 ml of plant extract were taken. The reagent was added followed by 2 drops of 10% thiourea solution. The tubes were incubated at 37◦C for 3 h. The ozone formed was dissolved by the addition of 2.5 ml of 85% sulphuric acid.DNPH reagent and thiourea were added to the blank after the addition of sulphuric acid. After cooling the tubes, the absorbance was read spectrophotometrically at 540 nm.The concentration of ascorbate in the sample was calculated and expressed in terms of mg/g of sample.

D) Estimation of α -Tocopherol (Rosenberg, 1992)

Principle

The Emmerie-Engel reaction is based on the reduction of ferric to ferrous ions by tocopherols, which, with 2,2'-dipyridyl, forms a red color. Tocopherols and carotenes are first extracted with xylene and read at 460nm to measure carotenes. Correlation is made for the carotenes after adding ferric chloride and read at 520nm.

Reagents

1. Absolute alcohol

2. Xylene

3. 2, 2'-dipyridyl (1.2g in one liter of n-propanol)
4. Ferric chloride solution (1.2g in one-liter ethanol)
5. Standard solution (D, L-α-tocopherol, 10mg/L in absolute alcohol)
6. Sulphuric acid (0.1N)

Procedure

Into 3 stoppered centrifuge tubes, 1.5ml of plant extract, 1.5ml of the standard, and 1.5ml of water were pipetted out separately. To all the tubes, 1.5 ml of ethanol and 1.5 ml of xylene were added, mixed well, and centrifuged. The xylene (1.0 mL) layer was transferred into another stoppered tube. To each tube, 1.0 mL of dipyridyl reagent was added and mixed well. The mixture (1.5ml) was pipetted out into a cuvette and the extinction was read at 460nm. Ferric chloride solution (0.33 mL) was added to all the tubes and mixed well. The red color developed was read exactly after 15 minutes at 520nm in a spectrophotometer.

APPENDIX VII

In Vitro Antioxidant Radical Scavenging Assays

A) DPPH Radical Scavenging Assay (Senguttuvan *et al.,* 2014)

Principle

DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a commercially available stable free radical, which is purple. The antioxidant molecules present in the herbal extracts, when incubated, react with DPPH and convert it into di-phenyl hydrazine, which is yellow. The degree of discoloration of purple to yellow was measured at 517 nm, which is a measure of the scavenging potential of plant extracts

Reagents

1. DPPH solution: 0.004g of DPPH (0.004%) in 100 ml of Methanol was made in a volumetric flask. Cover it with 2-3 layers of Aluminium foil and store it in the dark. Prepared fresh before use.
2. Stock Standard Solution: Ascorbic acid was used as a standard. 10 mg of ascorbic acid dissolved in 10 ml of methanol. Dilutions of this solution with distilled water 117 were prepared to give the concentration of 10, 25, 50, 100, 150, and 200µl.
3. Stock solutions of the sample were prepared by dissolving 10 mg of dried extract in 10 ml of methanol (1:1).

Procedure

Different concentrations of methanolic extract like 10, 25, 50, 100, 150, and 200 µg/ml were taken in a series of test tubes. Made it up to 3 ml using methanol. 50 µl of DPPH solution to all the test tubes, shaken well and incubated in the dark for about 20- 30 minutes. Methanol serves as the control and DPPH in methanol solution acts as blank without plant extracts.The changes in the absorbance of the plant samples were measured at 517 nm using a spectrophotometer. Results were compared with the standard ascorbic acid. The ability of DPPH radical scavenging activity was calculated by using the following formula:

$$DPPH\ scavenging\ effect\ (\%\ of\ inhibition) = \frac{(A0-A1)}{A0}\ X\ 100$$

here, A_0 is the absorbance of the control, and A_1 is the absorbance of the sample extracts. The IC_{50} (the microgram of extract to scavenge 50% of the radicals) value was calculated using linear regression analysis.

B) FRAP (Ferric Reducing Antioxidant Power) Activity (Pallab *et al*., 2013)

Principle

The reducing power increases with increasing amounts of the extract, when potassium ferricyanide reacts with ferric chloride in the presence of antioxidants, potassium ferrocyanide, and ferrous chloride are found as a product. The presence of reducers converts the Fe_{3+}/ferricyanide complex into ferrous.

Reagents

1. Standard Stock solution: 10 mg of gallic acid is dissolved in 10 ml of distilled water and different concentrations of 10, 25, 50, 100, 150, and 200 µg/ml were prepared.
2. 0.2M Phosphate Buffer (pH 6.6)
3. Potassium ferricyanide solution (1%): 1g of Potassium ferricyanide (K_4 [Fe $(CN)_6$] $3H_2O$) was dissolved in 100 ml of distilled water.
4. Trichloroacetic acid (CCl_3COOH): 10g of Trichloroacetic acid was dissolved in 100 ml of distilled water (If liquid 10 ml in 90 ml of water).
5. Ferric chloride (0.1%): 0.1g of ferric chloride ($FeCl_3$) was dissolved in 100 ml of distilled water.

Procedure

Different concentrations like 10, 25, 50, 100, 150, and 200µl of sample and standard solution were taken. 2.5 ml of phosphate buffer was added and mixed with 2.5 ml of 1% Potassium ferricyanide solution. The mixture was kept at 50°C in a water bath for 20 minutes. After cooling, 2.5 ml of 10% Trichloroacetic acid was added and centrifuged at 3000 rpm for 10 minutes. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 1 ml of 0.1% ferric chloride and kept for 10 minutes.

Control was prepared similarly, excluding the sample. The absorbance was measured at 700 nm in a spectrophotometer.

C) ABTS Radical Scavenging Activity (Chintalapani *et al*., 2018)

Principle

A ferryl myoglobin radical is formed from metmyoglobin and hydrogen peroxide. The ferryl myoglobin radical can oxidize ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation (ABTS+) which is green in colour and can be measured at 405 nm. Antioxidants suppress this reaction by electron donation radical scavenging and inhibit the formation of the coloured ABTS radical.

Reagents

1. 2. 3. Preparation of Standard stock solution: 10 mg of gallic acid dissolved in 10 ml of distilled water. Dilutions of this solution with distilled water were prepared to give the concentration of 10, 25, 50, 100, 150 and 200 µg/ml.
2. ABTS stock solution: The stock solutions of 7 mM ABTS and 2.45 mM potassium persulfate ($K_2S_2O_8$) were prepared in an ethanol-water (1:1) mixture.
3. ABTS reagent: Prepared by mixing equal volumes of ABTS and stock solutions. The mixture was allowed to react at room temperature for 12–16 hours in the dark

   to generate ABTS radical cation (ABTS+). The ABTS radical solution was then diluted with an ethanol-water mixture to obtain an absorbance of 0.700±0.020 at 734 nm.

Procedure

Different concentrations like 10, 25, 50, 100, 150, and 200 µg/ml of sample and standard solution were taken. 3.9 ml of ABTS reagent was added. The mixture was allowed to incubate at room temperature for about 6 min, and the absorbance was recorded at 734 nm. The percentage inhibition was calculated according to the formula:

$$Scavenging\ effect\ (\%) = \frac{(Absorbance\ of\ Control - absorbance\ of\ Sample)}{(Absorbance\ of\ Control)} \text{ X } 100$$

The antioxidant capacity of the test sample was expressed as IC_{50} (anti-radical activity), the concentration necessary for a 50% reduction of ABTS.

APPENDIX VIII

Ethical Clearance Certificate

Avinashilingam Institute for Home Science and Higher Education for Women

Deemed to be University Estd. u/s 3 of UGC Act 1956, Category A by MHRD
Re-accredited with A++ Grade by NAAC. CGPA 3.65/4, Category I by UGC
Coimbatore - 641 043, Tamil Nadu, India

(Reg. No. 623/PO/ReBi/S/02/CCSEA)

Certificate

This is to certify that the project proposal no **AIW:IAEC.2023:09** entitled **Antiurolithiatic activity of methanolic stem exract of *Spermacocce articularis* L.F** submitted by **Ms. Gopika. S** has been approved/recommended by the IAEC of **Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore** (Organization) in its meeting held on **14/03/2023** (date) and **30 Albino rat (Wistar)** (Number and Species if animals) have been sanctioned under this proposal for a duration of next **12** months.

Authorized by	Name	Signature	Date
Chairman:	Dr. Anitha Subash	.....	14/3/2023
Member Secretary:	Dr. R. Nirmaladevi	.....	14/3/2023
Main Nominee of CCSEA:	Dr. V. M. Berlin Grace	.....	14-03-2023

APPENDIX IX

Analysis of Haematological

Following the treatment period, animals were anesthetized using ketamine hydrochloride, and blood samples were collected from the retro-orbital sinus using a capillary tube into EDTA-coated centrifuge tubes for haematological analysis. Various parameters were estimated, including red blood cell (RBC) and white blood cell (WBC) counts, and haemoglobin concentration.

A) Enumeration of Red Blood Cells (RBC)

For the enumeration of RBCs, RBC diluting fluid was used. Blood was drawn into a red blood cell pipette up to the 0.5 mark, and the RBC diluting fluid was added up to mark II. The contents were thoroughly mixed and then transferred onto a haemocytometer. After allowing the mixture to settle for approximately 2 minutes, RBCs were counted in the four large corner squares under a microscope using a 45X (high-power) objective. The result was expressed as the number of cells per liter, specifically in the unit: Cells × 10^{12}/L.

B) Enumeration of White Blood Cells (WBC)

For the enumeration of white blood cells (WBCs), Turk's fluid was used as the diluting reagent, prepared by mixing 2 mL of acetic acid with 100 mL of distilled water and adding 10 drops of 3% methylene blue. Using a WBC pipette, well-mixed blood was drawn up to the 0.5 mark, followed by the addition of Turk's fluid up to mark II. The contents were thoroughly mixed and transferred onto a haemocytometer. After allowing the cells to settle for approximately 2 minutes, WBCs were counted in the four large corner squares under a microscope using a 10X (low-power) objective. The final result was expressed as the number of cells per 10 mm^3 of blood.

C) Estimation of Haemoglobin

The estimation of haemoglobin concentration is based on the principle that haemoglobin in the blood is converted to acid hematin upon reaction with Drabkin's reagent or a similar haemoglobin reagent. This reaction produces a stable colored compound whose intensity correlates directly with the haemoglobin content. A

measured volume of blood is added to the reagent and allowed to react for a specified period. The resulting color intensity is then measured using a spectrophotometer at a wavelength of approximately 540 nm. The absorbance obtained is compared against a standard calibration curve to determine the haemoglobin concentration. The results are expressed in grams per deciliter (g/dL).

Analysis of Serum

D) Estimation of BUN (Blood Urea Nitrogen)

The estimation of blood urea nitrogen (BUN) is based on the principle that urea is enzymatically hydrolyzed by urease to release ammonia. This ammonia then reacts with diacetyl monoxime in the presence of an acidic ferric solution—typically containing sulfuric acid and ferric ions—to form a pink-colored complex. The intensity of the color produced is directly proportional to the concentration of BUN in the sample. In the procedure, the sample is first mixed with a urease solution and incubated to allow hydrolysis. Following this, diacetyl monoxime and the acidic ferric reagent are added to initiate color development. The mixture is then heated, usually by boiling for about 20 minutes, and subsequently cooled. The absorbance of the developed color is measured at a wavelength between 520 and 540 nm, and the BUN concentration is determined accordingly.

E) Estimation of Creatinine

The estimation of serum creatinine is based on the Jaffe reaction, where creatinine reacts with picric acid in an alkaline medium to form a reddish-orange colored complex. The intensity and rate of color development are directly proportional to the creatinine concentration in the sample. This colorimetric method involves mixing 500 µL of picric acid solution and 500 µL of sodium hydroxide solution in a 5 mL test tube, followed by the addition of 100 µL of the serum sample. The mixture is thoroughly mixed, and the absorbance is measured immediately at 492 nm using a spectrophotometer with a 1 cm light path. The creatinine concentration is determined by comparing the sample's absorbance to that of a standard creatinine solution (2 mg/100 mL). The normal reference range for serum creatinine is typically 0.6–1.1 mg/dL.

F) Estimation of Uric Acid

Uric acid, the final product of purine metabolism, plays a key role in conditions such as gout, where elevated serum uric acid levels (hyperuricemia) lead to the deposition of monosodium urate crystals around joints. The estimation of uric acid is performed using an enzymatic photometric method involving TOOS (N-ethyl-N-(hydroxy-3-sulfopropyl)-m-toluidine). The principle is based on the enzymatic oxidation of uric acid by uricase, which produces allantoin, carbon dioxide, and hydrogen peroxide. The hydrogen peroxide then reacts with TOOS and 4-aminoantipyrine in the presence of peroxidase (POD) to form a pink-colored indamine dye. For the procedure, 800 µL of Reagent 1 is taken in a 2 mL centrifuge tube, followed by the addition of 20 µL of serum. The mixture is incubated at 30°C for 5 minutes. Then, 200 µL of Reagent 2 is added, mixed thoroughly, and incubated again at 37°C for 5 minutes. The absorbance is measured spectrophotometrically, and uric acid concentration is determined. The normal reference range for serum uric acid is 1.9–8.2 mg/dL.

APPENDIX X

Determination of Antioxidant Enzymes and Lipid Peroxidation

A) Estimation of Superoxide Dismutase (SOD) (Kakkar *et al*., 1984)

The estimation of antioxidant enzyme activity, such as superoxide dismutase (SOD), is based on the principle of inhibition of epinephrine auto-oxidation to adrenochrome. This reaction occurs under alkaline conditions and can be monitored by measuring the increase in absorbance at 480 nm.

In the procedure, liver tissue is first homogenized and then mixed with ethanol and chloroform. After centrifugation, the supernatant is collected and used for the assay. The reaction mixture typically contains carbonate buffer (0.05 M, pH 10.2), EDTA (0.49 M), and epinephrine (3 mM). The rate of adrenochrome formation is measured by recording the change in optical density per minute at 480 nm. The enzyme activity is expressed as units per milligram of protein (U/mg protein), with one unit defined as the amount of enzyme required to inhibit the rate of epinephrine auto-oxidation by 50%.

B) Estimation of Catalase (CAT) (Sinha *et al*.,1972)

The estimation of catalase activity is based on the principle that catalase catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. The remaining H_2O_2 is then quantified by its reaction with dichromate in acetic acid to form chromic acetate, a colored complex measurable at 570 nm.

In the procedure, a tissue homogenate is mixed with phosphate buffer (0.01 M, pH 7.0) and hydrogen peroxide (0.2 M), initiating the enzymatic reaction. After a specified incubation period, the reaction is stopped by adding the dichromate-acetic acid reagent. The mixture is then heated for 10 minutes to allow for the complete formation of chromic acetate. The absorbance of the resulting solution is measured at 570 nm, and catalase activity is expressed in units per milligram of protein (U/mg), reflecting the enzyme's efficiency in breaking down H_2O_2.

C) Glutathione Peroxidase (GPX) (Rotruck *et al*.,1973)

The estimation of glutathione peroxidase (GPx) activity is based on the principle of measuring the oxidation of reduced glutathione (GSH) in the presence of hydrogen peroxide (H_2O_2). The decrease in GSH levels is determined using DTNB (Ellman's reagent), which reacts with remaining GSH to form a yellow-colored complex measurable at 412 nm.

