CHAPTER 3

MATERIALS AND METHODS

3.1 PHYTOCHEMISTRY

3.1.1 Selection of Plant Material

Phyllanthus wightianus Muell. Arg., an ethnomedicine which is used by Malayali tribals in the Javadi Hills of Vellore District, Tamil Nadu, India, was selected for the present study.

3.1.2 Ethnobotanical Uses

Phyllanthus wightianus belongs to the family of Euphorbiaceae and is locally known as ‘Elumbotti’ by the Malayali tribals from the Vellore District of Tamil Nadu in India. They use plant paste for bone setting and to treat diarrhoea.

3.1.3 Collection of Plant Material

Whole plant material in required quantity has been collected during the months of December and January from the Javadi Hills (± 866 MSL) in the Vellore District. A voucher specimen (MBV & OSP 18632) was identified and authenticated by Dr. M.B. Viswanathan and has been deposited in the Herbarium of the Sri Paramakalyani Centre for Environmental Sciences, Manonmaniam Sundaranar University, Alwarkurichi, Tamil Nadu, India.

3.1.4 Description


Subshrubs, villous, up to 1.5 m high; branches spirally arranged towards stem tips. Leaves alternate, membranous, green above, pale green beneath, obliquely elliptic to oblong, obliquely truncate at base, entire, ciliate, obtuse, apiculate at apex, subsessile, puberulous above, pubescent beneath, 1.5 – 3 x 0.7 – 1.7 cm; stipules lanceolate, acuminate, persistent, c. 1.5 mm long. Flowers red, solitary, axillary. Male: pedicels densely puberulous, c. 2.6 cm long. Tepals 4 – 7, oblong-ovate or lanceolate, bluntly acuminate, glabrous within, villous without, ciliate along margins, c. 3 x 1.5 mm. Disc segments 4 – 7, truncate, dotted, c. 1.5 mm long. Stamens usually 2, rarely 3; filaments connate, 1 or 0.5 mm long; anthers 2 or 3, subglobose, dehiscing transversely, 0.5 or 1 x 1.5 mm. Female: pedicels densely puberulous, c. 2.7 cm long. Tepals 5 or 6, ovate, acuminate, glabrous within, villous without, ciliate along margins, c. 1 x 2 mm. Ovary globose, 3-lobed, hairy, c.1 mm long; styles 3, each 2-fid, divided up to base, glabrous, c. 1.2 mm long. Capsules globose, 3-valved, hairy, c. 6 x 6 mm; hairs soft, short, dense, branched, multicellular. Seeds 6, 3-gonous, appressed hairy, c. 3 x 1.5 mm.

Distribution: Tamil Nadu and Karnataka in India.

3.1.5 Chemicals

Chemicals (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India, and S.D. Fine Chemicals, Mumbai, India) used were of AR Grade. Silica gel (ACME’s and S.D. Fine Chemicals, Mumbai) 60 – 120 mesh and 100 – 200 mesh for Column Chromatography (CC) and Silica gelG were used for thin layer Chromatography
Pre-coated aluminium plates coated with silica gel 60 F254 (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India) for 0.2 mm layer thickness were used for HPTLC analysis.

3.1.6 Extraction

Shade-dried and coarsely powdered whole plant material was successively extracted (hexane, chloroform and methanol) using Soxhlet apparatus. The solvent extracts were recovered, concentrated under reduced pressure and the last traces of the solvents were removed in vacuo.

3.1.7 Preliminary Phytochemical Screening

All the extracts such as hexane, chloroform and methanol of *P. wightianus* (whole plant) were subjected to routine qualitative chemical analysis to identify the nature of phytochemical constituents present in it (Brindha *et al.*, 1982.).

