METHODOLOGY

4.1 PHYSICOCHEMICAL INVESTIGATION

4.1.1 Materials and methods

Solvents:

Light petroleum ether (40-60 ^{0}C), chloroform, methanol of AR grade solvents and distilled water were used.

Chemicals and Reagents:

Laboratory grade chemicals were used for routine work. Analytical grade reagents were used for analytical work.

4.1.2 Collection and authentication

In the present study, the whole plant of *Biophytum sensitivum* (Linn.) DC. and bark of *Syzygium cumini* (Linn.) Skeels were collected from the local areas of Goa and also Belgaum, Karnataka. The plants were authenticated by Dr. Harsha Hegde, Research Officer, Regional Medical Research Centre, Indian Council for Medical Research, Belgaum.

The plants of *Biophytum sensitivum* and bark of *Syzygium cumini* were then washed with water to remove physical impurities like soil and dirt, dried at room temperature and subjected to botanical evaluation with different parameters. The parameters that were used for evaluation were nature, odour, colour, taste, size, shape, width, and length.

4.1.3 Botanical and powder evaluation

Botanical evaluation of whole plant of *Biophytum sensitivum* (Linn.) DC:

Botanical name : *Biophytum sensitivum* (Linn.) DC.

Family : Oxalidaceae

Part of the plant used : Whole plant

The results of the examination of powdered drug of whole plant of *Biophytum sensitivum* (L.) DC. are given in table no.9.

Botanical evaluation of bark of *Syzygium cumini* (Linn.) Skeels:

Botanical name : *Syzygium cumini* (Linn.) Skeels

Family : Myrtaceae

Part of the plant used : Bark.

The results of the examination of powdered drug of the bark of *Syzygium cumini* (L.) Skeels are given in table no.10.

4.1.4 Physical evaluation

After botanical evaluation, the dried plants were subjected to size reduction with a crusher to get coarse powder and then passed through sieve no. 40 to get uniform powder. Then, the powder was subjected to standardization with different parameters as per literature.

4.1.4.1 Extractive Values:

a) **Determination of methanol-soluble extractive value**:

Procedure: About 5 g of the powdered drug was weighed in a weighing bottle and transferred to a dry 250 ml conical flask. A 100 ml graduated flask was filled to the delivery mark with methanol. The weighing bottle was washed and the washings were poured out together with the remainder of the solvent into the conical flask. The flask was corked and set aside for 24 h, shaking frequently (Maceration) and filtered into a 50 ml cylinder. When sufficient filtrate had been collected, 25 ml of the filtrate was transferred to a weighed, thin porcelain dish, as used for the ash values determinations. It was then evaporated to dryness on a water-bath and the drying was then completed in an oven at 100 ^{0}C. Further it was cooled in a desiccator and

weighed. The percentage w/w of extractive with reference to the air-dried drug was calculated.

Calculation:

25 ml of methanolic extract gives	=	‘x’ g of residue
100 ml of methanolic extract gives	=	4x g of residue
5 g of air-dried drug gives	=	4x g of methanolic Soluble residue
100 g of air-dried drug gives	=	80x g of methanolic Soluble residue
Methanol-soluble Extractive Value of the sample	=	80x %

b) Determination of Water-soluble extractive value and

c) Chloroform soluble extractive value:

Procedure: The procedure for determination of water and chloroform soluble extractive values is same as methanol extractive value, wherein water (few drops of chloroform was used as a preservative) and chloroform respectively, was used in place of methanol.

4.1.4.2 Moisture content (loss on drying):

Procedure: About 1.5 g of the powdered drug was taken in porcelain dish and dried in an oven at 105 ^{0}C and allowed to cool in a desiccator. The loss in weight was recorded as moisture content.

Calculation:

Weight of powdered drug + china dish = ‘x’ g

Weight of powdered drug + china dish = ‘y’ g

(After 2 h at 105^0 C in the oven)

Loss in weight = x – y = ‘z’ g

4.1.4.3 Ash Content:

a) Determination of Total Ash Value:

Procedure: A tarred silica crucible was weighed and ignited. About 2 g of the powdered drug was weighed into the crucible. The crucible was supported on a pipe-clay triangle placed on a ring of retort stand. It was heated with a burner, using a flame about 2 cm high and supporting the dish about 7 cm above the flame, it was heated till vapors almost ceased to be evolved; the crucible was lowered and heated more strongly until all the carbon was burnt off and it was allowed to cool in a desiccator. Weight of ash and calculation of the percentage of total ash with reference to the air-dried sample of the crude drug was performed.

Calculation:

Weight of the empty dish = ‘x’ g

Weight of the drug taken = ‘y’ g

Weight of the dish + ash = ‘z’ g

(After complete incineration)

‘y’ g of the crude drug gives = (z - x) g of the ash

100 g of the crude gives = $\frac{100 \times (z - x) \text{ g of the ash}}{y}$

Total ash value of the sample = $\frac{100 \times (z - x)}{y}$ %

b) Determination of Acid-insoluble Ash Value:

Procedure: As per the steps mentioned in the procedure for determination of total ash value of crude drug, the process was proceeded. Further: Using 25 ml. of dilute hydrochloric acid, the ash from the dish used for total ash was washed into 100 ml beaker. A wire gauge over a Bunsen burner was placed and boiled for five minutes. Then it was filtered through an 'ashless' filter paper; the residue was washed twice with hot water. The filter paper and residue were put together into the crucible; heated gently until vapors ceased to evolve and then more strongly until all carbon had been removed. Weighing of the residue and calculation of acid-insoluble ash of the crude drug with reference to the air-dried sample of the crude drug was done.