In this method, the tissue homogenate is incubated with sodium phosphate buffer (0.32 M, pH 7.0), EDTA, sodium azide, GSH, and H_2O_2. After incubation, the reaction is stopped by adding trichloroacetic acid (TCA), and the mixture is centrifuged. The supernatant is then reacted with DTNB, and the absorbance is measured at 412 nm. The enzyme activity is expressed as micrograms of GSH utilized per milligram of protein (µg/mg protein), indicating the antioxidant capacity of the sample.

D) Reduced Glutathione (GSH) (Ellman *et al.,* 1959)

The estimation of glutathione peroxidase (GPx) activity is based on the principle of measuring the oxidation of reduced glutathione (GSH) in the presence of hydrogen peroxide (H_2O_2). The decrease in GSH levels is determined using DTNB (Ellman's reagent), which reacts with remaining GSH to form a yellow-colored complex measurable at 412 nm.

In this method, the tissue homogenate is incubated with sodium phosphate buffer (0.32 M, pH 7.0), EDTA, sodium azide, GSH, and H_2O_2. After incubation, the reaction is stopped by adding trichloroacetic acid (TCA), and the mixture is centrifuged. The supernatant is then reacted with DTNB, and the absorbance is measured at 412 nm. The enzyme activity is expressed as micrograms of GSH utilized per milligram of protein (µg/mg protein), indicating the antioxidant capacity of the sample.

E) Estimation of Lipid Peroxidation (LPO) (Ohkawa *et al.,* 1979)

The estimation of lipid peroxidation is commonly assessed by measuring malondialdehyde (MDA), a byproduct of lipid degradation. The principle is based on

the reaction of MDA with thiobarbituric acid (TBA) to form a pink-colored chromogen, which can be quantified spectrophotometrically at 532 nm.

In this method, the tissue homogenate is mixed with a TCA-TBA-HCl reagent containing 15% trichloroacetic acid (TCA), 0.375% TBA, and 0.25 N hydrochloric acid, or with reagents such as 4% SDS, 20% acetic acid, and 0.8% TBA. The mixture is then heated at 85°C for one hour to allow the reaction to proceed. After cooling, the samples are centrifuged, and the absorbance of the supernatant is measured at 532 nm. The amount of MDA is calculated using 1,1,3,3-tetraethoxypropane as a standard and expressed as nanomoles of MDA per milligram of protein (nmol MDA/mg protein), serving as an index of oxidative stress and lipid peroxidation in the sample.

F) Estimation of Protein - Folin–Ciocalteu Reaction (Lowry's method)

Procedure:

To estimate protein concentration using the Lowry method, a series of standard BSA solutions ranging from 20 to 100 µg/mL were first prepared. Then, 0.1 mL of each standard and plant sample was pipetted into labelled test tubes. To each tube, 5.0 mL of freshly prepared alkaline copper solution was added, mixed thoroughly, and allowed to stand at room temperature for 10 minutes to facilitate the biuret reaction. Following this, 0.5 mL of Folin–Ciocalteu reagent was added rapidly to each tube. The contents were mixed immediately and incubated in the dark for 30 minutes to allow color development. Absorbance was then measured at 750–765 nm using a spectrophotometer. A standard curve was plotted based on the BSA standards, and the protein concentration of unknown samples was determined by comparing their absorbance values to the standard curve.

APPENDIX XI

Cytotoxicity analysis (Melendez *et al.*, 2022)

Reagents

1. MTT Powder (the solution is filtered through a 0.2µm filter and stored at 28°C for frequent use or frozen for extended periods)
2. DMSO
3. CO_2 incubator
4. Tecan Plate reader

Preparation of test solutions

Test sample preparation

For cytotoxicity studies, 32 mg/ml stocks were prepared using DMSO. Serial two-fold dilutions were prepared from 3.2 mg/ml to 10 mg/ml using DMEM plain media for treatment.

Cell lines and culture medium

HEK 293 Cell line was procured from ATCC, stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin(100IU/ml), streptomycin (100µg/ml) in a humidified atmosphere of 5% CO_2 at 37ºC until confluent. The cell was dissociated with cell dissociating solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The viability of the cells is checked and centrifuged. Further, 50,000 cells /well were seeded in a 96-well plate and incubated for 24 hrs at 37ºC, 5 % CO_2 incubator. Source of reagents: DMEM, FBS, PenStrep, Trypsin-procured from Invitrogen.

Procedure

The monolayer cell culture was trypsinized, and the cell count was adjusted to $5x10^5$ cells/ml using respective media containing 10% FBS. To each well of the 96-well microtiter plate, 100 µl of the diluted cell suspension (50,000 cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked

off, the monolayer was once with medium, and 100µl of different test concentrations of test drugs were added onto the partial monolayer in microtiter plates. The plates were then incubated at 37ºC for 24 hours in a 5% CO2 atmosphere. After incubation, the test solutions in the wells were discarded, and 100 µl of MTT (5mg/10 ml of MTT in 1X PBS) was added to each well. The plates were incubated for 4 h at 37ºC in a 5% CO_2 atmosphere. The supernatant was removed, 100µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula, and the concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.

Calculation

$$\%\ Inhibition = \frac{(OD\ of\ Control - OD\ of\ sample)}{OD\ of\ Control}\ X\ 100$$

APPENDIX XII

GC-MS Analysis (Tyagi and Agarwal, 2017)

Working Principle

The GC-MS work is based on the separation of individual components from a mixture upon heating, the heated gases are passed through a column with an inert helium gas. The separated substances emerge from the column, and they flow into the mass spectrometry

The GC-MS analysis was carried out using Agilent Technologies GC-MS (GC 7890A, MS 5975C) with a Fused silica 15m x 0.2 mm IDx1µm of capillary column. The instrument was set to an initial temperature of 110 ºC and maintained at this temperature for 2 min. At the end of this period, the oven temperature rose to 280 ºC, at the rate of an increase of 5ºC/min, and was maintained for 9 min. The injection port temperature was ensured as 250ºC, and the Helium flow rate as 1 ml/minute. The ionization voltage was 70 eV. The particle-free diluted crude extracts (1 µl) were taken in a syringe and were injected in split mode as 10:1. Mass spectral scan range was set at 30-450(m/z). The percentage composition of the crude extract constituents was expressed as a percentage by peak area. The identification and characterization of chemical compounds in various crude extracts were based on GC retention time.

Interpretation of the components

Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC-MS, compounds present in the plant's sample were identified. Interpretation of the mass spectrum GC-MS was conducted using the database of the National Institute of Standards and Technology (NIST), which has more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained

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Appendix L2
(Item No 5 of Check List)
Details of Research Publications

S.No	Article	Journal	Other Details Vol/No/Page No/ Year	Published in UGC-CARE / Scopus Indexed/ Web of Science
1	GC-MS profiling, in vitro antioxidant and antiurolithiatic potential of ethanol leaf extract of *Spermacoce articularis* L.f.	Medicinal Plants-International Journal of Phytomedicines and Related Industries	15(4), pp. 804–820, 2023.	Scopus Indexed
2	Evaluation of Antiurolithiatic Potential of Methanolic Stem Extract of *Spermacocce articularis* L.f.: An *In vitro* and *In vivo* Approach.	Pharmacognosy Journal	16(4): 770-778. 2024.	Scopus Indexed

*Proof of list of Journals from the Internet to be attached along with copies of reprints.

Scholar : [signature]

Supervisor : [signature] 2/9/2024

Library have checked
HoD A. Vijayalakshmi 20/9/24

Checked By:
[signature] 20/9/2024
Dean of Respective School

The scholar Miss S. Gopika (Reg. No. 18PHBOP001) has published her in the following journals:
1. Medicinal Plants - International Journal of Phytomedicines and Related Industries - indexed in Scopus and
2. Pharmacognosy Journal - indexed in Scopus - paper accepted

This may be considered.

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Medicinal Plants
Vol. 15 (4), December 2023, 804-820

doi : 10.5958/0975-6892.2023.00082.5

Research Article

GC-MS profiling, *in vitro* antioxidant and antiurolithiatic potential of ethanol leaf extract of *Spermacoce articularis* L.f.

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ABSTRACT

The whole part of the plant or the bioactive compounds isolated from plants act as complementary and substitute medicine for various health ailments. The present study was designed to identify and evaluate the chief phytochemical constituents of ethanol leaf extract of *Spermacoce articularis* L.f., its antiurolithiatic activity, and antioxidative activity against free radicals. *S. articularis* is a species of flowering plant belonging to the family Rubiaceae, with many ethnomedicinal uses. Phytochemical screening of ethanol leaf extract revealed the presence of alkaloids, tannin, terpenoids, saponin, volatile oil, vitamin C, flavonoid, phenol, quinone, anthocyanin, carbohydrates, and acidic compounds. The quantitative analysis reported that the ethanol leaf extract exhibited the maximum total alkaloid content of 31.38±0.014 mg AE/g followed by tannin of 31.27±0.245 mg TAE/g, polyphenol of 16.73±0.051 mg GAE/g, ascorbic acid of 15.28±0.004 mg AA/g and terpenoid of 11.01±0.004 mg linalool/g. The extract showed a phenol content of 23.63±0.004 mg GAE/g and a flavonoid of 101.5±0.007 mg RE/g. The plant extracts and their overall phenolic and flavonoid contents showed significant antioxidant activity through DPPH free radical scavenging assay, ferric reducing power assay (FRAP), and ABTS radical scavenging activity. IC_{50} values obtained by DPPH activity of *S. articularis* ethanol leaf extract was found to be 23.55±1.270 µg AA/ml, reducing power was found to be 37.49±0.132 µg GAE/ml and ABTS was 22.47±0.637 µg GAE/ml. Gas chromatography-mass spectrometry results revealed the presence of 12 major phytoconstituents in the ethanol leaf extract with the help of peak area (%) and retention time. *S. articularis* ethanol leaf extract can inhibit the nucleation of CaOx crystals, from the results obtained, it is observed that standard cystone showed the highest inhibition of 65.22±0.003% at 600 µg/ml, whereas the ethanol leaf extract showed the inhibition effect of 18.2±0.001% at 600 µg/ml. The findings of the study revealed that the leaves of *S. articularis* contains various phytochemicals which is responsible for diverse medicinal potential of this plant.

Keywords: *Spermacoce articularis* L.f., antioxidant, antiurolithiatic, GCMS, phytoconstituents

INTRODUCTION

Medicinal plants due to their therapeutic potential, have been used by humans in medicine for decades. Phytomedicine at present is a significant science for both ancient and modern medical systems. Phytochemical investigations are based on the exploration of plants for their use in the production of novel therapeutic drugs. Research on medicinal plants has also led to the discovery of potential novel drugs that are being used to treat a range of

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illnesses (Gnanadeebam and Viswanathan, 2014). Phytochemicals, also known as secondary metabolites, are naturally formed compounds seen in plants that benefit human health. Some of the secondary metabolites are alkaloids, flavonoids, tannins, phenolics, saponins, steroids, glycosides, terpenes, etc. These secondary metabolites protect plants from disease and contribute to plants colour, aroma and flavour. Further, they also protect human health from various diseases when they are taken in the diet (De Silva *et al*., 2017).

Since the start of the twenty-first century, a lot of research has been done on antioxidants based on their origin and functions. A group of substances that can function as antioxidant inhibitors and break the chain of free radical reactions by complexing with them are called antioxidants. These substances occur synthetically or naturally. A molecule that acts as an antioxidant must have the capacity in scavenging the radicals and creating fresh, stable ones in addition to inhibiting the mechanism of production (Christodoulou *et al*., 2022). To stimulate favourable biological activities in the living system, antioxidants are utilized widely in the field of pharmaceuticals, cosmetics and food industry. An enormous number of antioxidants have been discovered from natural sources with their unique properties. The variation in the antioxidative and biological effects of these antioxidants is mainly because of the differences in chemical structure, number, and position of phenolic hydroxyl groups (Mohammad *et al*., 2022). Many advanced techniques were employed to identify and quantify the bioactive compounds of plant sources. One such valuable tool is gas chromatography-mass spectrometry (GC-MS) used in the identification of non-polar components, volatile oils, fatty acids, lipids, and alkaloids (Jayakar *et al*., 2020)

One of the widespread diseases affecting humans is the formation of kidney stones and it is believed to be the third most common problem. Nearly 4-15% of the human population, all over the world suffer from the urinary stone problem. In India, 12% of the population is expected to have urinary stones. Stone formation takes place due to the accumulation of solute components from urine, including calcium, oxalate, phosphate, and uric acid. Calcium oxalate has been identified as a significant reason for urolithiasis in India (Pinjarkar Rupam *et al*., 2017). According to previous studies, the phytoconstituents such as a-amyrin, b-amyrin, lupeol saponins, and flavonoids prove to possess significant anti-urolithiasis and diuretic effects.

A family of flowering plants known as Rubiaceae includes the genus *Spermacoce* (False Buttonweed), which has about 150-200 species. These species are primarily found in temperate and tropical areas of the southern hemisphere, including Australia and America (Kala *et al*., 2013). In Ayurveda *S. articularis* (fam: Rubiaceae) is known as "Madanaghanti" and in Tamil it is called "Nathaisuri." The herb is used traditionally to treat eye disorders such as ophthalmia, inflammation of the eye, blindness, sores, and conjunctivitis, and is also used to cure fever, inflammation of gums, and spleen complaints. Leaves extract is used as an astringent in hemorrhoids and gallstones, seeds as a demulcent in diarrhea and dysentery, roots as a mouthwash to cure toothache, and decoction of the herb in the treatment of headaches. The herb has abundant calcium and phosphorus and mainly possesses beta-sitosterol, ursolic acid, iso-rhamnetin, and D-mannitol (Dahiya and Solanki, 2011). Thus, to prove the efficacy of *S. articularis* ethanol leaf extract, this study was conducted to assess the chemical composition, antioxidant, and anti-urolithiasis activities of the plant.

MATERIALS AND METHODS

Collection and authentication of plant

The whole plant *S. articularis* was collected from Attappady, Kerala. The plant samples were identified and authenticated taxonomically by the Botanical Survey of India, Southern Region, Coimbatore, Tamilnadu, India.

Preparation of plant extract

Soxhlation

Fresh leaves of *S. articularis* were collected from Attappady, Kerala. It was chopped up, cleaned in tap water and then dried in the shade for about ten days at room temperature. An electric blender was used to refine the dried leaf sample. The powdered samples were weighed (10g), and packed tightly in Whatman No.1 filter paper, for extraction using ethanol solvent (100 ml) by soxhlet apparatus. The solvent-extracted (ethanol) fractions were subjected to a rotary evaporator. The

dried extract was used for GC - MS characterization, quantitative estimation, and *in vitro* studies.

Preliminary phytochemical screening

The ethanol leaf extract of *S. articularis* was analyzed for the presence of alkaloids, flavonoids, saponins, steroids, tannins, glycosides, terpenoids, starch, cellulose, oil and fat, proteins, carbohydrates, volatile oils, resins, vitamin c, catechins, anthraquinones, and coumarins according to standard methods of Harborne (1998).

Quantitative estimation of phytoconstituents

Estimation of total alkaloid

A sample extract of 0.1 ml was taken and about 5 ml of phosphate buffer (pH 4.7) and 5 ml of Bromocresol Green solution (BCG) were added and the mixture was shaken with 1, 2, 3, and 4 ml of chloroform. A blank solution was prepared as the above procedure, and the absorbance of the compound in chloroform was measured at 470 nm. Concentration was calculated using atropine as standard and expressed as mg of AE/g of extract (Bromocresol Green Method).