3.1.8 Qualitative Analysis

3.1.8.1 Determination of Extractive Value

This method was used to determine the amount of active constituents present in each plant solvent extract such as hexane, chloroform and methanol. Plant material weighing 100 g was successively extracted with organic solvents in the order of increasing polarity using a Soxhlet apparatus, following the British Pharmacopoeia (Anonymous, 1993). The percentage solubility for the extracts was calculated. The extractive values of the crude drugs thus calculated are very much useful in their evaluation where the constituents of a drug can not be readily estimated by any other means.
3.1.8.2 Fluorescent Analysis

Organic solvents such as hexane, benzene, chloroform, ethyl acetate, alcohol and acetone, water, 1 N HCl and 1:1% H2SO4, HNO3 and alkaline solutions of aqueous and alcoholic 1 N NaOH were treated individually with desired quantity preferably one g each of the drug powder in separate test tubes. After 24 hours, fluorescence of each extraction observed under day light and UV light (254 nm) was recorded.

3.1.8.3 Determination of Ash Value

The presence of ash in the plant materials can be determined by three different ways such as determination of total ash, acid insoluble ash and water soluble ash (Anonymous, 1996).

Total Ash

Total ash was determined by the method of the Association of Official Analytical Chemists (Horwitz, 1980). This method was designed to measure the amount of material remaining after ignition. Physiological ash is derived from the plant tissue itself and non-physiological ash is the residue after ignition of the extraneous matter (e.g. sand and soil) adhering to the surface.

About 2 g of the plant powder was accurately weighed in a tarred silica crucible previously ignited and weighed. The powder was scattered in a fine even layer on the bottom of the crucible. It was ignited by gradually increasing the heat to 500°C. The ignition was repeated until constant weight was obtained, cooled in a desiccator and weighed. Percentage of ash with reference to the air-dried drug was calculated.
Acid Insoluble Ash

The total ash obtained above was boiled for 5 min with 25 ml of dilute hydrochloric acid. The insoluble matter was collected on an ash less filter paper and washed with hot water. Then, it was ignited and weighed after cooling in a desiccator. The percentage of acid insoluble ash was calculated with reference to the air-dried drug and tabulated.

Water Soluble Ash

The total ash obtained as above was boiled with 25 ml of water for 5 min. The insoluble matter was collected in an ash less filter paper and washed with hot water. This was then ignited to constant weight at temperature not exceeding 450°C. The weight of insoluble matter was subtracted from the weight of total ash. The difference in weight represents the water soluble ash. The percentage of it was calculated with reference to air-dried drug. The above tests were repeated thrice and the average value was calculated.

3.1.8.4 Determination of Loss of Weight on Drying

An excess of water in medicinal plant materials will lead to microbial growth, insect attack and deterioration by hydrolysis. Hence, loss of weight on drying was determined. This test was used to determine both water and volatile matter.

A known quantity of 500g of fresh plant material was weighed and dried under shade until a constant weight was obtained. The loss of weight on drying was calculated.

3.1.8.5 Tests for Inorganic Elements

The total ash value was determined for the plant material and the total ash content represents the inorganic salts, which usually consist of carbonates, phosphates and silicates of sodium, potassium, magnesium, calcium, etc. These salts may be
occurring naturally or may be present as impurity or adulterant. Hence, the following tests were performed using the total ash. Tests for arsenic, borate, copper, calcium, magnesium, lead, iron, potassium, mercury, sodium, sulphate, phosphate, chloride, carbonate and nitrates were analyzed and the results were tabulated.

3.1.9 Quantitative Analysis of Inorganic Elements (Salts and Minerals)

Apart from that, column chromatography of the methanol extract when eluted continuously with ethyl acetate: methanol 3:1 yielded fractions 1, 2, 3, and 4 with a high content of salts. Therefore, the plant material was subjected to inorganic mineral analysis to know the quantity of minerals present in 1 g of plant material.

Preparation of Sample Solution for Inorganic Mineral Analysis

The plant material (1 g) was digested with 10 ml of nitric acid and left over night. It was then heated on a hot plate until the reddish brown fumes ceased and cooled. A small volume of perchloric acid was added and transferred to a 50 ml volumetric flask and made up to volume with double distilled water (McKenzie, 1982).