Calculation:

Weight of the empty crucible = 'x' g

Weight of the drug taken = 'y' g

Weight of the crude drug + ash = 'z' g

(After complete incineration)

Weight of the residue = 'a' g

(Acid-insoluble ash)

'y' g of the air-dried drug gives = 'a' g of acid - insoluble ash

100 g of the air-dried drug gives = $\frac{(100 \times a)}{y}$ g of the acid - insoluble ash

Acid-insoluble ash value of the sample = $\frac{100 \times a}{y}$ %

c) Determination of Water – soluble Ash Value:

Procedure: The Procedure was same as above wherein water was used, in place of dilute hydrochloric acid.

4.1.4.4 <u>Fluorescence analysis of the drug</u>:

Procedure: The piece of the drug or its powder was examined directly under the lamp.[10,218-221]

The results of the Quality Control tests of whole plant of *Biophytum sensitivum* (L.) DC. and bark of *Syzygium cumini* (L.) Skeels are given in table no.11 and table no.12 respectively.

4.1.5 Extraction

The shade dried whole plants of *Biophytum sensitivum* (family: Oxalidaceae) and bark of *Syzygium cumini* (family: Myrtaceae) were reduced to course powder and around 200 g of powdered plant material was subjected to successively hot continuous extraction (Soxhlet extractor) with petroleum ether (only for the purpose of defatting), chloroform and methanol. Each time before extracting with the next solvent, the powdered material was dried in hot air oven below 50 ^{0}C. Finally, the marc was macerated with distilled water and few drops of chloroform was added as a preservative for more than 24 hours to obtain the aqueous extract. Each extract was then distilled to dryness under reduced pressure using Buchi Rota evaporator. The extract obtained with each solvent was weighed and its percentage in terms of the air-dried weight of the plant material was calculated. And also the colour and consistency of the extract was noted.

The results of the successive solvent extraction of air-dried plant material of the whole plant of *Biophytum sensitivum* (L.) DC. and bark of *Syzygium cumini* (L.) Skeels are given in table no.13 and table no.14 respectively.

4.2 HISTOLOGICAL CHARACTERISTICS

Examination of T.S/ Powdered Drugs by Microscopical Examination:

- ✓ The T.S/ powder was cleared with clearing reagent, chloral hydrate.
- ✓ The cleared T.S/ powder was stained with staining reagents, phloroglucinol and concentrated hydrochloric acid (1:1) and stained with iodine solution (for determination of starch)
- ✓ The mount was made free from air bubbles to determine –
  - The type of cells.
  - The nature of cell walls present and
  - Cell contents.[218]

The results of the microscopic and powder characteristics of whole plant of *Biophytum sensitivum* (L.) DC. and bark of *Syzygium cumini* (L.) Skeels are given in figure no.3, 4, 5 and 6 respectively.

4.3 PHYTOCHEMICAL INVESTIGATION

4.3.1 Materials and methods

Chemicals and Reagents:

Laboratory grade chemicals were used for routine work. Analytical grade reagents were used for analytical work.

Adsorbents:

Silica gel GF_{254} (RFCL Ltd., Rankem Production, New Delhi) was used for TLC, Silica gel column (200-400 mesh) was used for column chromatography, Silica gel 60 GF_{254} (E.Merck) was used for HPTLC.

4.3.2 Phytochemical screening

The extracts obtained were then subjected to qualitative chemical examination for the identification of various plant constituents. The various tests and reagents used are given below:

1) TESTS FOR CARBOHYDRATES:

a) **Molish's test (General test):** To 2-3 ml of aqueous extract, few drops of alpha-napthol solution in alcohol was added, shaken and conc. H_2SO_4 was added from sides of the test tube. Violet ring was formed at the junction of two liquids.

b) **Tests for reducing sugars:**

   a. **Fehling's test:** 1 ml Fehling's A and 1ml Fehling's B solutions was mixed, boiled for one minute. Equal volume of test solution was added and heated in boiling water bath for 5-10 min. First a yellow, then brick red precipitate was observed.

   b. **Benedict's test:** Equal volume of Benedict's reagent and test solution in test tube was mixed and heated in boiling water bath for 5 min. Solution appeared green, yellow or red depending on amount of reducing sugar present in test solution.

c) **Tests for Monosaccharides:**

   a. **Barfoed's test:** Equal volume of Barfoed's reagent and test solution was mixed and heated for 1-2 min. in boiling water bath and cooled. Red precipitate was observed.

d) **Test for Pentose sugars:** Pentose are components of certain gums.

a. Equal amount of test solution and HCl was mixed and heated. A crystal of phloroglucinol was added. Red colour appeared.

e) **Test for Hexose sugars:**

a. **Tollen's phloroglucinol test for galactose:** 2.5ml conc. HCl and 4 ml. 0.5% phloroglucinol was mixed. 1-2 ml test solution was added and heated. Yellow to red color appeared.

b. **Cobalt-chloride test:** 3 ml test solution was mixed with 2 ml cobalt chloride. Boiled and cooled. Few drops of NaOH solution was added. Solution appeared greenish blue (glucose) or purplish (fructose) or upper layer greenish blue and lower layer purplish (mixture of glucose and fructose).

f) **Tests for Non-reducing sugars:**

a. Test solution did not give response to Fehling's and Benedict's tests.

b. Hydrolyze test solution. Fehling's and Benedict's tests were positive.

g) **Test for Non-Reducing Polysaccharides (Starch):**

a. **Iodine test:** 3 ml test solution and few drops of dilute Iodine solution was mixed. Blue color appeared; it disappeared on boiling and reappeared on cooling.

b. **Tannic acid test for starch:** With 20% tannic acid, test solution gave precipitate.