Estimation of total tannin

To the sample extract of 0.1 ml, 0.9 ml of distilled water was added and the volume was made up to 1 ml. This solution was then treated with 0.5 ml of Folin's Ciocalteu reagent (1:2) and 5 ml of 35% sodium carbonate and kept at room temperature for 5 min. At 640 nm, the intensity of the blue colour was measured. The tannin content of the extract was determined using gallic acid as a standard. The total tannin content was expressed in GAE mg/g of extract (Folin and Ciocalteu Method, 1927).

Estimation of ascorbic acid (vitamin C)

To 1 ml of sample extract, 0.5 ml of Dinitrophenyl hydrazine followed by 2 drops of thiourea was added and kept for incubation at 37 °C for 3 hours. After incubation, to dissolve the orange-red crystals, 2.5 ml of 80% sulphuric acid was added. At 540 nm, the absorbance was measured and a standard graph was plotted. From the standard graph, vitamin C content was calculated (Roe and Keuther, 1943).

Estimation of total polyphenolic content

The Folin's Ciocalteu method was used to determine the total polyphenolic content spectrophotometrically against gallic acid as the standard. A plant extract of 0.5ml was taken to which 2.5 ml of distilled water was added followed by the addition of 0.5 ml of Folin's Ciocalteu reagent in drops. 7.5% of 2.5 ml sodium carbonate was added and the tubes were allowed to stand at room temperature for 60 minutes. Absorbance was measured at 765 nm and the same procedure was repeated for the standard solution. The total polyphenol content was expressed as the GAE/g of the leaf extract (Singleton *et al.*, 1999).

Estimation of total terpenoid content

One ml of plant extract was mixed with 2 ml of chloroform. After completely vortexing the sample mixture, it was left undisturbed for 3 min. To that 200 µl of concentrated sulphuric acid was added. It was incubated for 1.5 to 2 hours in the dark at room temperature. A reddish-brown precipitate was formed from which the supernatant was carefully decanted. For the precipitate to dissolve 3 ml of methanol was added and vortexed. Absorbance was read at 538 nm. The total terpenoid content of the extract was calculated as linalool/g of the extract (Ghorai *et al.,* 2012).

Antioxidant activity study

The antioxidant activities of the ethanol leaf extract were determined by DPPH, reducing power assay, and ABTS radical cation decolorization assay.

Estimation of total phenolic content

The total phenolic content (TPC) of the extract was determined by following the procedure of Kim *et al.* (2003). To 1 ml of extract, 1 ml of Folin's Ciocalteu reagent was added. After 5 minutes, 7% sodium carbonate solution of 10 ml and distilled water of 13 ml were added and properly mixed. The absorbance was measured at 750 nm after 90 min of incubation in the dark. The TPC was determined using standard gallic acid. Phenolic compound estimation was carried out in triplicates. The TPC was expressed as a GAE/g of leaf extract.

Estimation of total flavonoid content

Park *et al.* (2008) method was followed to determine the total flavonoid content. To 0.3 ml of leaf extract, add 3.4 ml of 30% methanol, 0.15 ml of sodium nitrite (0.5 M), and 0.15 ml of aluminium chloride (0.3 M). After 5 minutes, 1

ml of sodium hydroxide (1 M) was added. At 506 nm absorbance was measured against the blank and the same procedure was followed for the standard rutin. The total flavonoid content (TFC) was expressed as rutin/g of the sample.

DPPH (2,2 - diPhenyl-1-Picrylhydrazyl) assay

The free radical scavenging activity of the fractions was measured *in vitro* by 2,2 - diphenyl-1-picrylhydrazyl (DPPH) assay by the procedure of Blois, 1958 with slight modifications. To different concentrations of the extract, about 50 µl of 0.2 mmol/l solution of DPPH in methanol was prepared and added. After vortexing, the mixture was incubated for 30 min in the dark at room temperature. Methanol served as a reference and blank was prepared in the same manner without the sample extract. Using a spectrophotometer, the plant sample's absorbance was determined at 517 nm. A higher level of radical scavenging activity is indicated by a lower absorbance value. Results were compared with the standard antioxidant ascorbic acid. The ability of DPPH radical scavenging activity was calculated by using the following formula:

DPPH scavenging effect (% of inhibition) = (A_0-A_1) X 100/A_0

where, A_0 is the absorbance of the control, and A_1 is the absorbance of the sample extracts. Using linear regression analysis, the IC_{50} (the microgram of extract to scavenge 50% of the radicals) value was calculated. Antioxidant activity is greater when the IC_{50} value is lower.

FRAP (Ferric Reducing Antioxidant Power) assay

The ferric-reducing ability of *S. articularis* was determined by following the method of Benzie and Strain (1996). Different plant extract concentrations were mixed with 1 ml of 0.2 mol/L phosphate buffer (pH 6.6) and 1 ml of freshly prepared 1% potassium ferricyanide. The mixture was incubated at 50 °C in a water bath for 20 minutes. To that 10% of 1 ml trichloro acetic acid was added and the mixture was centrifuged for 10 min at 3000 rpm. 2 ml of supernatant was mixed with 2 ml of distilled water and 0.5 ml of 1% ferric chloride. The absorbance was read at 700 nm. Ascorbic acid served as a positive control and sample-free mixture as a blank. The higher absorbance of the reaction indicates the greater reducing power.

ABTS (2,2' -AzinoBis, 3-ethylbenzThiazoline-6-Sulphonic acid) assay

The cation scavenging activity of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) also known as ABTS, was carried out. A dark-coloured solution containing ABTS radical cations was produced by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate solution and the mixture was kept undisturbed in the dark overnight. The ABTS radical cation was diluted with 50% methanol before use in the assay, resulting in an initial absorbance of about 0.70±0.02 at 735 nm, with temperature control set at 30°C. Different concentrations of the sample was mixed with 3 ml of ABTS solution and the decrease in absorbance was measured. The results were compared with that of the standard antioxidant gallic acid. The percentage of the scavenging effect was calculated according to the formula:

Scavenging effect (%) = [(Absorbance $_{Control}$ - absorbance $_{Sample}$) / (Absorbance $_{Control}$)] X 100

The antioxidant capacity of test samples was expressed as IC_{50} (anti-radical activity), the concentration necessary for a 50% reduction of ABTS (Re *et al*.,1999).

***In-vitro* antiurolithiatic activity**

Nucleation assay

The effect of leaf extract on calcium oxalate (CaOx) crystal formation was determined using nucleation assay. Buffer containing Tris-HCl of 0.05 mol/l and sodium chloride (NaCl) of 0.15 mol/l was prepared and maintained at pH 6.5. 5 mmol/l of calcium chloride ($CaCl_2$) and 7.5 mmol/l of sodium oxalate ($Na_2C_2O_4$) solution was prepared using the buffer. Different concentrations (100, 200, 300, 400, 500, and 600 µl) of ethanol leaf extract were mixed with 3 ml calcium chloride solution followed by the addition of 3 ml sodium oxalate solution. The solution was kept at incubation for 30 min at 37ºC. The absorbance of the mixture was measured at 620 nm. Percent inhibition of nucleation by ethanol leaf extract was calculated using the formula and compared to that calculated for the standard cystone (Hennequin *et al.*, 1993).

% Inhibition = (1- OD $_{Sample}$ / OD $_{Control}$) X 100

Microscopic evaluation

After which a few drops of the mixture from different concentrations were placed in different slides and the reduction of crystal size and morphology of CaOx crystals formed in the presence and absence of *S. articularis* ethanol leaf extract was determined using a trinocular microscope at 1000x magnification.

GC-MS analysis

The GC-MS analysis was carried out using Agilent Technologies GC-MS (GC-7890A, MS 5975C) with Fused silica 15m x 0.2 mm ID x 1 µm of the capillary column. For 2 min the instrument was set to an initial temperature of 110ºC and maintained. Then, the temperature was raised to 280ºC, at the rate of an increase of 5ºC/min, and maintained for 9 min. The temperature of the injection port was ensured as 250ºC and the Helium flow rate as 1 ml/min. The ionization voltage was 70eV. In a syringe the particle-free diluted crude extracts (1µL) were taken and were injected in split mode as 10:1. Mass spectral scan range was set at 30-450 (m/z). The crude extract constituents' composition was expressed as a percentage by peak area. Based on GC retention duration, chemical components in different crude extracts were identified and described. Using NIST Ver.2.1 MS data library the spectrum obtained through GC-MS were compared with the compounds present in the plant's sample and identified. National Institute of Standard and Technology (NIST) having more than 62,000 patterns was used in the interpretation of the mass spectrum GC-MS. A comparison was made between the spectra of the unknown components and the spectrum of known components stored in the NIST library. The name, molecular weights, and structures of the compounds were determined (Adams, 2001; Stein *et al*., 2002).

Statistical analysis

The number of trials (n) in each experiment was three, and the results were provided as mean ± SD. Thus, P values were not calculated.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

Table 1 illustrates the results of the qualitative phyto-chemical assay. Phytochemical screening of ethanol leaf extract has exhibited the presence of alkaloid, tannin, terpenoids, saponin, volatile oil, and vitamin C in high amounts and flavonoid, phenol, quinone, anthocyanin, carbohydrates, and acidic compounds in moderate levels. The extract revealed the absence of steroids, starch, oil and fat, amino acids, resins, catechins, anthraquinones, coumarins, and cardiac glycoside. Our results showed similarity to the results of Anupriya *et al.* (2016) who reported the presence of various active components such as alkaloids, carbohydrates, coumarins, cyanins, flavonoids, glycosides, phenols, quinones, saponins, steroids, tannins, terpenoids and triterpenoids in *S. hispida* whole plant methanol extract. A phytochemical analysis of *Borreria sp*. whole plant hexane, ethyl acetate, acetone, and methanol extract revealed the presence of several secondary metabolites, including phenolics, alkaloids, flavonoids, tannins, saponins, and terpenoids. All kinds of chemical components were present in *B. laevicaulis,* while phenol,

Table 1: Qualitative phytochemical screening of *S. articularis* L.f. ethanol leaf extract

S.No.	Phytochemicals	*S. articularis* ethanol leaf extract
1	Alkaloid	++
2	Flavonoid	+
3	Tannin	++
4	Phenol	+
5	Steroids	-
6	Terpenoids	++
7	Quinone	+
8	Starch	-
9	Oil and Fat	-
10	Anthocyanin	+
11	Saponin	++
12	Proteins	++
13	Carbohydrates	+
14	Volatile oil	++
15	Amino acids	-
16	Resins	-
17	Vitamin C	++
18	Catechins	-
19	Anthraquinones	-
20	Coumarins	-
21	Acidic compounds	+
22	Cardiac Glycosides	-

++ Present, + Slightly Present, - Absent

flavonoids, and tannins were found to be present in *B. latifolia, B. remotifolia, B. exilis* and *R. brasiliensis*. Terpenoids were reported to be present in *R. brasiliensis* along with the other constituents (Wong *et al.*, 2015). Similarly, Islam *et al.* (2015) reported the presence of various active components such as alkaloids, glycosides, flavonoids, steroids and polyphenols in crude methanol extract of *Mussaenda roxburghii* leaves and Mishra *et al.* (2021) reported the presence of alkaloids, saponins, flavonoids, glycosides, phenols, tannins and steroids in hexane, acetone, chloroform, methanol and aqueous root extracts of *Rubia cordifolia.*

Quantitative phytochemical analysis

The quantitative analysis was done on major phytochemicals such as alkaloids, polyphenols, ascorbic acid, terpenoids, and tannins by standard procedures. The quantity of phytochemicals present in ethanol leaf extract of *S. articularis* is presented in Table 2. The extract showed the maximum quantity of total alkaloid and total tannin content of 31.38±0.014 mg AE/g and 31.27±0.245 mg TAE/g followed by polyphenol content of 16.73±0.051 mg GAE/g, ascorbic acid content of 15.28±0.004 mg AA/ g and terpenoid content of 11.01±0.004 mg linalool/g. The earlier reports on *S. hispida* methanol extract of the whole plant expressed a tannin content of 11.04 mg/g and an alkaloid content of 10.42 mg/g (Anupriya *et al.*, 2016). Our results were similar to previous reports where tannin content showed a significant amount in ethanol leaf extracts of 148.23±0.510 mg TAE/g in *Ardissia solanaceae* (Chopade *et al.*, 2023) and 2.74 ± 0.15 mg CE/g in *S. italica* (Towanou *et al.*, 2023).

Antioxidant activity

Phenols and flavonoids' strong ability to donate hydrogen is the cause of their antioxidant effects (Shimanda *et al.*, 1992).

Determination of total phenolic content

The phenolic content of any plant is directly related to their antioxidant properties. Phenolic compounds can scavenge free radicals and act as reducing agents and hydrogen donors (Wojdylo *et al.*, 2007).

The Folin's Ciocalteu method using gallic acid as the standard was used to calculate the total phenolic content in the ethanol leaf extract of *S. articularis* L.f. and the results are shown in Table 3. The calibration curve was constructed using the absorbance values that were obtained at various gallic acid concentrations. Using the regression equation of calibration curve $Y=0.821x - 0.001$; $R^2 = 0.9938$, the total phenolic content of the extract was calculated and expressed as gallic acid equivalents (GAE/ g) of the plant extract. The sample was found to contain 23.63± 0.004 mg GAE/g of phenolic content.

Our results coincide with the results of Ankad *et al.* (2015) who reported a significant TPC value of 9.17±0.46, 8.90±0.45, 9.44±0.47 and 10.88±0.54 mg TAE/g in ethanol leaf extract of *S. hispida, S. mauritiana, S. stricta, and S. ocymoides* respectively. The ethanol leaf extracts showed the highest concentration of phenolics 10.138±0.010 mg GAE/g in *Ardissia solanaceae* (Chopade *et al.*, 2023), 3179.91±223.11 mg GAE/100 g in *S. italica* (Towanou *et al.*, 2023) and 80.03±2.59 mg GAE / g in *Citrus acida* (Jain *et al.*, 2021) which are in par with our results. Similarly, in another study, the TPC value for the n-butanol fraction of *S. latifolia* whole plant extract was reported to be 57.97±1.99 mg GAE/g of extract (Luo *et al.*, 2020).

Determination of total flavonoid content

Table 2: Quantitative determination of phytochemicals in ethanol leaf extract of *S. articularis* L.f.

S.No.	Phytoconstituents	*S. articularis* ethanol leaf extract
1	Alkaloid (Atropine/g)	31.38±0.0147
2	Polyphenol (GAE/g)	16.73±0.051
3	Ascorbic acid (AA/g)	15.28±0.004
4	Tannin (TAE/g)	31.27±0.227
5	Terpenoid (Linalool/g)	11.019±0.004

Values are mean ± SD

Table 3: Total phenol, flavonoid content, and IC_{50} values of DPPH, ABTS, and FRAP in *S. articularis* L.f. ethanol leaf extract

S.No.	Antioxidant assays	*S. articularis* ethanol leaf extract
1	TPC (GAE mg/g)	23.63±0.004
2	TFC (RE mg/g)	101.5±0.007
3	DPPH (%)	23.55±1.270
4	ABTS (%)	22.48±0.637
5	FRAP (µg/ml)	37.5±0.135

Values are mean ± SD

TFC value of crude extracts of *S. articularis* are presented in Table 3. Rutin served as the standard and the total flavonoid content was determined. The calibration curve of rutin was linear with Y=0.057x + 0.0086; R^2=0.9915. The sample was found to contain 101.5±0.007 mg RE/g extract.