Determination of Sodium and Potassium by Flame Photometry (Anonymous, 1996)

The instrument used was Systronics Flame Photometer. A series of standard solutions containing the element to be determined in increasing concentrations within the concentration range recommended for the instrument was prepared. Nitric acid and perchloric acid used for the preparation of the sample solution of the plant material were also added in the same concentrations to the standard solutions. The appropriate filter was chosen, water was sprayed into the flame and the galvanometer reading was adjusted to zero. The most concentrated solution was then sprayed into the flame and the galvanometer reading was recorded. Again, water was sprayed till
the galvanometer reading was zero. Then the standard solution was sprayed into the flame and the procedure was repeated thrice for each concentration. A calibration curve was prepared by plotting the mean of three readings of each standard against the concentration. The sample solution prepared above was then aspirated into the flame thrice followed by recording the galvanometer reading. The apparatus was washed thoroughly with water after each aspiration. Using the mean of three readings, the concentration of the element being examined was determined from the calibration curve. To confirm the concentration thus obtained, the operation was repeated with the standard solution of the same concentration as that of the solution being examined.

**Determination of Calcium, Cobalt, Copper, Iron, Magnesium and Manganese by Atomic Absorption Spectroscopy** (Anonymous, 1996)

The instrument used was Perkin Elmer Atomic Absorption Spectrophotometer. Three standard solutions of the element to be determined covering the concentration range recommended for the instrument for the element were prepared. Nitric acid and perchloric acid used in the preparation of the solution of the substance being examined were also added to the standard solutions in the same concentration. After calibration of the instrument, each standard solution was introduced into the flame for three times and the steady readings were recorded. The apparatus was thoroughly washed after each introduction. A calibration curve was prepared by plotting the mean of each group of three readings against the concentration. The plant extract prepared above was then introduced into the flame. The reading recorded and the apparatus washed with water. The sequence was then repeated twice. Using the mean of the three readings, the concentration of the element was determined from the calibration curve. The process was repeated for determination of other elements using different lamps.
3.1.10 Separation Methods

3.1.10.1 Paper Chromatography

Amino acids were identified following Jayaraman (1981) and Sadasivam and Manickam (1996). The mobile phase used for identification of amino acids was a mixture of n-butanol, glacial acetic acid and water in the ratio of 4:1:5 v/v. SD's Amino acid reference collection (Kit of 24 items) was used. The collection contains 24 chromatographically homogenous amino acids. Each vial contains 1 g except 3 (3, 4–dihydroxy phenyl)–DL–alanine and L-hydroxy proline which contains 0.1 g each.

The twenty four standard amino acids were dissolved with distilled water at a concentration of 1 mg/ml in different test tubes. Very dilute hydrochloric acid was used for dissolving tyrosine and phenylalanine and dilute sodium hydroxide was used to dissolve tryptophan.

A known quantity of plant material was ground using a mortar and pestle with 10 fold volume of 70% ethanol. The contents were shaken at 55°C for 30 min. The contents were centrifuged and the supernatant was collected. The extraction was repeated again twice. The supernatants were pooled and evaporated to dryness under vacuum. The residue was dissolved in a known volume of absolute ethanol or water for analysis. ninhydrin solution (1 mg/ml) in acetone was used as spraying agent. The chromatography sheet was cut into a convenient size, a line was drawn about 5 cm away from one end and points were marked at regular intervals of 3 cm.

A small volume of the each standard amino acid and sample were applied using a microsyringe. The spots were allowed to dry fast using a stream of hot air from a dryer. The sheet was then placed inside of a chamber containing the mobile phase and the chromatogram was developed. The paper was then removed and the solvent front was recorded. The chromatogram was dried and sprayed with ninhydrin
reagent using an atomizer. The paper was dried at room temperature and then in an Oven for 2-3 min. Amino acids appeared as purple spots whereas hydroxy proline and proline give yellow spots. The spots were marked and their Rf values were calculated. The amino acids present in the sample were then identified by comparing the Rf obtained with that of the authentic amino acids and also by co-chromatography.