2) TESTS FOR PROTEINS:

a) **Biuret test (General test):** To 3 ml test solution, 4% NaOH and few drops of 1% $CuSO_4$ solution was added. Violet or pink color appeared.

b) **Xanthoprotein test (for protein containing tyrosine or tryptophan):** 3 ml test solution was mixed with 1 ml conc. H_2SO_4. White precipitate was formed. It was then boiled. Precipitate turned yellow. NH_4OH was added, precipitate turned orange.

c) **Test for proteins containing sulphur:** 5 ml test solution was mixed with 2 ml 40% NaOH and 2 drops 10% lead acetate solution. Boiled. Solution turned black or brownish due to PbS formation.

d) **Precipitation test:** The test solution gave white colloidal precipitate with following reagents: (a) absolute alcohol, (b) 5% $HgCl_2$ solution, (c) 5% $CuSO_4$ solution, (d) 5% lead acetate, (e) 5% ammonium sulphate.

3) TESTS FOR AMINO ACIDS:

a) **Ninhydrin test (General test):** 3 ml of test solution was heated and 3 drops of 5% Ninhydrin solution was added and kept in boiling water bath 10 min. Purple or bluish color appeared.

b) **Test for tyrosine:** 3 ml of test solution was heated and 3 drops of millon's reagent were added. Solution showed dark red color.

c) **Test for tryptophan:** To 3ml test solution few drops of glyoxalic acid and conc. H_2SO_4 was added. Reddish violet ring appeared at junction of the two layers.

d) **Test for cysteine:** To 5 ml test solution few drops of 40% NaOH and 10% lead acetate solution was added. Boiled. Black precipitate of lead sulphate formed.

4) TESTS FOR FATS AND OILS:

a) **Solubility test:** Oils were soluble in ether, benzene and chloroform, but insoluble in 90% ethanol and in water. (Exception – Castor oil, soluble in alcohol).

b) Filter paper got permanently stained with oils.

5) TESTS FOR STEROIDS:

a) **Salkowski reaction:** To 2 ml of extract, 2 ml chloroform and 2 ml conc. H_2SO_4 was added. Shaken well. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence.

b) **Liebermann-Burchard reaction:** 2 ml extract was mixed with chloroform. 1-2 ml of acetic anhydride was added and 2 drops conc. H_2SO_4 were added from the sides of test tube. First red, then blue and finally green color appeared.

c) **Liebermann's reaction:** 3 ml extract was mixed with 3 ml acetic anhydride. Heated and cooled. Few drops of conc. H_2SO_4 were added. Blue color appeared.

6) TESTS FOR VOLATILE OILS:

a) Volatile oils had characteristic odour.

b) Filter paper was not permanently stained with volatile oil.

c) **Solubility test:** Volatile oils were soluble in 90% alcohol.

7) TESTS FOR GLYCOSIDES:

a) **Tests for cardiac glycosides:**

   a. **Baljet's test:** A thick section showed yellow to orange color with sodium picrate

b. **Legal's test (Test for cardenoloids):** To aqueous or alcoholic extract, 1 ml, pyridine and 1 ml sodium nitroprusside was added. Pink to red color appeared.

c. **Test for deoxysugars (Keller-Killiani test):** To 2 ml extract, glacial acetic acid, one drop 5% $FeCl_3$ and conc. H_2SO_4 were added. Reddish brown color appeared at junction of the two liquid layers and upper layer appeared bluish green.

b) Tests for Anthraquinone Glycosides:

a. **Borntrager's test for Anthraquinone glycosides:** To 3 ml extract, dil. H_2SO_4 was added. Boiled and filtered. To cold filtrate, an equal volume of benzene or chloroform was added. Shaken well. The organic solvent was separated. Ammonia was added. Ammonical layer turned pink or red.

b. **Modified Borntrager's test for C-glycosides:** To 5 ml extract 5 ml 5% $FeCl_3$ and 5 ml dil. HCl was added. Heated for 5 min. in boiling water bath. Cooled and benzene or any organic solvent was added. Shaken well. The organic layer was separated, equal volume dilute ammonia was added. Ammonia layer showed pinkish red color.

c) Tests for Saponin glycosides:

a. **Foam test:** The drug extract or dry powder was shaken vigorously with water. Persistent foam was observed.

d) Tests for Cyanogenetic glycosides:

a. **Grignard reaction or sodium picrate test:** A filter paper strip was soaked first in 10% picric acid, then in 10% sodium carbonate, and dried. In a conical flask moistened

powdered drug was placed and corked; the above filter paper strip was placed in the slit of the cork. The filter paper turned brick red or maroon.

e) Tests for Coumarin glycosides:

a. Coumarin glycosides had aromatic odour.

b. Alcoholic extract when made alkaline showed blue or green fluorescence.

c. Moistened dry powder was taken in a test tube. Test tube was covered with filter paper soaked in dilute NaOH and kept in water bath. After sometime filter paper was exposed to UV light. It showed yellowish- green fluorescence.

f) Tests for Flavonoids:

a. Shinoda test: To dry powder or extract, 5 ml 95% ethanol, few drops conc. HCl and 0.5g magnesium turnings was added. Pink color was observed.

b. To small quantity of residue, lead acetate solution was added. Yellow colored precipitate was formed.

c. Addition of increasing amount of sodium hydroxide to the residue showed yellow colouration, which decolourised after addition of acid.