Similar results were obtained by Ankad *et al.*, 2015, who reported the highest TFC of 5.98±0.30, 5.62±0.28, 6.18±0.31 and 6.31±0.32 mg QE/g in ethanol leaf extract of *S. hispida, S. mauritiana, S. stricta and S. ocymoides* respectively. The flavonoid content in the *Mitragyna speciosa* leaf methanol extract was found to be 91.12 ± 17.27 mg CE/g (Parthasarathy *et al.*, 2009). The crude methanol and ethanol leaf extracts of *Bathysa gymnocarpa* (Araujo *et al.*, 2023) and *Ardissia solanaceae* (Chopade *et al.*, 2023) revealed the total flavonoid content (TFC) of 70.07±1.5 mg RE/g extract and of 371.91± 0.167 mg QE/g which coincides with our present study. Similarly, Towanou *et al.*, 2023 and Jain *et al.*, 2021 reported a total flavonoid content of 898.36 ± 4.04 mg QE/g and 181.47±3.65 mg QE/g in *S. italica* and *Citrus acida* ethanol leaf extracts. The TFC value for the ethyl acetate fraction of *S. latifolia* whole plant extract was found to be 168.16±0.65mg RE/g extract (Luo *et al.*, 2020).

DPPH radical scavenging activity

In the DPPH assay, the anti-oxidants were able to reduce the stable radical DPPH from purple colour to yellow-coloured diphenyl-picryl hydrazine. The result of antioxidant DPPH of *S. articularis* ethanol leaf extract is shown in Figure 1 and Table 3. The extract and standard ascorbic acid showed dose-dependent quenching of DPPH in which the calibration curve was linear with Y=0.1588x + 46.258; R^2=0.9952. DPPH radical scavenging ability was also evaluated based on their IC_{50} values. The IC_{50} is the concentration of an antioxidant-containing substance required to scavenge 50% of the initial DPPH radicals. The lower the IC_{50} value, the more potent the DPPH scavenging substance, and this implies a higher antioxidant activity. The IC_{50} value was found to be 23.55±1.270% µg AA/ml whereas the standard ascorbic acid showed 53.05 ± 1.67% µg/ml indicating the powerful antioxidant potential of *S. articularis* ethanol leaf extract.

According to Luo *et al.* (2020) the DPPH radical-scavenging capacities of the whole plant extracts of *S.*

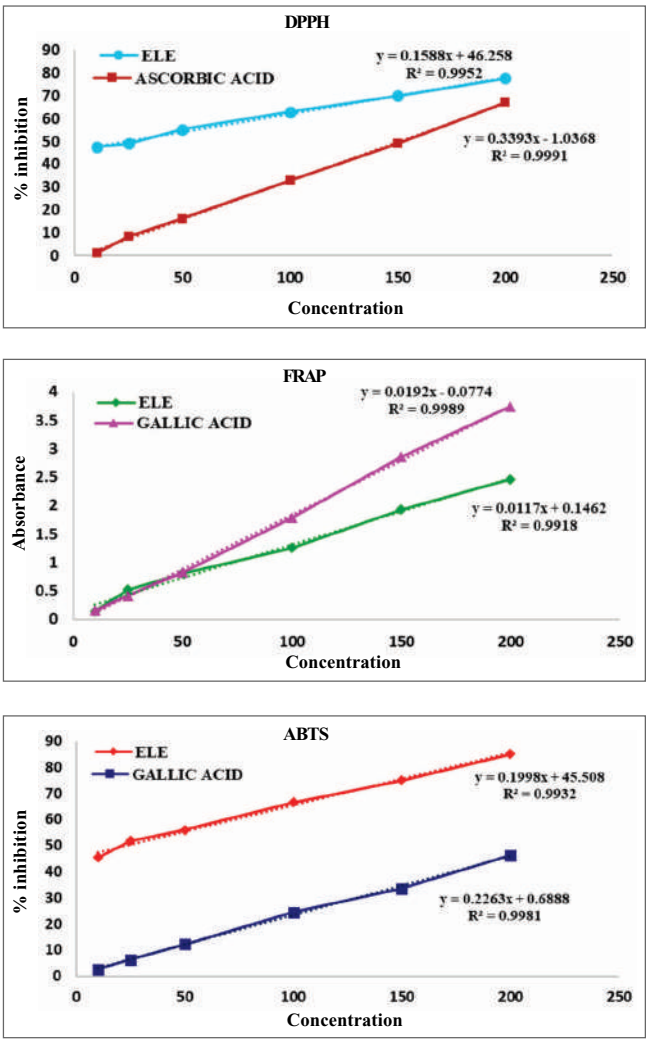


Figure 1: DPPH, FRAP and ABTS radical scavenging activity of ethanol leaf extract of *S. articularis* L.f.

latifolia, expressed IC_{50} values of 70.22±0.62, 51.44±0.44, 72.12±0.18 and 2252±2.09 µg AA/ml for petroleum ether, ethyl acetate, n-butanol, and aqueous extracts respectively. Mozibullah *et al.* (2023) reported that the calculated IC_{50} value of leaf methanolic and ethanolic extracts of *Ixora chinensis* was found to be 39 and 18.52 µg GAE/ml. The IC_{50} value obtained is in line with earlier published studies (Zengin *et al.,* 2023; Oso and Olowooker, 2018; Islam *et al.,* 2015) in ethanol leaf extracts of various plants. The IC_{50} value was found to be 40.44±1.41 µg AA /ml in *C. acida* ethanol leaf extract (Jain *et al.*, 2021) and 78.1±0.87 µg AA/ml in *Rubia cordifolia* methanol root extract (Mishra *et al.*, 2021). *Mussaenda erythrophylla* IC_{50} values with ethyl

acetate and methanol extract of the stem were found to be 159.86 µg AA/ml and 174.35 µg AA/ml respectively (Eswaraiah and Satyanarayana, 2010). The IC_{50} value of stem ethyl acetate extract of *Coffea benghalensis* was found to be 3.22±0.17% µg BHT/ml (Sagor *et al.,* 2021) showing high potent antioxidant activities.

FRAP (Ferric Reducing Antioxidant Power) assay

Ferric ion reducing power (FRAP) assay, is an electron transfer-based assay, where the reduction of ferricyanide complex in the reaction mixture to the ferrous (Fe^{2+}) form takes place due to the availability of reducing agents in the samples that contain antioxidants (Huda-Faujan *et al*., 2009). Here, depending on the reducing power of the samples, the test solution's yellow colour is reduced to various shades of green and blue. As shown in Figure 1 and Table 3, the *S. articularis* ethanol leaf extract exhibited significant reducing power that increased with extract concentration, whereas standard gallic acid showed stronger activity of 54.02±0.101 µg/ml. The calibration curve was linear with Y=0.0117x + 0.1462; R^2=0.9918 and the IC_{50} value of the ethanol leaf extract was 37.49±0.132 µg GAE/ml.

Our results agree with the report of Huda-Faujan *et al*. (2009) who reported that the ferric-reducing abilities of both extract and standard ascorbic acid increased in a concentration-dependent manner. Similar results were observed by Sagor *et al.* (2021) and Mishra *et al.* (2021) who reported the strongest reducing power at 400 µg/ml and 500 µg/ml concentrations of stem ethyl acetate extract of *Coffea benghalensis* and methanol root extract of *Rubia cordifolia*. The whole plant extract of *S. latifolia* ethyl acetate fraction exhibited the strongest reducing power of 8.36±0.10 mmol Fe (II) AA/g, followed by 6.11±0.20 mmol Fe (II) AA/g in n-butanol fraction, 5.89±0.09 mmol Fe (II) AA/g in petroleum ether fraction and 1.49±0.25 mmol Fe (II) AA/g extract in the aqueous fraction (Luo *et al*., 2020). The IC_{50} values of leaf methanol extract of *Paliurus spina-christi* Mill and *C. alata* (Linn.) Roxb was found to be 664.85 ± 0.49 mg TE/g and 649.36 ± 113.14l mg AA/100g by Zengin *et al.*, 2023 and Oso and Olowookere, 2018.

ABTS radical scavenging activity

Similar to FRAP, ABTS•+ is also an electron transfer-based assay, in which the antioxidants are reduced from a dark blue colour 2,2-azino-bis (3-ethylbenzothiazoline -6-sulfonate) radical cation (ABTS•+), into colourless ABTS, which can be measured spectrophotometrically. Plant extract and standard showed a concentration-dependent increase in ABTS radical scavenging activity as seen in Figure 1 and Table 3. The scavenging activity of plant extracts was higher than standard gallic acid. The IC_{50} value of the gallic acid was 46.94±0.494 µg/ml and the ethanol leaf extract were 22.47±0.637% µg GAE/ml which showed linear calibration of y=0.1998x + 45.508; R^2=0.9932. The results proved the presence of antioxidants as there was a decrease in the absorbance and decolourization was linear with increasing antioxidant capacity of the extract.

The results are in accordance with Luo *et al.* (2020) who reported the IC_{50} values of 14.36±0.13, 10.94±0.11, 15.40±0.09 and 162.22±1.39 µg AA/ml for petroleum ether fraction, ethyl acetate fraction, n-butanol fraction and aqueous fraction, respectively in the whole plant crude extracts of *S. latifolia*. The IC_{50} values of *Paliurus spina-christi* Mill and *C. olitorius* leaf methanol extract was found to be 1171.58 ± 25.83 mg TE/g (Zengin *et al.*, 2023) and 33.19 µg AA/ml (Chigurupati *et al*., 2020). The IC_{50} value of ethanol extract was found to be 58.46±2.04 µg /ml in *C. acida* leaf (Jain *et al.*, 2021) and 58.15% and 70.25% mg AA /ml in *G. carpinifolia* leaf and stem (Adebiyi *et al.*, 2017) respectively.

***In vitro* antiurolithiatic activity**

Nucleation assay

The crystallization is initiated due to urine supersaturation as their concentrations become greater than the thermodynamic solubility for those stone materials (Worcester and Coe, 2008). This condition leads to the process of nucleation where stone-forming salts in a supersaturated solution are linked with microscopic particles. The stone formation may also occur by the irregular urinary composition that produces unequalness between promoters and inhibitors in the kidneys (Chinnala *et al*., 2013).

The effect of inhibition of nucleation activity of *S. articularis* ethanol leaf extract at different concentrations is presented in Figure 2. Data represents that % inhibition of calcium oxalate crystal formation after 30 min of

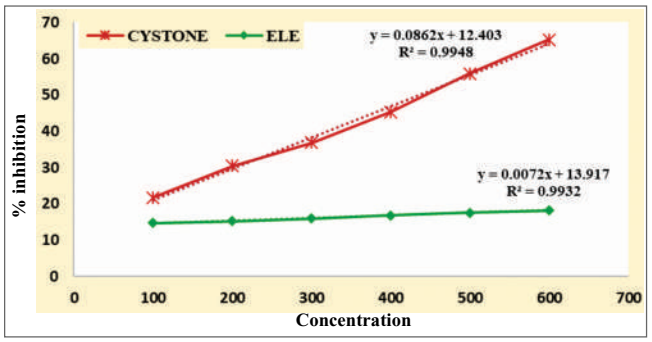


Figure 2: Effect of different concentrations of ethanol leaf extract of *S. articularis* L.f. on CaOx crystal nucleation

incubation, was directly proportional to the increase in the concentration of the plant extract, with minimum inhibition of 14.77±0.001% at 100 µg/ml to a maximum inhibition of 18.2±0.001% at 600 µg/ml. Cystone showed the highest inhibition of 65.22±0.003% at 600 µg/ml. The percentage inhibition in the presence of *S. articularis* ethanol leaf extract was observed and compared with the control and cystone. The presence of the herb extract reduced the turbidity of the solution compared to the control, showing that oxalate crystallization was less in the presence of the extract.

From Figure 3, it is observed that in the light microscopic images of A (control) where the ethanol leaf extract was not added, the number of crystals was maximum. Images C, D, E, F, G, and H which represent the plant extract showed minimum inhibition effect at different concentrations from 100 µg/ml till 600 µg/ml when compared with image B of cystone at 600 µg/ml. This indicated that there is a dose-dependent manner of crystals formed after 30 min of incubation. In the crystal growth experiment shown in the nucleation assay, at constant time against different concentrations, in the presence and absence of ethanol leaf extract determined the rate of the growth of crystals.

Hewagama and Hewawasam (2022) in their previous study conducted on *in vitro* antiurolithiatic activity, reported the maximum percentage of inhibition of whole plant hexane extract of *Aegle marmelos* at the concentration of 1000 µg/ml (83.56±0.06%) and the ethanol extract of *A. marmelos* at 1000 µg/ml also showed a significant percentage inhibition of (82.74±0.68%). In the *Daucus carota* ethanol root extract at 1000 µg/ml, a percent decrease in nucleation was observed in the reaction mixture of 56.1±1.55% which was significantly higher than that produced by cystone of 41.67±1.03% (Bawari *et al.*, 2018). Ethanolic extract of *Sterculia urens* leaves showed a significant percentage inhibition of 35% at 600 µg/ml which was significantly higher than that produced by cystone of 27% at 600 µg/ml (Swarnalatha, 2022). *In vitro* antiurolithiatic activity of *Aerva lanata* leaves ethanol extract showed 87.93% of inhibition at 500 µg/ml which was compared with cystone of 90.64±1.05 at 500 µg/ml

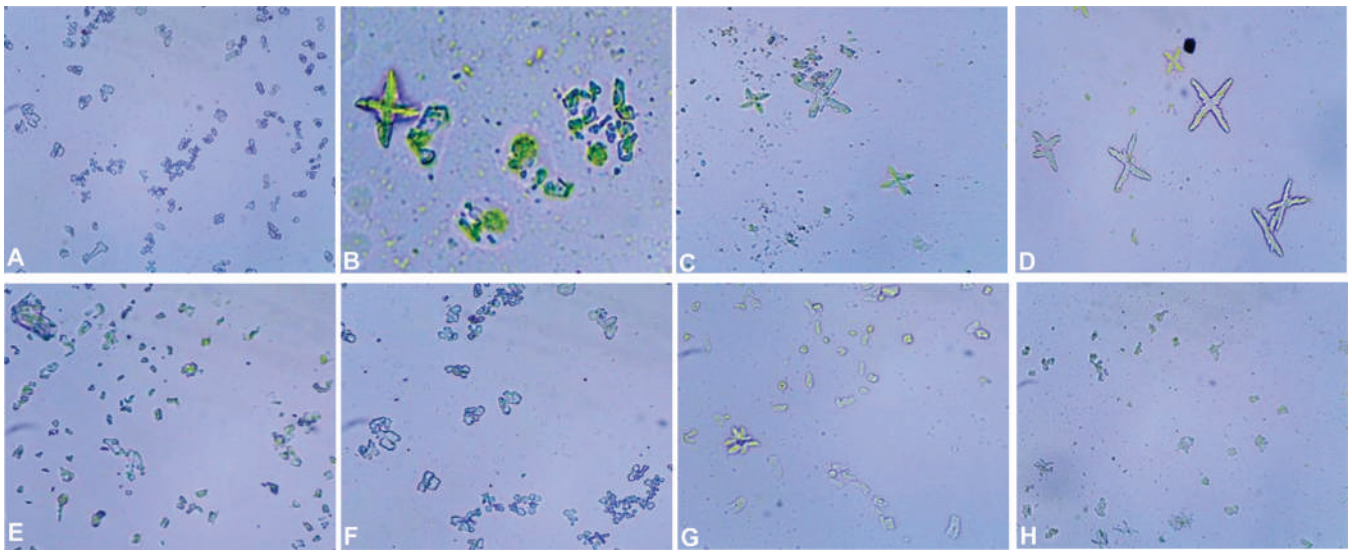


Figure 3: Micrograph of *S. articularis* L.f. in the presence and absence of ethanol leaf extract. A-Control, B-Cystone, C to H represents *S. articularis* L.f. ethanol leaf extract at different (100, 200, 300, 400, 500 and 600 µg/ml) concentrations

(Vishnupriya and Tamilselvi, 2022). The findings of Solomon *et al*. (2019) and our results were comparable where % inhibition of the leaf ethanol extract of *Maerua angolensis* was found to be 22 mg cystone/ml at the highest concentration of 30 mg/ml.