3.1.10.2 Thin Layer Chromatography

A slurry of the adsorbent (Silica gel*) was prepared with water (1:2). Dried and clean glass plates (20 cm x 5 cm) were laid in a row as a template, the suspension was poured into Stahl TLC spreader, which was adjusted to 0.25 mm thickness and coated in a single passage on the spreader over them. The prepared plates were allowed to air-dry and placed in an Oven at 110°C for 30 min after drying towards activation. Then, they were transferred into a dust free chamber. Aluminium plates coated with silica gel° F254 (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India) were also used. The application of spots was done using capillary tubes about 2 cm above the bottom of the plate.

Chromatograms were detected with vanillin-H₂SO₄ reagent (1 g vanillin dissolved in 100 ml H₂SO₄, heated at 110°C after spraying). The fractions of similar TLC patterns were combined, concentrated and re-chromatographed repeatedly over silica gel to isolate pure compounds.

3.1.10.3 Column Chromatography

The adsorbent used was Silica gel 60 – 120 mesh and finer than 200 mesh. The columns of different sizes were used for the isolation of constituents in the present investigation. The column elutes were collected in fractions with components of small elution volumes from 25 to 50 ml. The fractions were analyzed
using Thin Layer Chromatography. Fractions containing the individual pure components were combined and the eluting solvents removed using rotary evaporator.

3.1.10.4 High Performance Thin Layer Chromatography (HPTLC)

HPTLC fingerprinting was performed on CAMAG TLC scanner3 instrument, equipped with Linomat IV applicator and CATS 3.1 Software. The various extracts used for taking HPTLC fingerprints were hexane, chloroform and methanol extracts of the test plant.

**Stationary Phase**

Aluminium sheets pre-coated with silica gel 60 F254 (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India) of 0.2 mm layer thickness were used as the stationary phase.

**Mobile Phase**

The HPTLC chromatograms for the different solvent extracts were developed with the following solvent systems:

**Hexane Extract**

Developing System : Hexane: Toluene 3:2
Scanning wave length : 200 nm
Marker compound : Friedelin

**Chloroform Extract**

Developing System : Chloroform
Scanning Wave Length : 200 nm
Marker compound : Lupeol

**Methanol Extract**

Developing System : Ethyl acetate
Scanning Wave Length : 254 nm

Marker compounds : Bergenin and gallic acid

Fingerprints were obtained by development in CAMAG twin trough glass chamber. The developed TLC plates were dipped in vanillin-H$_2$SO$_4$ reagent and heated at 110°C for 5 min. The development of spots on the TLC plates was given (Fig. 8).

**Preparation of Extracts**

The dried whole plant material was powdered and passed through 60 mesh. A quantity of 5 g powder was successively extracted with hexane, chloroform and methanol in Soxhlet apparatus. Each extract was concentrated and made up to 50 ml in a standard flask with respective solvents. All the marker compounds such as friedelin, lupeol, bergenin and gallic acid, each 10 mg was dissolved in 10 ml of suitable solvent (friedelin and lupeol were dissolved with chloroform, while bergenin and gallic acid were dissolved with methanol). A 10 μl of the extract solution and marker compound solutions were spotted for the chromatogram. The scanning wavelength was 200 nm for hexane and chloroform extracts while for methanol extract it was 254 nm.

**3.1.10.5 High Performance Liquid Chromatography (HPLC) of Tannins and Lignans**

HPLC analysis of total tannins and lignans was done using Shimadzu HPLC-LC 2010 CHT with class VP version 6.2–6.3 with auto injector.

**Operational Conditions**

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Shimadzu LC-2010 (Quaternary gradient)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationery phase</td>
<td>Lichrocart RP 18E (02/04)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>: Binary Gradient</td>
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<tr>
<td>---------------------</td>
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</tr>
<tr>
<td>Pump A</td>
<td>: 0.05% Orthophosphoric acid in acetonitrile</td>
</tr>
<tr>
<td>Pump B</td>
<td>: 0.05% Orthophosphoric acid in water gradient elution</td>
</tr>
<tr>
<td>Flow rate</td>
<td>: 1.0 ml/minute</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>: 220 &amp; 254 nm</td>
</tr>
</tbody>
</table>