8) TESTS FOR ALKALOIDS:

The aqueous, alcoholic and chloroform extracts were evaporated separately. To the residue, dilute HCl was added. Shaken well and filtered. With the filtrate, following tests were performed:

Dragendorff's test: To 2-3 ml. filterate, few drops of Dragendorff's reagent was added. Orange brown ppt was formed.

Mayer's test: 2-3 ml filtrate with few drops of Mayer's reagent gave ppt.

Hager's test: 2-3 ml filtrate with Hager's reagent gave yellow precipitate.

Wagner's test: 2-3 ml filtrates with few drops Wagner's reagent gave reddish brown precipitate.

Murexide test for purine alkaloids: To 3-4 ml. test solution, 3-4 drops of conc. HNO_3 was added. Evaporated to dryness. Cooled and 2 drops of NH_4OH were added. Purple colour was observed.

9) TESTS FOR TANNINS AND PHENOLIC COMPOUNDS:

To 2-3 ml of aqueous or alcoholic extract, few drops of following reagents were added:

a) **5% $FeCl_3$ solution:** deep blue-black color.

b) **Lead acetate solution:** white precipitate.

c) **Gelatin solution:** white precipitate.

d) **Bromine water:** decoloration of bromine water.

e) **Dilute Iodine solution:** transient red colour.

f) **Dilute HNO_3:** reddish to yellow colour.

g) **Dil. Potassium permanganate solution:** decolouration.[218]

The results of the qualitative chemical test for extracts of *Biophytum sensitivum* (L.) DC. and *Syzygium cumini* (L.) Skeels are given in table no.15 and table no.16 respectively.

4.3.3 Chromatographic studies

4.3.3.1 Thin Layer Chromatography:

Thin Layer Chromatography (TLC) studies were carried out for various extracts to confirm the presence of different phytoconstituents in these extracts. The extract was applied as a small spot at the origin of thin sorbent layer supported on a glass plate. The mobile phase migrated through the stationary phase by capillary action. The separation of solutes took place due to their differential adsorption coefficient with respect to both mobile and stationary phases. Each separated component had same migration time but different migration distance. The plates were activated at 110 ^{0}C for 1 h.

The Retardation Factor (R_f) was calculated using following formula,

$$R_f = \frac{\text{Distance travelled by solute from the origin}}{\text{Distance travelled by solvent from the origin}}$$

The details of TLC are as follows:

Table 7: *Biophytum sensitivum* TLC details

Adsorbent	Silica gel GF 254 (activated)
Thickness	0.4 mm
Plate size	10 x 20 cm
Activation temp.	110 ^{0}C for 1 h
Volume of spot	20 µl
Solvent system	Chloroform: Glacial Acetic Acid: Acetone (7.5: 2.5: 1)

Table 8: *Syzygium cumini* TLC details

Adsorbent	Silica gel GF 254 (activated)
Thickness	0.4 mm
Plate size	10 x 20 cm
Activation temp.	110 ^{0}C for 1 h
Volume of spot	20 µl
Solvent system	Ethyl acetate: Chloroform (9.5:0.5)

The spots were observed in iodine chamber and UV (366 nm) visualizing agent.[222-224]

The results of the TLC of methanolic extract of *Biophytum sensitivum* (L.) DC. and *Syzygium cumini* (L.) Skeels are given in figure no. 7 and figure no. 8 respectively.

4.3.3.2 Column Chromatography

This was done to isolate and purify the constituents present in the extracts.

Packing of Column:

- ✓ Dried glass column was held in place by retort stand and was sealed with glass-wool.
- ✓ The column was packed with n-hexane and silica gel as adsorbent and the column was tapped in order to avoid air-bubbles.
- ✓ 5ml of the extract was introduced into column then solvent mixture (eluent) in proper ratio was added into the column.

Several fractions were obtained, concentrated and their purity was determined by using thin-layer chromatography. The impure fractions were further processed for re-chromatography using a different solvent mixture.

The results of the column chromatography (R_f values and weight % for isolated compounds) of *Biophytum sensitivum* (L.) DC. and *Syzygium cumini* (L.) Skeels are given in table no. 17 and table no. 18 respectively.

4.3.3.3 HPTLC analysis[225]

Various steps involved in HPTLC were:

1. Selection of HPTLC plate
2. Sample preparation
3. Application of sample
4. Detection
5. Scanning
6. Documentation

1. Selection of HPTLC Plates

Precoated TLC layer: Silica gel GF_{254} plates

Size of the plate: 10 x 10 cm

Support material: Aluminum sheet (0.1 mm thickness).

2. Sample preparation

Isolated compound by preparative TLC dissolved in methanol.

3. Application of Sample

The purified isolated sample was applied in the form of a band using CAMAG LINOMAT V an automatic sample application

device, maintaining a band width-6mm, space-3mm, sec/µl-5. Quantity of sample applied was 5 µl.