GC-MS analysis

The best method for identifying the bioactive components, such as long-chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids, amino, and nitro compounds found in different plant species, is GC-MS. As a result, the detection methods associated with gas chromatography (GC) and mass spectrometry (MS) have evolved into sophisticated techniques for analyzing different chemicals of medicinal importance. The compounds with their peak area (%), retention time (RT), molecular formula, molecular weight, and structure were presented in Table 4 and Figure 4. The ethanol leaf extract of *S. articularis* revealed 43 different

Table 4: GC – MS spectral analysis of ethanol leaf extract of *S. articularis* L.f.

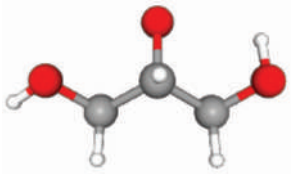
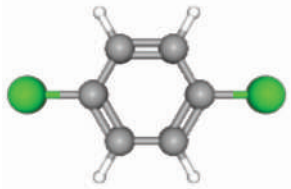
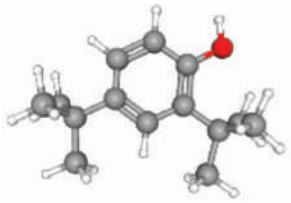
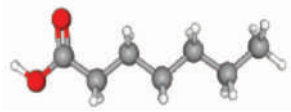
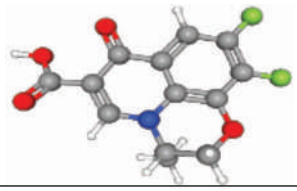
S.No.	Rt (Min)	Peak area (%)	Name of the compound	Molecular formula	Molecular wt. G/Mol	Structure of the compound
1	4.365	1.63	Glycerin	$C_3H_8O_3$	92.09	
2	4.820	1.25	Benzene, 1,4-Dichloro-	$C_6H_4Cl_2$	147.00	
3	9.664	0.17	Phenol, 2,4-Bis (1,1-Dimethylethyl)	$C_{14}H_{22}O$	206.32	
4	10.097	0.43	Dodecanoic Acid	$C_{12}H_{24}O_2$	200.32	
5	10.342	0.23	Undecanoic Acid, Ethyl Ester	$C_{13}H_{26}O_2$	214.34	
6	10.686	0.96	Heptanoic Acid	$C_7H_{14}O_2$	130.18	
7	10.930	0.19	Ofloxacin Q acid	$C_{13}H_9F_2NO_4$	281.21	

Table 4 contd...

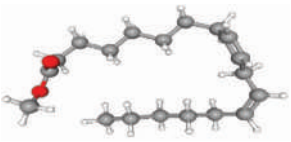
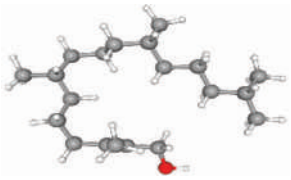
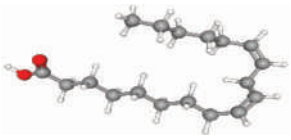
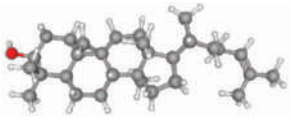
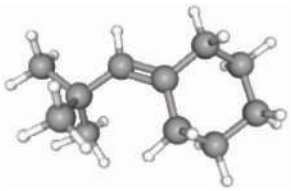
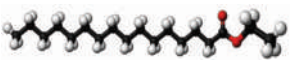
S.No.	Rt (Min)	Peak area (%)	Name of the compound	Molecular formula	Molecular wt. G/Mol	Structure of the compound
8	11.641	0.30	Tridecanoic acid	$C_{13}H_{26}O_2$	214.34	
9	11.841	0.30	Octadecanoic Acid, Ethyl Ester	$C_{13}H_{26}O_2$	214.34	
10	12.208	0.35	2-Tetradecanone	$C_{14}H_{28}O$	212.37	
11	12.764	0.32	Methyl Hexadecanoate	$C_{17}H_{34}O_2$	270.5	
12	13.075	17.39	Hexadecanoic Acid	$C_{16}H_{32}O_2$	256.42	
13	13.219	6.90	Undecanoic Acid, Ethyl Ester	$C_{13}H_{26}O_2$	214.34	
14	13.874	1.35	Methyl linoleate	$C_{19}H_{34}O_2$	294.5	
15	13.974	0.73	Phytol	$C_{20}H_{40}O$	296.5	
16	14.197	33.28	Linoleic Acid	$C_{18}H_{32}O_2$	280.4	
17	14.463	2.68	Ethyl Decanoate	$C_{12}H_{24}O_2$	200.32	
18	15.008	2.65	Cycloartenol	$C_{30}H_{50}O$	426.7	
19	15.419	2.71	Neopentylidene cyclo hexane	$C_{11}H_{20}$	152.28	
20	15.608	1.22	Ethyl Palmitate	$C_{18}H_{36}O_2$	284.5	

Table 4 contd...

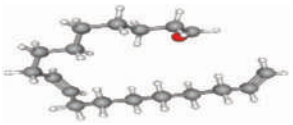
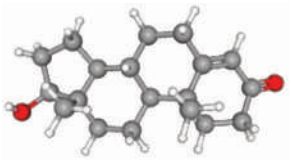
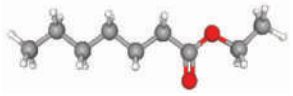
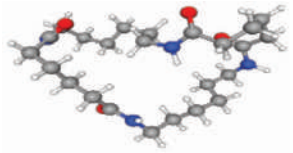
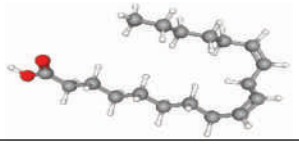
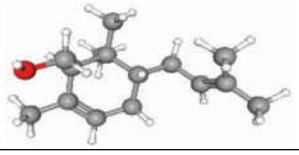
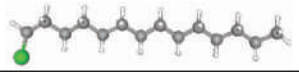
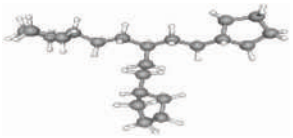
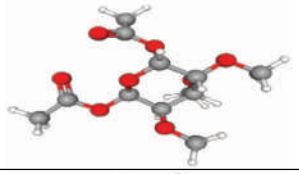
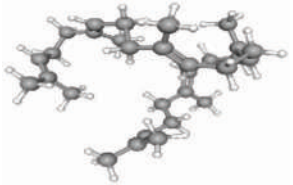
S.No.	Rt (Min)	Peak area (%)	Name of the compound	Molecular formula	Molecular wt. G/Mol	Structure of the compound
21	15.763	0.59	9,17-Octadecadienal, (Z)-	$C_{18}H_{32}O$	264.4	
22	16.052	0.94	Testosterone	$C_{19}H_{28}O_2$	288.4	
23	16.152	0.26	Ethyl Heptanoate	$C_9H_{18}O_2$	158.24	
24	16.285	0.55	1,8,15,22-Tetraaza-2,7,16,21-Cyclooctacosanetetrone	$C_{24}H_{44}N_4O_4$	452.6	
25	16.385	0.17	(S) (+)-Z-13-Methyl-11-Pentadecen-1-Ol Acetate	$C_{18}H_{34}O_2$	282.5	
26	16.463	0.46	Linoleic Acid	$C_{18}H_{32}O_2$	280.4	
27	16.963	0.55	2,4,4-Trimethyl-3-Hydroxy-methyl-5a-(3-Methyl-But-2-Enyl)-Cyclohexene	$C_{15}H_{26}O$	222.37	
28	17.207	1.16	1-Chlorotetradecane	$C_{14}H_{29}Cl$	232.83	
29	304.6	0.63	1,5-Dicyclopentyl-3-(2-Cyclopentylethyl) Pentane	$C_{22}H_{40}$	304.6	
30	17.207	17.718	D-Xylopyranose, 5-C-(Acetyloxy)-2,3,4-Tri-O-Methyl-, Acetate	$C_{12}H_{20}O_8$	292.28	
31	17.852	1.32	Squalene	$C_{30}H_{50}$	410.7	

Table 4 contd...

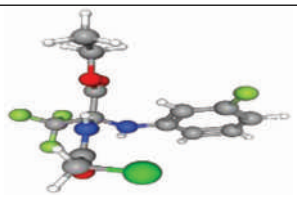
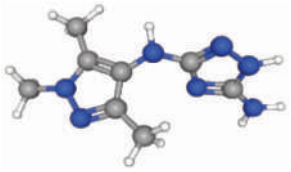
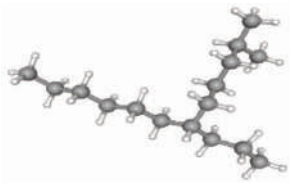
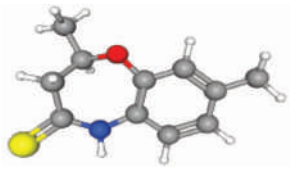
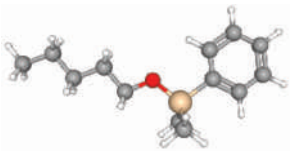
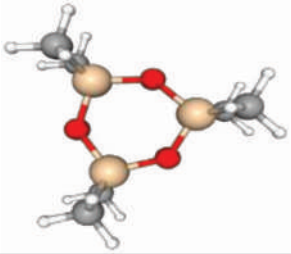
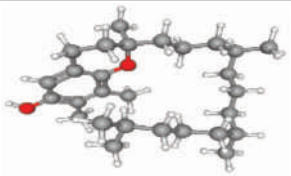
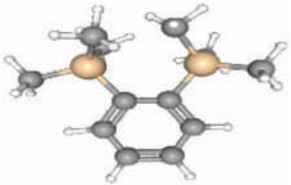
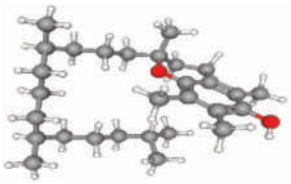
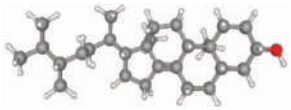
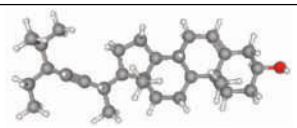
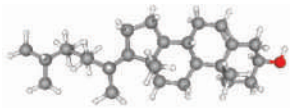
S.No.	Rt (Min)	Peak area (%)	Name of the compound	Molecular formula	Molecular wt. G/Mol	Structure of the compound
32	18.085	0.35	Ethyl 2-(2-Chloroacetamido)-3,3,3-Trifluoro-2-(3-Fluoroanilino) Propionate	$C_{13}H_{13}ClF_4N_2O_3$	356.70	
33	18.207	0.26	1,2,4-Triazol-3-Amine, 5-(1,3,5-Trimethyl-4-Pyrazolyl) Amino	$C_8H_{13}N_7$	207.24	
34	18.341	0.27	Dodecane, 2-Methyl-6-Propyl	$C_{16}H_{34}$	226.44	
35	18.518	0.40	Benz[B]-1,4-Oxazepine-4(5H)-Thione, 2,3-Dihydro-2,8-Dimethyl	$C_{11}H_{13}NOS$	207.29	
36	18.729	0.27	1-Dimethyl (Phenyl) Silyloxypentane	$C_{13}H_{22}OSi$	222.40	
37	19.140	0.25	Hexamethyl cyclotrisiloxane	$C_6H_{18}O_3Si_3$	222.46	
38	19.385	0.23	Gamma -Tocopherol	$C_{28}H_{48}O_2$	416.7	
39	19.829	0.29	1,2-Bis (Trimethylsilyl) Benzene	$C_{12}H_{22}Si_2$	222.47	

Table 4 contd...

S.No.	Rt (Min)	Peak area (%)	Name of the compound	Molecular formula	Molecular wt. G/Mol	Structure of the compound
40	20.207	0.59	Vitamin E	$C_{29}H_{50}O_2$	430.7	
41	21.362	0.46	Alpha - Ergostenol	$C_{28}H_{48}O$	400.7	
42	21.640	0.59	Stigmasterol	$C_{29}H_{48}O$	412.7	
43	22.385	0.93	Campesterol	$C_{28}H_{48}O$	400.7	

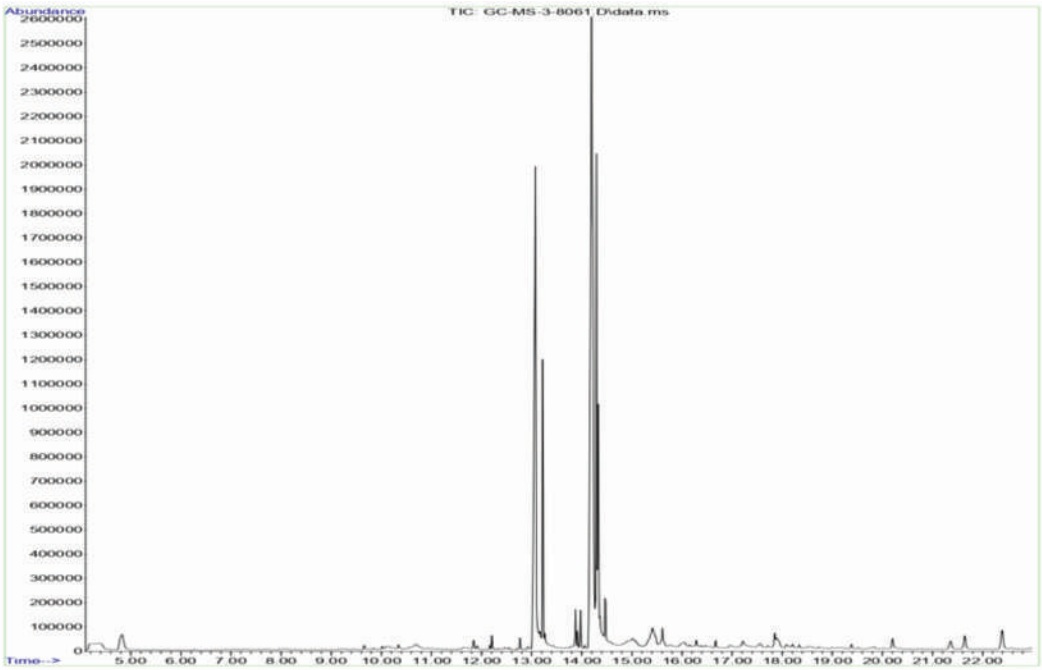


Figure 4: GC-MS Chromatogram of ethanol leaf extract of *Spermacoce articularis* L.f.

compounds and based on the maximum peak area (%) and retention time 12 compounds such as Linoleic acid (33.28%, RT-14.197), Hexadecanoic acid (17.39%, RT-13.075), D-xylopyranose, 5-C-(acetyloxy)-2,3,4-Tri-O-methyl-, acetate (17.718%, RT-17.207), undecanoic acid, ethyl ester (6.90%, RT-13.219), ethyl decanoate (2.68%, RT-14.463), cycloartenol (2.65%, RT-15.008), neopentylidene cyclo hexane (2.71%, RT-15.419), glycerin (1.63%, RT-4.365), 9,12-octadecadienoic acid (Z, Z)-, methyl ester (1.35%, RT-13.874), ethyl palmitate (1.22%, RT-15.608), 1-chlorotetradecane (1.16%, RT-17.207), squalene (1.32%, RT-17.852) were identified.