4. HPTLC development

Biophytum sensitivum

Mobile Phase: The mobile phase employed in the HPTLC profile was chloroform: glacial acetic acid: acetone (7.5: 2.5: 1). The plates were developed by placing in presaturated or preconditioned tank (12 cm height) with mobile phase until it reaches to previously marked plate (7 cm). The plates were dried by evaporating the solvent either at room temperature or by spraying hot air by air dryer.

Syzygium cumini

Mobile Phase: The mobile phase employed in the HPTLC profile was ethyl acetate: chloroform (9.5:0.5). The plates were developed by placing in presaturated or preconditioned tank (12 cm height) with mobile phase until it reaches to previously marked plate (7 cm). The plates were dried by evaporating the solvent either at room temperature or by spraying hot air by air dryer.

5. Detection/ Scanning

Instrument: CAMAG TLC Scanner III, densitometric evaluation system with Win CATS software was used for scanning of thin layer chromatogram objects in reflectance or transmission mode by absorbance or by fluorescence at 254 or 366 nm respectively.

6. Documentation/ Finger print

The fingerprint of HPTLC profile was taken using the computer-printing machine.

7. Evaluation

R_f value of various samples was evaluated using the following formula.

$$R_f = \frac{\text{Distance travelled by solute from the origin}}{\text{Distance travelled by solvent from the origin}}$$

HPTLC of isolated compound was performed using CAMAG TLC Scanner III and Linomat-V. HPTLC chromatogram are given in fig. 9 A, 10 A, 11 A, 12 A and 13 A respectively.

4.3.4 Characterization of Isolated Compounds

4.3.4.1 IR spectrum

The FTIR absorption spectrum was recorded on Shimadzu IRAFFINITY-1 spectrophotometer by using KBr pallets. Spectras are given in fig. 9 B, 10 B, 11 B, 12 B and 13 B respectively.

4.3.4.2 NMR spectrum

The ^{1}H NMR and ^{13}C NMR was recorded on Bruker Avance II 400 NMR spectrometer, chemical shifts expressed as δ values. Spectras are given in fig. 9 C, D, 10 C, D, 11 C, D, 12 C, D and 13 C, D respectively.

4.4 PHARMACOLOGICAL INVESTIGATION

4.4.1 Acute Toxicity Studies

Literature survey found that acute toxicity study of *Biophytum sensitivum* was found to be 2000 mg/kg body weight in animals and stem bark of *Syzygium cumini* was found to be 5000 mg/kg respectively as safe.[159,207] The dose administered was 200 mg/kg p.o. for extracts of *Biophytum sensitivum* and 500 mg/kg p.o. for extracts of *Syzygium cumini*.

4.4.2 Screening of Diuretic Activity in Rats

4.4.2.1 Diagnostic kits:

Sodium, Potassium (Pariksha Biotech Private Ltd., Hyderabad), Chloride (ERBA diagnostics Mannheim, GmbH).

4.4.2.2 Instruments:

UV-Spectrophotometer (UV-1800, Shimadzu Corporation, Japan)

4.4.2.3 Materials and methods:

Animals Stock: Wistar albino rats of either sex weighing between 150-200 g were obtained from P.E.S's Rajaram and Tarabai Bandekar College of Pharmacy, Ponda, Goa. Animals were housed into groups of 6-8 per cage at a temperature of 25±1 ^{0}C and relative humidity of 45-55%. Animals had free access to food and water, however, food and water was withdrawn 18 h before the experiment. The Institutional Animal Ethics Committee approved the protocol of this study.

4.4.2.4 Preparation of dosage form: The emulsion of extracts (chloroform and methanol) were prepared by triturating the accurately weighed quantity of the extract with 1% Tween 80 in a glass mortar, with gradual addition of distilled water, to make up the required volume. Furosemide Tablets I.P. 40 mg (Lasix 40 mg, Aventis Pharma Ltd., B. no. 0211063, Mfg October 2011, Exp. September 2015) and aqueous extract of *B. sensitivum* and *S. cumini* was diluted with distilled water.

4.4.2.5 Biochemical Estimation:

4.4.2.5.1 Sodium:

Principle: The sodium and the protein in the serum are precipitated with magnesium uranyl acetate. After separation by centrifugation the excess of uranyl ions in the supernatant react with potassium ferrricyanide forming a colored complex which absorbance varies

inversely to the concentration of sodium in the sample.

Sodium Assay

Step-1: Precipitation of Sodium and serum proteins:

Pipette into clean dry test tubes labelled as Standard (S) and Test (T):

	(S)	(T)
R1 Precipitating Reagent	1000 µl	1000 µl
R4 Standard	10 µl	-
Serum Sample	-	10 µl

Shake vigorously and incubate at room temperature for 5 minutes. Then Centrifuge at 2000-3000 rpm for 2 minutes to obtain a clear supernatant.

Step- 2: Colour development

Pipette into clean dry test tubes labelled as Blank (B), Standard (S) and Test (T):

	(B)	(S)	(T)
R2 Colour Reagent	1000 µl	1000 µl	1000 µl
Supernatant from Step 1	-	20 µl	20 µl
R1 Precipating Reagent	20 µl	-	-

Mix well and allow it to stand at room temperature for 5 minutes. Then measure the absorbance of Blank (B), standard (S), and Test (T) on a photocolorimeter with green filter or on a spectrophotometer at 530nm (505 - 530 nm) within 10 minutes.