Among them, linoleic acid and 9,12-octadecadienoic acid belonged to omega-6 fatty acids, hexadecanoic acid, and ethyl palmitate belonged to long-chain fatty acids, undecanoic acid, ethyl ester and ethyl decanoate belonged to fatty acid ester, cycloartenol, and squalene belonged to triterpene, neopentylidene cyclohexane belonged to alkanes and glycerin belonged to the class alcohol.

The results are in accordance with the study of Soosairaj *et al.* (2013) who reported the GC-MS analysis of *Spermacoce articularis* whole plant ethanol extract showed 25 compounds in which the major compounds with the highest peak area were 2-Benzylidene- 3- oxo4-(octlsulfany1)-2,3- dihydrothiophene-1 dioxide (27.71), tridecanoic acid [CAS] (17.46), 3,7- dimethyl- 3- hydroxy-4-isopropyl-6- octadiene (6.73), octadecanoic acid (CAS) (5.60), methyl-threo-9, 10-dichloro- octadecanoate.

Vadivel and Gopalakrishnan (2011) reported the presence of four major phytochemical constituents, such as (-)-quinic acid (32.87%), 4-((1E)-3-hydroxy-1-propenyl)-2-methoxy phenol (8.30%), 1,2,3-benzenetriol (7.70%) and naphthalene, decahydro-2 -methoxy-(7.20 %) in *Mussaenda frondosa* whole plant ethanol extract.

Similarly, the studies of Senthil *et al.,* 2016 revealed the presence of 20 compounds in the ethanol leaf extract of *Ipomoea sepiaria* such as 1-dodecene; 1-hexadecanol; 1-hexadecene; 2-hexadecanol; dibutyl phthalate; 9-hexacosene; 3,7,11,15- Tetramethyl-2-hexadecen-1-ol; cyclononasiloxane, octadecamethyl-; 1- monolinoleoyl glycerol trimethylsilyl ether; octasiloxane, 1,1,3,3,5,5,7,7,9, 9,11,11,13, 13,15,15- hexadecamethyl-; cyclodecasiloxane, eicosamethyl-; hexasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11-dodecamethyl-; 13-docosenamide, (Z)-; squalene; octadecane, 3-ethyl-5- (2-ethylbutyl)-; glycine, N-[(3a,5a, 7a,12a)-24-oxo-3,7,12- tris[(trimethylsilyl)oxy] cholan- 24-yl]-, methyl ester; campesterol; stigmasterol; hexatriacontane; c-sitosterol by GC-MS analysis.

CONCLUSION

Numerous contemporary medicines are also extracted from natural sources. Due to their novel uses, plant-derived pharmaceutical substances have recently emerged as excellent sources. Therapeutic plants are a hotspot for a mixture of various medications. There is a growing awareness of correlating the active principles of medicinal plants with their biological activities. Today, the antioxidant properties of plants have become an area of vast interest due to their therapeutic use as natural supplements alternative to synthetic ones. The results obtained in the present study showed that the ethanol leaf extract of *Spermacoce articularis* L.f. exhibited a significant phytochemical constituent and contained the maximum antioxidant compound which can scavenge different reactive oxygen species (ROS) and free radicals under *in vitro* conditions. Thus, the present study suggests that *Spermacoce articularis* L.f. can be used as a good source of natural antioxidants for health benefits and further pharmacological evaluation of isolated compounds may be carried out to develop new drugs for well-being.

Conflict of interest

The authors declare no conflict of interest

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Original Article

Evaluation of Antiurolithiatic Potential of Methanolic Stem Extract of *Spermacocce articularis* L.f.: An *In vitro* and *In vivo* Approach

S Gopika, MK Nisha*, E Gaayathiri Devi, A Raja Rajeswari, R Vasandhlakshmi

ABSTRACT

Context: Polygenic urolithiasis has a complicated etiology and even more varied therapeutic outcomes. *Spermacoce articularis* L.f. has been used historically for stone treatments in several traditional medical systems. **Aim:** The current study aimed to investigate the *in vitro* and *in vivo* anti-urolithiatic potential of *Spermacoce articularis* Stem Extract (SASE). **Methods:** *In vitro* antiurolithiatic potential on the CaOx crystallization was evaluated using nucleation and aggregation assays. *In vivo*, activity was assessed on renal calculi-induced Wistar rats by polyethylene glycol (0.75%) in drinking water for 14 days. SASE and cystone with two experimental doses (250 and 500 mg/kg, p.o.) were dispensed for ten days. Various biochemical parameters were assessed in the kidneys' serum, urine, and histological sections. In addition, SASE inhibited CaOx crystallization by reducing the density of crystals, triggering the breakdown of CaOx crystals, and hindering their growth. Cystone demonstrated comparable outcomes. **Results:** Upon treatment with SASE, urinary, serum, kidney homogenates, and antioxidants were significantly improved ($p<0.05$) to normal levels. The histopathology of the kidney section showed no damaged cells of SASE treated and Cystone treated compared with that of control animals. **Conclusion:** This research validates the traditional idea and suggests that SASE is advantageous in preventing the growth of urinary stones.
Keywords: Calcium oxalate, Polyethylene glycol, *Spermacoce articularis*, Urolithiasis, *In vivo.*

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INTRODUCTION

Urolithiasis or urinary stone formation in the urinary system is an extremely painful condition that has plagued people since ancient times.This multifactorial disorder is mainly caused by the abnormality in metabolism and urinary tract, urinary infection is a combination of genetic, metabolic, and epidemiological risk factors[1].Low consumption of fruits, vegetables, and fluids, as well as high intake of animal proteins, salt, and sweetened beverages, have a significant impact on urolithiasis incidence.An abnormal rise in the excretion of compounds such as calcium, cystine, oxalate, phosphate, uric acid, urate, and xanthine, or a decrease in urinary level causes stone formation.[2] The primary inhibitors of stone formation in the urinary tract are magnesium and citrate, and stone formation results from low or absent concentrations of these inhibitors in the urine.[3]

According to reports, calcium oxalate and calcium phosphate makeup 80% of renal stones, with struvite-containing magnesium ammonium phosphate accounting for 10%.[4]Urolithiasis has been managed with a variety of treatment approaches and their combinations, including diet, diuretics, probiotic therapy, expulsion therapy, and chelating agents.[5]Current-day medical expenses are either higher or accompanied by adverse effects. The healthcare system may incur significant expenses and face severe problems when intervention treatments are utilized for therapy. Hence, in many countries, including India, phytotherapeutic agents are widely used as complementary and alternative medicines for the treatment of urolithiasis. The majority of Ayurvedic treatments were derived from plants and have been proven effective.These plants are a cheap source of drugs, widely available, affordable, and thought to be quite safe with few to no adverse effects. In Ayurveda, *S. articularis* L.f. which belongs to the family, Rubiaceae, is known as "Madanaghanti" and in Tamil "Nathaisuri"possesses various pharmacological properties, that cure inflammation of gums, and spleen complaints, used as an astringent in hemorrhoids and gallstones, demulcent in diarrhea and dysentery, and decoction of herb in the treatment of headaches.[6] Although *S. articularis* is used traditionally there is no report on the antiurolithiatic potential. Thus, the present objective was to investigate and validate the Antiurolithiatic property of *S. articularis* extract against calcium oxalate crystallization and PEG-induced kidney calculi rat model.

MATERIALS AND METHODS

Plant material and extraction

The fresh stem of *Spermacoce articularis* L.f. was collected from Attappady, Kerala. The plant samples were identified and authenticated taxonomically by the Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamil Nadu, India, with Voucher No: BSI/SRC/5/232022/Tech. The fresh stem was cleaned, chopped, and shade-dried for about ten days at room temperature and powdered. In the Soxhlet apparatus, the refined stem samples (10g), were packed tightly in Whatman No. 1 filter paper for extraction with methanol solvent (100ml). The methanol-extracted fractions were concentrated to a crude using a rotary evaporator under reduced pressure at 37°C and the extract was used for *in vitro* and *in vivo* studies.

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Evaluation of *In-vitro* antiurolithiatic activity

Nucleation assay

The effect of stem extract on calcium oxalate (CaOx) crystal development was examined using nucleation assay.Buffer containing Tris-HCl of 0.05 mol/l and sodium chloride (NaCl) of 0.15 mol/l was prepared and maintained at pH 6.5. 5 mmol/l of calcium chloride ($CaCl_2$) and 7.5 mmol/l of sodium oxalate ($Na_2C_2O_4$) solution was prepared using the buffer. Different concentrations (100, 200, 300, 400, 500, and 600 µl) of SASE were mixed with 3 ml calcium chloride and sodium oxalate solution each and were kept in incubation at 37°C for 30 min. The absorbance (OD) of the mixture was observed (620 nm). The percentage inhibition (%) of nucleation by SASE was obtained using the formula and compared to the standard cystone.[7]

$$\% \text{ Inhibition} = (1 - OD_{Sample} / OD_{Control}) \times 100$$

Aggregation assay

Dissolution of the aggregated CaOx crystal by the stem extract was analyzed using the standard procedure[8], with a minor modification. Calcium chloride and sodium oxalate were combined to get CaOx monohydrate crystals at 50 mmol/l. Compensated the solution in a water bath at 60°C for 1 hour, and cooled to 37°C all night. Extracted the crystals using centrifugation and evaporated at 37°C. CaOx crystals were employed at a final concentration of 0.8mg/ml, buffered with 0.05mol/l Tris and 0.15mol/l NaCl at pH 6.5. Experiments were carried out at 37°C in the presence or absence of a sample. The inhibitory percentage of aggregation (Ir) was then estimated by calculating the turbidity of the sample to that acquired in the control using the formula

Ir = (1- Sample turbidity /Control turbidity) x 100

Microscopic evaluation

After which, a few drops of the mixture from different concentrations were placed in different slides and the reduction of crystal size and morphology of CaOx crystals formed with the presence and absence of *S.articularis* stem methanol extract was determined using a trinocular microscope at 1000 x magnification.

Evaluation of *in vivo* anti-urolithiatic activity

Experimental Animals

Thirty healthy adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing 150–200 g were acquired from the animal house of Biogene lab, Bangalore. Rats were housed under controlled environmental conditions (Temperature 28±2°C; 12:12 h light: dark cycle; 50±10% humidity)in plastic cages with filter tops. All rats were fed with commercial rat pellets (SAI Animal Feed Ltd, Bangalore) and were given water ad libitum. Before experimental work, the rats were acclimated in the animal house for 10 days.

The experimental protocol was approved 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).

Acute toxicity assay

The acute oral toxicity study was carried out as per Organization for Economic Co-operation and Development (OECD) Guidelines 425. One animal is utilised in a step by step process. The stem methanol extract was administered at the dose level of 2000 mg/kg and observed mortality after 24 hr. One-tenth of the median lethal dose (LD_{50})was taken as an effective dose.[9]

Ethylene *glycol-induced urolithiasis in Wistar albino rats*

Ethylene glycol 0.75% (0.75 ml of ethylene glycol in 100 ml of drinking water) to rats for 28 days for the production of calcium oxalate stone in rats. Such an induced urolithiasis model was used to study the antiurolithiatic activity in male Wistar albino rats. The adult male Wistar albino rats were divided into 5 groups, from Group I to Group V, each with 6 rats,Group 1 was reserved as Normal control, whereas all other groups were induced with urolithiasis (stone).Group II served as lithiatic control and received vehicle 1% tween 80.Group III to V received a daily oral solution of 0.75% ethylene glycol till day 14 days and each group were treated with standard cystone (100 mg/kg), SASE low dose (PEG+L.D 250 mg/kg) and SASE high dose (PEG+H.D 500 mg/kg) respectively from 15 to 28 days. Group IV and V were served as curative treatment groups.

Collection and analysis of urine, blood and serum

After all the treatment, all rats were encased in individual metabolic cages. During urine collection, the animals had no restriction to drinking water. On the 28th-day treatment, the pH of the urine samples (24 hours) was determined using a digital pH meter shortly after the collection, anda drop of strong hydrochloric acid was added before storing the collected urine at 4°C. It was then analyzed for calcium[10], phosphate[11], oxalate[12], urea[13], uric acid[14] and citrate[15] were performed by using standard methods. The hematological parameters like RBC, WBC and Hb percentage were estimated.

Serum samples were obtained by the retro-orbital collection of blood from the eyes of the rats using 5% isoflurane on day 28.A cervical dislocation procedure was used to slaughter rats for histopathological examination. Separated the serum from the blood using a cooling centrifuge at 10,000 rpm for 10 minutes to separate the serum from the blood, and the samples were labelled. The serum was stored in a refrigerator and measured for creatinine and uric acid levels[16].

Measurement of Body and Kidney Weight

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

Determination of antioxidant enzymes and lipid peroxidation

Preparation of tissue homogenate

SASE post-treated liver of the sacrificed rats was isolated, cleansed with normal saline (0.9%), and stored for 12 h for *in vivo* antioxidant studies.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)[17], Catalase (CAT)[18], Glutathione peroxidize (GPX)[19], Reduced glutathione (GSH)[20] and Lipid peroxidation (LPO)[21]liver tissues of all the tested rats were determined. Protein estimation was carried out using Folin-Ciocalteau and Biuret reaction combination[22]. The results of the antioxidants SOD, and CAT were expressed as U/mg protein, and GPX, GSH, and LPO were expressed as µmol/mg protein, µg/mg protein, and nmol/mg protein respectively.

Histopathological analysis of kidneys

On day 29 rats were anesthetized, sacrificed through an incision on their abdomen, and both 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%. Then, the kidney tissue portion was fixed in 10% neutral formalin(pH 7.4) solution. One of the two kidneys that were isolated was immersed in paraffin films using a traditional method, then sliced into 5 µm thin sections by a rotary vertical microtome. A hematoxylin and eosin solution was used to stain the sections to indicate changes in the histopathological analysis and crystal deposition.[16] Hematological parameters like hemoglobin content, total erythrocyte count,and leukocyte count were analyzed in the blood.[23]

Statistical analysis

The results of biochemical paraeters are expressed as mean ± SEM. One-way analysis of variance was used to determine the significance of the urine and blood serum analysis, followed by Dunnett's test. A significance level of P< 0.05 was established for statistical significant.