Calculations:

$$Sodium\ in\ mmol/L = \frac{\text{Abs of B} - \text{Abs. of T}}{\text{Abs of B} - \text{Abs of S}} x\ 150$$

4.4.2.5.2 Potassium:

Principle: Potassium ions in a protein-free alkaline medium reacts with sodium tetraphenylboron to produce a finely dispersed colloidal suspension of potassium tetraphenylboron. The turbidity produced is proportional to the potassium concentration in the sample.

Potassium Assay

Pipette into two clean dry test tubes labelled Standard (S) and Test (T)

	(S)	**(T)**
R3 Boron Reagent	1000 µl	1000 µl
R4 Standard	50 µl	-
Serum Sample	-	50 µl

Mix well and Incubate for 5 minutes at RT. Read absorbance of the Standard (Abs.S) and Test Sample (Abs.T) against Distilled water at 620 nm.

Calculations:

$$Potassium\ in\ mmol/L = \frac{\text{Abs. T}}{\text{Abs. S}} x\ 5.0$$

4.4.2.5.2 Chloride:

Principle: When chloride is mixed with a solution of undissociated mercuric thiocyanate, the chloride preferentially combines with mercury forming mercuric chloride.

The thiocyanate that is released then combines with ferric ions present in the solution forming strongly coloured ferric thiocyanate with an absorption maxima at 480 nm.

Assay procedure

Wavelength 492 (470 - 630) nm

Cuvette 1 cm

	Reagent blank	**Standard (Cal.)**	**Sample**
Reagent 1	1000 µl	1000 µl	1000 µl
Sample	-	-	10 µl
Standard (Cal.)	-	10 µl	-
Distilled water	10 µl	-	-

Mix and incubate 5 min. at 37 ^{0}C. Measure absorbance at of the sample A_{sam} and standard A_{st} against reagent blank.

Calculation

$$Chloride\ mmol/L = \frac{\Delta A_{sam}}{\Delta A_{st}} \times C_{st}$$

C_{st} = standard (calibrator) concentration

4.4.2.6 Diuretic activity:

Animals were divided in total of five groups (n = 6 in each group). All animals were deprived of food and water 18 h prior to the experiment. On the day of experiment, the dosing were scheduled as follows:

B. sensitivum

Group I: Normal saline.

Group II: Furosemide 10 mg/kg p.o. as reference diuretic drug.

Group III: Chloroform extract 200 mg/kg p.o.

Group IV: Methanol extract 200 mg/kg p.o.

Group V: Aqueous extract 200 mg/kg p.o.

S. cumini

Group I: Normal saline.

Group II: Furosemide 10 mg/kg p.o. as reference diuretic drug.

Group III: Chloroform extract 500 mg/kg p.o.

Group IV: Methanol extract 500 mg/kg p.o.

Group V: Aqueous extract 500 mg/kg p.o.

Immediately after the dosing, animals were placed in metabolic cages and urine was collected up to 5 h after dosing. Room temperature was maintained up to 25± 0.5 ^{0}C.

During this period no water or food was made available to the animals. Diuretic activity was assessed by measuring the following parameters:

- ✓ Total urine volume.
- ✓ Urine concentration of Na^+, K^+ and Cl^-.[226,227]

The effect of different extracts of *Biophytum sensitivum* (L.) DC and *Syzygium cumini* (L.) Skeels exhibiting diuretic activity are shown in Table no. 24 – 25.

4.4.3 Screening of Nephroprotective Activity in Rats

4.4.3.1 Drugs and Chemicals:

5,5'-Dithio-bis-(nitrobenzoic acids) (DTNB), Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, (±) epinephrine were purchased from Sigma-Aldrich Co., USA.

4.4.3.2 Diagnostic kits:

Urea, Uric acid and Blood urea nitrogen (BUN), (Manufactured by Span Diagnostics Ltd., Sachin, Surat), Creatinine (ERBA diagnostics Mannheim, GmbH).

4.4.3.3 Instruments:

Refrigerator centrifuge (REMI-C-24BL Remi Electrotechnik Ltd, Vasai), UV-Spectrophotometer (UV-1800, Shimadzu Corporation, Japan), Research centrifuge (REMI-C-24BL Remi Electrotechnik Ltd, Vasai), Homogenizer (Remi Motors, Mumbai) and Hot air oven (180 BSS, Pathak Electical Works, Mumbai).

4.4.3.4 Materials and methods:

Animals Stock: Wistar albino rats of either sex weighing between 150-200 g were obtained from P.E.S's Rajaram and Tarabai Bandekar College of Pharmacy, Ponda, Goa. Animals were housed into groups of 6-8 per cage at a temperature of 25±1 ^{0}C and relative humidity of 45-55%. Animals had free access to food and water, however, food and water was withdrawn 18 h before the experiment. The Institutional Animal Ethics Committee approved the protocol of this study.

4.4.3.5 Preparation of dosage form:

The emulsion of extracts (chloroform and methanol) were prepared by triturating the accurately weighed quantity of the extract with 1% Tween 80 in a glass mortar, with gradual addition of distilled water, to

make up the required volume. Aqueous extract of *B. sensitivum* and *S. cumini* was diluted with distilled water.

4.4.3.6 <u>Biochemical Estimation:</u>

4.4.3.6.1 Creatinine:

Principle: Creatinine reacts with alkaline picrate to produce an orange-yellow colour (Jaffe's reaction). Specificity of the assay has been improved by the introduction of an initial rate method. The absorbance of the orange-yellow colour formed is directly proportional to creatinine concentration and is measured photometrically at 500-520 nm.