RESULTS AND DISCUSSION

Evaluation of *In vitro* antiurolithiatic activity

Nucleation assay

The effect of inhibition on the nucleation of calcium oxalate crystals using the extract was determined by a spectrophotometric assay.[24]The inclusion of SASE reduced the turbidity of the solution by disintegrating the oxalate crystallization after 30 minutes of incubation. An increase in the percentage of calcium oxalate crystallization'inhibition was observed with an increase in the content of plant extracts. All the SASE concentrations showed a significant reduction (P<0.01) in the nucleation of CaOx crystals when compared with cystone. The SASE at 600 µg/ml showed the highest inhibition of 67.16±0.002% compared to cystone 65.22 ± 0.003% at 600 µg/ml presented in (Figure 1). The images displayed in (Figure 2A-H)observed under the light microscope exposed a paramount of crystal formation in the negative control, followed by a significant reduction in the sizeand number of crystalsat different concentrations (100 µg/ml-600 µg/ml) of SASE indicating dose-dependent inhibitory effect in par with the standard cystone at 600 µg/ml. It is observed that in the light microscopic image of A (the control) without SASE, the number of crystals was the highest. Figure 2C-H represents the plant extract showing maximum inhibition effect at different concentrations (100 µg/ml-600 µg/ml) when compared with Figure 2B of cystone at 600 µg/ml. This indicated a dose-dependent manner of crystals formed after 30 min of incubation. In the crystal growth experiment shown in the nucleation assay, at the constant time against different concentrations, the presence and absence of SASE determined the rate at which the crystals grow. Nucleation is the spontaneous crystallization of dissolved materials in a supersaturated solution due to a thermodynamically driven phase change. Significant inhibition of CaOx crystal nucleation was seen in the presence of the extract when compared to the presence of cystone. Reduction in the size of the crystals formed in the presence of the extract further exhibited the extract's growth inhibitory capabilities. This indicated that the extract in the CaOx crystallization assay has anti-crystallization activity. The extract may have anti-crystallization properties due to its capacity to form complexes with free calcium and oxalate ions, thereby preventing the formation of CaOx complexes. CaOx crystals should be reduced in size because they tend to spontaneously dissolve in urine.[25,26]

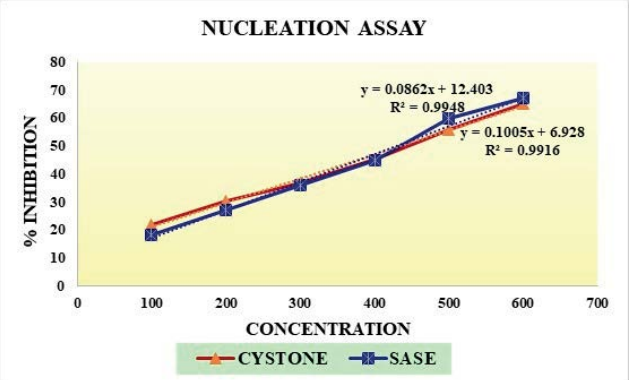

Figure 1: *In vitro* Antiurolithiatic Activity of SASE extract using Nucleation assay. Values are expressed as mean ± S.D.

Aggregation assay

At 600 µg/ml (88.4 ± 0.153%), SASE was found to have a more effective inhibitory effect on the aggregation of CaOx crystals than cystone (77.09 ± 0.007%). A concentration-dependent increase of SASE causes a reduction in CaOx crystal size and increases the inhibition percentage (Figure 3). In the images displayed in (Figure 4A-H)an increase in crystal growth was observed under a light microscope in the negative control, followed by a significant reduction in crystal size and number as SASE concentrations increased (100 µg/ml -600 µg/ml, suggesting a dose-dependent inhibitory effect comparable to cystone at 600µg/ml.

Same as the nucleation assay, in (Figure 4A-H) it is observed that in the light microscopic images of A (the control) without SASE, the number of crystals was the maximum. Figure 4C-H represent the plant extract showing maximum inhibition effect at various concentrations (100 µg/ml-600 µg/ml) when compared with Figure 4 B of cystone at 600 µg/ml. Incubation for 30 minutes resulted in crystals forming in a dose-dependent manner. A constant time against various concentrations of methanol stem extract determined the rate of crystal growth in the aggregation assay experiment. This study also exhibited strong inhibitory effect of SASE on the crystallization of CaOx. These results were in agreement with the previous reports studied in *Spermacoce articularis*[27]*Boldoa purpurascens*[28] *Ocimum bacilicum* seed extracts[29]*Daucus carota.*[30]Urinary supersaturation, which results from alterations in urine chemistry such as hyperoxaluria and hypercalciuria, crystallizes, aggregates, and eventually forms stones.[31]

In vivo Antiurolithiatic Activity

Acute toxicity study

The oral administration of SASE up to a dosage of 2000 mg/kg showed no signs of toxicity in rats, and for 14 days there was no death. Therefore, for the anti-urolithiatic investigation in the present study, the therapeutic dosage was determined to be 250 mg/kg and 500 mg/kg body weight.

Effect of SASE on the urine, blood and serum parameters

On par with the normal control group (Group I), the administration of PEG in the rats with induced urolithiasis (Group II) led to increased renal excretion of phosphate on the 28th day, along with hypercalciuria and hyperoxaluria. Urinary calcium levels (***p<0.05; 13.8±0.551), oxalate (***p<0.05;12±0.374), and phosphorus (p<0.05;7.21±0.242) were improbably elevated in animals induced with stones. However, the levels of calcium (nsp<0.05; 7.76±0.32), oxalate (**p<0.05; 6.67±0.759), and phosphorus (**p<0.05; 5.05 ±0.101) in the animals in the standard drug cystone-treated group were significantly lower. When compared to the disease control group, treatment with SASE (250 mg/kg) did not significantly lower the elevated levels of calcium (***p>0.05; 10.20±0.199) or oxalate (***p<0.05; 8.02±0.208) or phosphorus (p<0.05; 5.47±0.294). However, when SASE (500 mg/kg, p.o) was administered, the levels of calcium, oxalate, and phosphorus (*p<0.05; 8.69 ± 0.214, nsp<0.05; 5.57±0.395, p<0.05 4.37±0.144) in urine were reduced. Urine BUN, creatinine, and uric acid levels

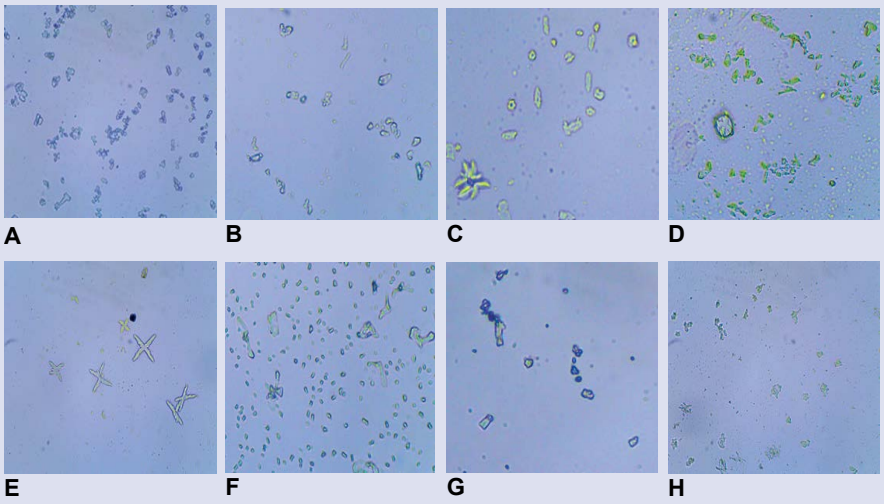

Figure 2: Nucleation Assay - Micrograph of *S. articularis* L.f. in the presence and absence of stem methanol extract. A-Control, B-cystone at 600 µg/ml, C to H represents *S. articularis* L.f. stem methanol extract at different (100, 200, 300, 400, 500, and 600 µg/ml) concentrations.

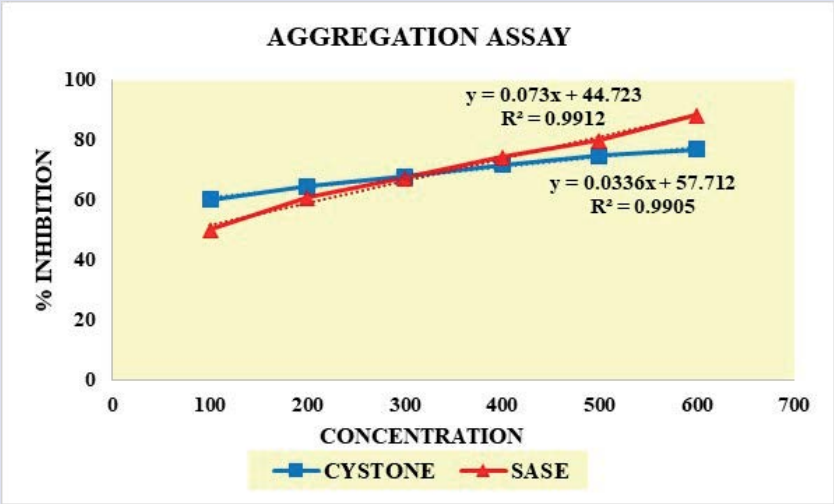

Figure 3: *In vitro* Antiurolithiatic Activity of SASE extract using Aggregation assay. Values are expressed as the mean ± S.D.

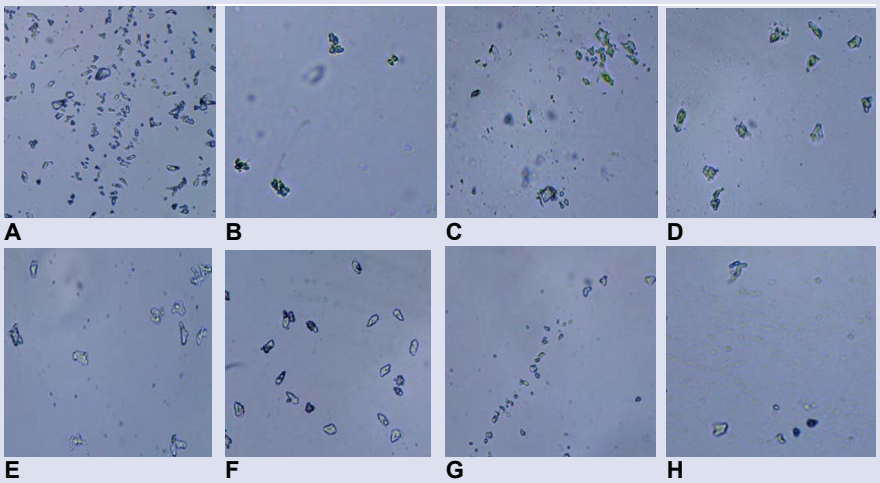

Figure 4: Aggregation Assay - Micrograph of *S. articularis* L.f. in the presence and absence of stem methanol extract. A-Control, B-cystone at 600 µg/ml, C to H represents *S. articularis* L.f. stem methanol extract at different (100, 200, 300, 400, 500 and 600 µg/ml) concentrations.

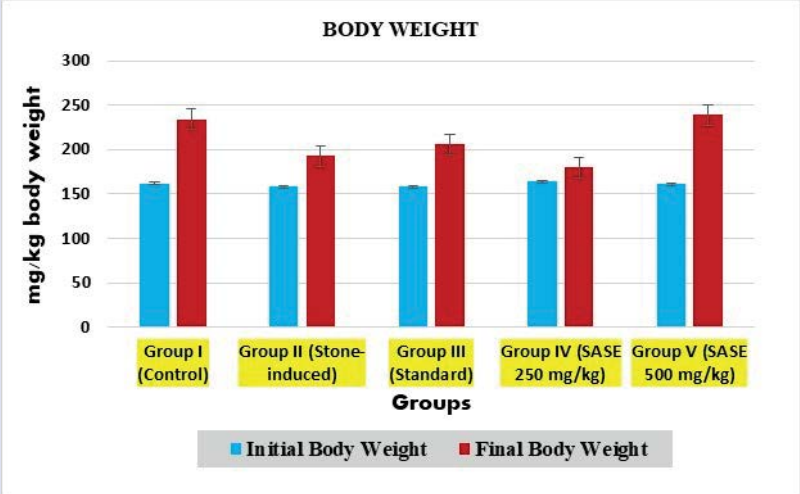

Figure 5: Body weight of control and treatment groups. Values are expressed as the mean ± S.D. Statistical significance (p) was calculated by one-way ANOVA followed by Dunnett's. ns- not significant **P< 0.05 calculated by comparing the treated group with the control group. n = 6 in each group.

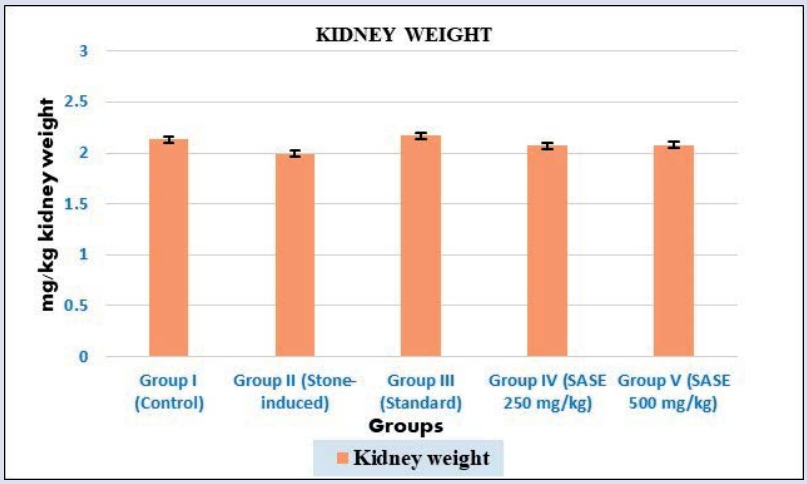

Figure 6: Kidney weight of control and treatment groups. Values are expressed as the mean ± S.D. Statistical significance (p) was calculated by one-way ANOVA followed by Dunnett's. ns- not significant **P< 0.05 calculated by comparing the treated group with the control group. n = 6 in each group.

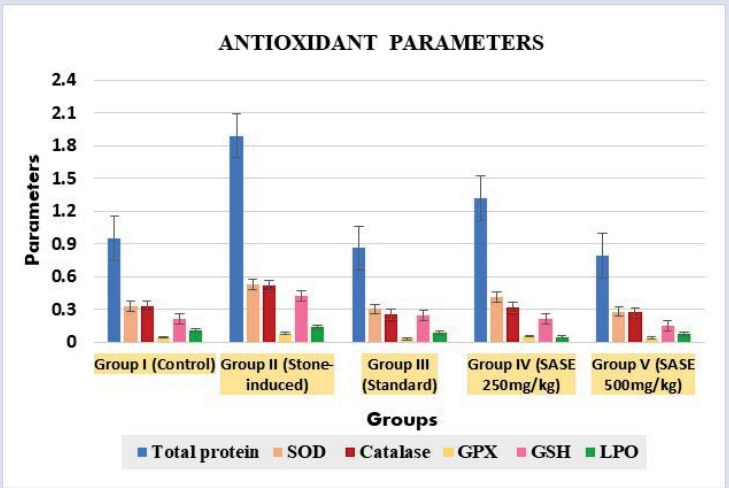

Figure 7: Effect of SASE on Antioxidant Parameters. Values are expressed as the mean ± S.D. Statistical significance (p) was calculated by one-way ANOVA followed by Dunnett's. ns- not significant *P<0.05, **P< 0.05, ***P<0.05 calculated by comparing treated group with control group. n = 6 in each group.