Assay Procedure:

Pipette	Standard	Test
Working Reagent	1000 μl	1000 μl
Standard	100 μl	----
Test	----	100 μl

Mix well and read initial absorbance (A_1) 20 seconds after mixing and final absorbance (A_2) 80 seconds after mixing.

Clinical Significance: Creatinine is a waste product formed in muscle from the high energy storage compound, creatinine phosphate. The amount of creatinine produced is fairly constant (unlike urea) and is primarily a function of muscle mass. It is removed from plasma by glomerular filtration and then excreted in urine without any appreciable reabsorption by the tubules. Creatinine is a useful indicator of renal function.

Increases Elevated creatinine level in serum is usually associated with various renal diseases. In the earlier stage of renal diseases, creatinine

clearance test is a sensitive index of impaired renal function.

Calculation: $\Delta A = A_2 - A_1$

Creatinine (mg/dl) = Abs. of Test/Abs. of Standard x Concentration of standard (mg/dl).[228]

4.4.3.6.2 Uric acid

Principle:

Uric acid + O_2 + H_2O (Urease) → Allantoin + CO_2 + H_2O_2

H_2O_2 + 4 - AAP + TBHB (Peroxidase) → Quinoneimine + H_2O

4 - AAP = 4 – Aminoantipyrine, TBHB = 2, 4, 6 - Tribromo -3-hydroxy benzoic acid.

Assay Procedure:

Pipette into test tubes marked	Blank	Standard	Test
Working Reagent	1000 µl	1000 µl	1000 µl
Distilled Water	20 µl	---	---
Standard	---	20 µl	---
Test	---	---	20 µl

Mixed and incubated for 5 minutes at 37 °C. The absorbance of standard and each test at 505 nm (500 - 540 nm) or 505/670 nm on bichromatic analyzers against reagent blank was recorded.

Clinical Significance: Uric acid is a metabolite of purines, nucleic acids and nucleoproteins. Consequently, abnormal levels may be indicative of a disorder in the metabolism of three substances.

Increases: Increased levels of serum uric acid are observed in renal

dysfunction, gout, leukemia, polycythemia, atherosclerosis, diabetes, hypothyroidism, or in some genetic diseases.

Decreases: Uric acid concentration decreases in patients with Wilson's disease.

Calculations: Uric Acid (mg/dl) = Abs. of Test/ Abs. of Standard x Concentration of standard (mg/ dl).[229]

4.4.3.6.3 Urea

Clinical significance: Urea is the major end product of protein metabolism in humans. It constitutes the largest fraction of the non-protein nitrogen component of the blood. Urea is produced in the liver and excreted through kidneys in the urine. Consequently, the circulating levels of urea depend upon protein intake, protein catabolism and kidney function.

Methodology:

Principle: The estimation of urea in serum involves the following enzyme catalyzed reactions

Urea + H_2O (urease) → $2NH_3$ + CO_2

NH_3 + α - KG + NADH (GLDH) → Glutamate + NAD

α - KG: α - Ketoglutarate, GLDH: Glutamate dehydrogenase

Procedure

Pipette in to test tubes marked	Standard	Test
Working Reagent	1000 µl	1000 µl
Standard	20 µl	--
Test	--	20 µl

Mixed well, and aspirate standard followed by samples.[230]

4.4.3.6.4 BUN

Principle: The estimation of urea in serum involves the following enzyme catalyzed reactions

Urea + H_2O (urease) $\rightarrow$ $2NH_3$ +CO_2

NH_3 + α - KG + NADH (GLDH) $\rightarrow$ Glutamate + NAD

α - KG: α - Ketoglutarate

GLDH: Glutamate dehydrogenase

The rate of decrease in absorbance is monitored at 340 nm and is directly proportional to urea concentration in the sample.

Clinical significance: Urea is the major end product of protein metabolism in humans. It constitutes the largest fraction of the non-protein nitrogen component of the blood. Urea is produced in the liver and excreted through the kidneys in the urine. Consequently the circulating levels of urea depend upon protein intake, protein catabolism and kidney function.

Increases: Elevated serum urea concentrations are observed in impaired kidney function liver diseases, congestive cardiac failure, diabetes infections and diseases which impair kidney function.

Assay procedure:

Pipette in to test tubes marked	Standard	Test
Working Reagent	1000 µl	1000 µl
Standard	20 µl	--
Test	--	20 µl

Calculation: Absorbance change (ΔA) for the standard and unknown samples was determined by using the formula, $\Delta A = A_1 - A_2$

BUN (mg/dl) = ΔA of Test / ΔA of Standard x Concentration of standard (mg/ dl).[231]

4.4.3.7 Evaluation of Gentamicin induced Nephroprotective activity:

Animals were divided in total of five groups (n = 6 in each group). All animals were deprived of food and water 18 h prior to the experiment. On the day of experiment, the dosing were scheduled as follows:

B. Sensitivum

Group I: Served as control, was given normal saline in the dose of 30 ml/Kg p.o., twice a day for 7 days.

Group II: Animals were treated with only Gentamicin (GM) 40 mg/kg i.p., twice a day for 7days.

Group III - V: Received 200 mg/kg p.o. *B. sensitivum* plant extracts along with Gentamicin (GM) in the dose of 40 mg/kg i.p., twice a day for 7 days.

S. cumini

Group I: Served as control, was given normal saline in the dose of 30 ml/Kg p.o., twice a day for 7 days.

Group II: Animals were treated with only Gentamicin (GM) 40 mg/kg i.p., twice a day for 7days.

Group III - V: Received 500 mg/kg p.o. *S. cumini* plant extracts along with Gentamicin (GM) in the dose of 40 mg/kg i.p., twice a day for 7 days.

Collection and analysis of serum sample:

After the experimental period, blood samples were collected from

retro-orbital plexus under light ether anesthesia in centrifuged tube and centrifuged at 3000 rpm for 10 minutes to obtained serum, the resulting serum collected was properly labeled, cleaned and dried micro-centrifuge tubes analysed immediately for creatinine[228], uric acid[229], urea[230] and BUN[231] by using kits using UV spectrophotometer.

4.4.3.8 Kidney homogenate analysis:

On 8th day, animals were sacrificed by chloroform entrapment. The abdomen was opened to remove both kidneys and washed in 0.9% cooled saline, kept on ice, one kidney stored in 10% formalin for histopathology study. Another one homogenized in cold phosphate buffer (0.1 M, pH 7.4). The homogenates were centrifuged at 10000 rpm for 10 min at 4ºC and post-mitochondrial supernatant (PMS) was used for the estimation of Lipid peroxidation. Another part of homogenize supernatant was centrifuged at 17000 rpm for 1 h at 4ºC. The supernatant obtained was used for further estimation of SOD, CAT and GSH. Histopathology of Kidneys are shown in fig no. 14 and 15.

4.4.3.8.1 Lipid peroxidation (LPO)

Principle: Oxidative stress is associated with peroxidation of cellular lipids, which is determined by measurement of thiobarbituric acid reacting substance (TBARS). The concentration of LPO products may reflect the degree of oxidative stress. The increased level of TBARS results in increased of OFRs, which attacks the polyunsaturated fatty acids in cell membranes and cause LPO. The malondialdehyde (MDA) content, a measure of lipid peroxidation was assayed in the form of TBARS.

Reagents used:

1. 15% Trichloroacetic acid (TCA)
2. 0.375% Thiobarbituric acid (TBA)
3. Phosphate buffer (pH 7.4)

4. 5 N HCl

Procedure: 500 µl of homogenate, 300 µl 15% TCA, 300 µl TBA, and 300 µl 5 N HCl was added. Boiled on water bath at 90 - 100° C, for 20 to 30 mins, the test tubes were cooled and centrifuged at 2000 rpm for 10 mins. The absorbance at 532 nm by using spectrophotometer was measured.[232]

4.4.3.8.2 Superoxide dismutase (SOD)

Principle: Superoxide dismutase is a metalloprotein and is the first enzyme involved in the antioxidant defence against ROS by lowering the steady state level oxygen. SOD scavenges the superoxide ions produced as cellular byproducts. SOD is a major defence for aerobic cells combating the toxic effect of superoxide radicals. The SOD activity is determined by the ability of the enzyme to inhibit auto oxidation of pyrogallol.

Reagents used:

1) M sodium carbonated buffer (pH 10.2)
2) Epinephrine

Procedure: 0.8 ml of 0.1 M sodium carbonated buffer (pH 10.2), 0.1ml of supernatant, 0.1 ml of epinephrine was added in quartz cuvette. The absorbance at 295 nm by using spectrophotometer was measured. The absorbance change for 0 min and 1 min was recorded.[233]

4.4.3.8.3 Catalase (CAT)

Principle: CAT is a hemeprotein, localized in the microperoxisomes. It reduces hydrogen peroxide produced by dismutation reaction and prevents generation of hydroxyl radicals thereby protecting the cellular constituents from oxidative damage in peroxisome. The enzyme catalyses the decomposition of H_2O_2 to H_2O and oxygen and thus protecting the cell from oxidative damage by H_2O.

Reagents used:

1) 50 MM phosphate buffer (pH 7.0)

2) H_2O_2 0.17 MM (30 % W/V)

Procedure: 1.95 ml phosphate buffer (50 MM, pH 7.0), 1.0 ml H_2O_2 (0.17 MM), 0.05 ml homogenate (10%, w/v) in a total volume of 3.0 ml was added. The absorbance at 240 nm by using spectrophotometer was measured. The absorbance change for 0 min and 1 min was recorded.[234]

4.4.3.8.4 Glutathione (GSH)

Principle: GSH is a major non-protein thiol and endogenous antioxidant that counters balance free radical mediated damage. It is involved in the protection of normal cell structure and function by maintaining the redox homeostasis, quenching of free radicals and by participating in detoxification reaction.

Reagents used:

1) 0.6mM DTNB

2) 10% Trichloroacetic acid (TCA)

3) Phosphate buffer (pH 8.0)

Procedure: 1ml of liver homogenate, 6 ml of Phosphate buffer (pH 8.0) and 1 ml of 0.6mM DTNB was added. Incubated at R.T. for 10 mins. The absorbance at 412 nm by using spectrophotometer was measured.[235]

4.4.3.9 Statistical analysis:

All the results analyzed using ANOVA followed by Dunnett's multiple comparison test.[236]

The effect of different extracts of *Biophytum sensitivum* (L.) DC and bark of *Syzygium cumini* (L.) Skeels exhibiting nephroprotective activity are shown in Table no. 26 – 29.