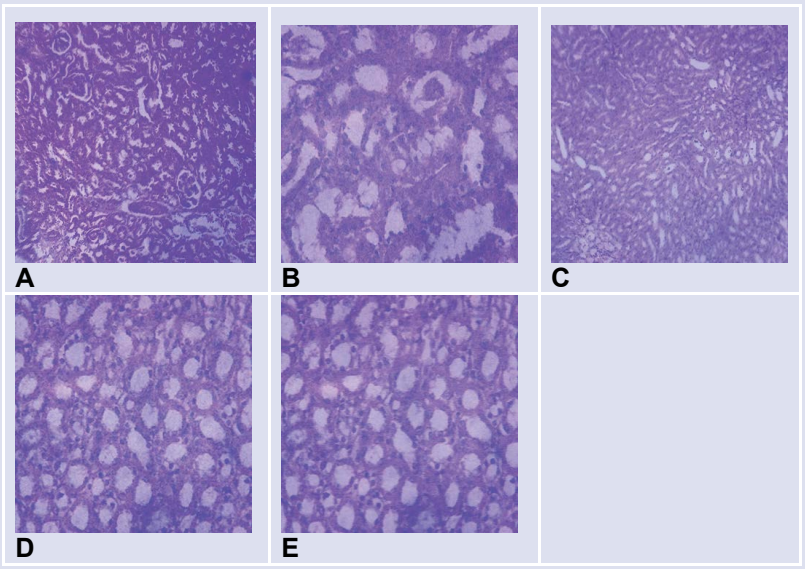

Figure 8: Histopathological analysis of kidney at 400X: A - Group I (Control), B - Group II Poly Ethylene glycol (1%) (Stone Induced), C - Group III cystone (Standard), D - Group IV Methanol stem extract 250 mg/kg body weight and E - Group V Methanol stem extract 500 mg/kg body weight.

Table 1: Effect of *S. articularis* stem methanol extract (SASE) on different parameters of urine analysis in Urolithiasis Male Wistar Rats. Values are expressed as the mean ± S.D. Statistical significance (p) calculated by one way ANOVA followed by dunnett's. ns- not significant, *P<0.05, **P< 0.05, ***P<0.05 calculated by comparing treated group with control group.

©	Urine Analysis (mg/dl)							
	Bun- Blood Urea Nitrogen	Creatinine	Uric acid	Oxalate	Citrate	Calcium	Magnesium	Phosphorus
Group I (Control)	18.4±1.1	1.2±0.346	0.733±0.0882	4.12±0.32	22.6±0.722	7.1±0.453	3.1±0.121	5.15±0.364
Group II (Stone-induced)	103±7.4***	7.87±0.677***	5.47±2.12*	12±0.374***	8.89±0.248***	13.8±0.551***	1.93±0.0924 ns	7.21±0.242
Group III (Standard)	36.5±6.96ns	2.47±0.467ns	2±0.208ns	6.67±0.759**	16.80±0.517***	7.76±0.32 ns	2.78±0.323	5.05±0.101
Group IV (SASE 250mg/kg)	73.3±4.11***	5.47±0.406***	2.57±0.674ns	8.02±0.208***	11.60±0.703***	10.20±0.199***	2.32±0.0577 ns	5.47±0.294
Group V (SASE 500mg/kg)	43.9±3.54*	2.47±0.348ns	1.9±0.436ns	5.57±0.395 ns	17.60±0.419***	8.69±0.214*	2.83±0.026 ns	4.37±0.144

Table 2: Effect of *S.rticularis* stem methanol extract (SASE) on different parameters of serum and haematology in Urolithiasis Male Wistar Rats. Values are expressed as the mean ± S.D. Statistical significance (p) calculated by one way ANOVA followed by dunnett's. ns- not significant *P<0.05, **P< 0.05, ***P<0.05 calculated by comparing treated group with control group.

Group	Serum (mg/dl)			Haematological parameters		
	Bun- Blood Urea Nitrogen	Creatinine	Uric acid	RBC ($X10^6$/µl)	WBC ($X10^3$/µl)	HB (g/dl)
Group I (Control)	54.3±3.48	0.867±0.176	3.1±0.173	6.07±0.172	11.3±0.876	15.6±0.458
Group II (Stone-induced)	153±7.26***	3.6±0.404***	11.7±0.586***	5.45±0.319ns	11.6±0.994	13.7±1.3
Group III (Standard)	58.3±10.4ns	1.07±0.12ns	3±0.361ns	6.16±0.274ns	13.4±0.762	13.2±0.907
Group IV (SASE 250mg/kg)	98.3±11.3*	1.73±0.521ns	7.3±0.608**	4.86±0.0437*	10±0.984	12.6±0.546
Group V (SASE 500mg/kg)	61±12.1ns	1.03±0.393ns	4.93±0.913ns	5.85±0.331ns	12.2±1.27	14.2±0.649

were significantly elevated in the rats given ethylene glycol treatment when compared to Group I and restored by SASE treatment in Group IV & V which was similar to Group III. Comparing group II to the control group (group I), urinary citrate and magnesium excretion was reduced after the administration of ethylene glycol. The levels of citrate and magnesium were nearly returned to normal after supplementing with SASE (250 and 500 mg/kg), which also markedly increased (P < 0.05) this parameter (Table 1). The concentration of BUN (153±7.26), creatinine (3.6±0.404), and uric acid (11.7±0.586) in the serum was highly increased (***p<0.05) in the stone induced group, indicating renal damage (Table 2). However, group V treated with SASE 500 mg/kg significantly reduced the concentrations of BUN, creatinine, and uric acid in contrast to group III and IV.

The addition of ammonium chloride accelerated the stone formation in the rat model of ethylene glycol-induced urolithiasis. There were lower levels of calcium, phosphate, and oxalate in urine excretion in extract-treated groups. Calcium phosphate crystals are formed when urine phosphate and oxalate levels are elevated, which creates the ideal condition for stone formation.[32] Urine that has a higher calcium content is more likely to precipitate and nucleate CaOx, which leads to the formation of crystals. Calcium excretion in urine and increased calcium deposition in the kidney are due to either increased absorption from the intestine or defective renal tubular reabsorption; patients with renal calcium stones were found to have hyper absorptive calcium.[33] Magnesium levels are low in rats that form stones. The production of calcium oxalate crystals in the urine is known to be inhibited by magnesium, because magnesium binds free oxalate and makes it more soluble, thus preventing calcium oxalate crystals from forming in urine. There have been reports that magnesium-containing diet interventions may reduce the supersaturation of lithogenic salts in the urine.[32]Citrate, a significant urolithiasis inhibitor, prevents calcium oxalate and phosphate from precipitating and aggregating by forming a soluble complex with calcium. This investigation supported our results where the administration of SASE and cystone resulted in an increase in citrate concentrate, which may have decreased calcium oxalate crystallization.[34] As compared to normal, the lithiatic group experiences a decrease in renal function due to elevated urine and serum creatinine, BUN, and uric acid levels. All groups showed increased hemoglobin, leukocytes and erythrocyte counts compared to stone-induced groups athematological analysis,after 28 days Table 2. A significant increase was observed to be 14.2±0.649, 12.2±1.27, and 5.85±0.331 respectively in Group V (500 mg/kg) when compared to the stone induced group. Similar result was found in the extract of *Pedalium murex*[23] with erythrocyte count of 6.21±1.02, leucocytes count of 605± 37.3 and haemoglobin amount of 14.1±1.23.

Measurement of Body and Kidney Weight

The mean body and kidney weight of the five experimental groups i.e., control (Group I), stone induced-PEG (Group II), PEG+STD (Group III), SASE-treated PEG+L.D 250 mg/kg (Group IV), PEG+H.D 500 mg/kg (Group V), were recorded practically the same for all the groups before the commencement of treatment (day 1) and the changes observed after 28 days are summarized in (Figure 5 & 6).It showed that animals in cystone and SASE-treated 500 mg/kg had a significant gain ($p<0.05$) in mean body weight than the stone-induced group after 28 days of treatment which coincides with the previous reports.[35,36,37]The results of wet kidney weight were significantly similar in the treated groups concerning normal control cystone-treated group (Group III), and plant extract-treated groups(Group IV and Group V). An increase in kidney weight in the PEG-treated animals (Group II) observed in the present study indicated stone formation. Compared to groups treated with extract, the disease control group's kidney weight was greater as a result of CaOx crystal precipitation.[38]Our results were in par with the previous findings of. [39,40]The cystone and plant extract treated groups exhibited antiurolithiatic activity, which may have prevented stone accumulation, therefore providing pain relief for the animals. This might be the cause of the considerable rise in body weight that results from increasing food consumption. Similar result was exhibited by *S. hispida* methanol extract (100 mg/kg), the rats body weight in normal group was found to be increasing (192.9±4.595) ($P< 0.05$) in contrast to cisplatin treated group (179.3±6.103) ($P <0.05$)[37] and hydroalcoholic extract of *Copaifera langsdorffii* leaf exhibited significant antiurolithiatic activity with the increase in body weight in plant extract-treated groups when compared to control groups.[41]

Effect of extract on antioxidants and lipid peroxidation activity in the experimental rats

The total protein level was significantly ($P<0.05$) increased to 1.89 ± 0.552 mg/dl in the Ethylene glycol-treated group Figure 7. This increased total protein level was considerably decreased after treating withcystone and with SASE based on extract concentration. The 250 and 500 mg/kg extract concentrations of SASE were observed equipotent($P<0.05$) to the standard cystone. For *in vivo* antioxidant activity, Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPS), and Reduced glutathione (GHS) were found to have decreased ($P<0.05$) and to have inclined ($p<0.05$) in the PEG-treated lithiasis rats (Group II) in contrast to the normal control rats (Group I). Antioxidants levelswere ($p<0.05$) increased and LPO was ($p<0.05$) reduced in the SASE(250 mg/kg) treated rats (Group III),SASE (500 mg/kg) treated rats (Group IV), and the standard cystone treated rats (Group V) when correlated with PEG treated lithiatic rats (Group II). In the experimental models, the 500 mg/kg SASE treatment decreased lipid peroxidation and enhanced antioxidant activities.

Increased LPO and GSH levels in the control group of our study indicated that hyperoxaluria promoted extensive ROS production. The extract has a dose-dependent antioxidant effect, as evidenced by a significant drop in the antioxidant levels of GSH, LPO, and catalase in the SASE-treated Groups IV and V. Renal epithelial cells are toxically exposed to oxalate, which causes lipid peroxidation (LPO) mediated by free radicals. The antioxidant response of SASE was in a dose-dependent manner similar to previous reports of *Musa paradisiaca* pseudo stem.[42]

Histopathological studies

Large spaces, increased tubular dilatation, and abnormal calcium oxalate crystal deposition were observed in the urolithiatic rats exposed to PEG. Less crystal deposition and dilation were seen in the treatment groups, and Group III's cystone treatment showed notable improvements, as shown in Figure 8C. SASE treatment Figure 8D &E with varying doses i.e. (Group IV and V), suggestively lowered the calcium oxalate crystals deposition, renal tubules dilation and interstitial inflammation compared to Group-II. The presence of rosette-like, polycrystalline CaOx crystals indicated that the particles have adhered to and retained in the renal tubules. On the other hand, SASE treatment significantly decreased the CaOx crystal deposits that was showed in urine microscopy and kidney histology.In the normal group, kidney tissues were examined microscopically and no cellular necrosis or inflammatory infiltration was visible in the interstitial space of the kidney tubules. Several inflammatory infiltrations were observed in the renal cortex and renal tubular epithelium of rats injected with PEG. Alternatively, SASE treatment reduced the large range of infiltrates. A dose of SASE treatment was shown to protect the renal tubular necrosis score (Group IV and V) compared to the PEG treated Group II.In the present study supplementation with SASE and cystone, restored oxalate and calcium levels in urine in both curative as well as preventive regimens as compared to untreated animals. Test findings revealed that SASE and standard cystone markedly reduced the stone size and crystalluria which exhibits considerable antiurolithiatic activity. PEG-treated rats for 28 days excreted abundant and aggregated CaOx crystals stones in their urine. According to the results, the SASE reduced the aggregation and thereby prevented the formation of stones, or broke down the calcium particles into subtle constituents that were not visible. Through excreting a smaller amount of debris from the kidney and decreasing the possibility of them being deposited in the urinary tract, this effect may help prevent the formation of urinary stones.[43,44]

CONCLUSION

Although advances in medical research have been made in recent decades, urolithiasis recurrence remains a severe issue for many

patients, prompting them to look for treatment alternatives, such as herbal therapies. The present results of this study, clearly demonstrated that *S. articularis* stem extract has the *in vitro* antiurolithiatic potential against CaOx stones. Furthermore, the extract was administered to reduce the progression of the disease in rats in urolithiasis model. Therefore, more research should be performed using SASE to identify the constituents exhibiting anti-urolithiatic activity in order to discover potential lead molecules for future studies into drug formulation.

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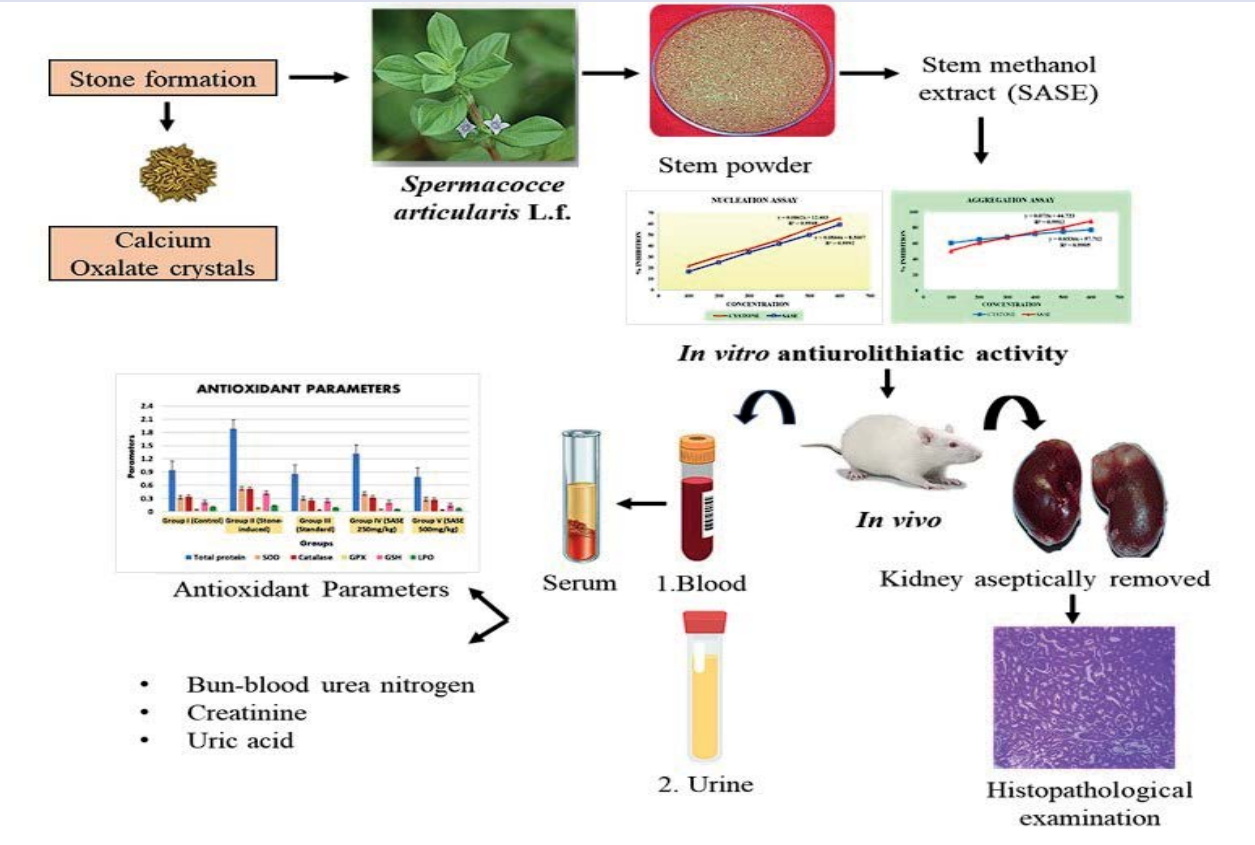
GRAPHICAL ABSTRACT



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