**Reagents and Chemicals**

| Tanins              | : Gallic acid (GA), corillagin (C), geranin (G) and ellagic acid (EA) were procured from SPIC Pharma, Chennai. |
| Acetonitrile        | : HPLC grade                          |
| Orthophosphoric acid| : AR grade                            |
| Water               | : HPLC grade                          |

**Estimation of Lignans**

| Lignans              | : Hypophyllanthin and Phyllanthin     |
| Instrument           | : Shimadzu 10 AD                      |
| Stationary Phase     | : Spherisorb ODS-2                    |
| Mobile phase         | : 65 CH₃OH:35 H₂O                     |
| Flow rate            | : 1.3 ml/minute                       |
| Detection wavelength | : 230 nm                              |
| Standard dilution    | : Hypophyllanthin – 0.25 mg/ml and Phyllanthin – 0.50 mg/ml in CH₃CN |
| Volume injected      | : 20 μl                               |
| Sample dilution      | : 200 mg in 20 ml                     |
**Standard Stock Solution**

An accurately weighed quantity of gallic acid (GA), corillagin (C), geraniin (G) and ellagic acid (EA) standards were dissolved in methanol to prepare a known concentration of 0.125, 0.25, 0.30 and 0.05 mg/ml respectively.

**Sample Preparation**

About 200 mg of sample was transferred to a 25 ml volumetric flask, dissolved with diluents by sonication and made up to volume with diluents and filtered the above solution through syringe filter.

**Estimation method**

Standard solutions such as gallic acid (GA), corillagin (C), geraniin (G) at 220 nm and ellagic acid (EA) at 254 nm were injected.

**Formula**

\[ \text{Sample area} \times \frac{\text{Standard concentration}}{\text{Sample concentration}} \times \text{standard purity} \]

**3.1.10.6 Gas Chromatography–Mass Spectrometry (GC-MS) Analysis of Lipids**

GC-MS was recorded on Agilent instrument by the direct inlet method.

**Operating Parameters**

- **Name of the instrument**: GCMS D 5973 Agilent
- **Detector**: Mass selective detector
- **Column specification**: DB₅– MS
- **Column thickness**: 0.25 μ (film thickness)
- **Column length**: 30 m
- **Internal diameter**: 0.25 mm
- **Column thickness**: 0.25 μm
- **Carrier gas**: Helium
Temperature programme : 70°C for 2 min, 10°C/min up to 280°C for 10 min
Injection temperature : 250°C
Flow Rate : 1 ml/minute
Library software : NIST

3.1.11 Identification of the Compounds

3.1.11.1 Melting Point

Melting points were recorded on an INLAB Melting point apparatus and paraffin was used as a solution.

3.1.11.2 Structural Elucidation by Spectral Analysis

The structure of the isolated compounds was elucidated by analyzing the spectral data and in comparison with compounds previously reported in literature.

3.1.11.3 Ultraviolet–Visible Spectroscopy

The absorption spectra were measured in very dilute solution against a solvent blank using an automatic recording spectrophotometer. For colorless compounds, measurements were made in the range of 200 to 400 nm (nanometers) and for coloured compounds, the range was 200 to 700 nm. The wavelengths of the maxima and minima of the absorption spectrum so obtained were recorded (in nm). The instrument used for taking UV–Visible spectra was Shimadzu UV-Vis Spectrophotometer Model UV–1601, with Spectroscopic grade methanol in the range of 200–600 nm.

3.1.11.4 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra were taken on Shimadzu FTIR grating spectrophotometer, model FTIR 8101 A, with potassium bromide (KBr disc).
3.1.11.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

The $^1$H and $^{13}$C NMR Spectra were taken on Bruker (400 Mz) or JOEL (400 Mz) instruments. The solvents used were (CDCl$_3$ or DMSO – d$_6$ with tetra methyl silane (TMS) as the internal standard chemical shifts were recorded $\delta$ in scale. Proton shifts in organic compounds ranged from 0 ppm to 14 ppm, i.e. from a $\delta$ value of 0 – 14. The spread of resonance for $^{13}$C was from 0 to 180 ppm.

3.1.11.6 X-ray Crystallography

Suitable single crystals of compound (6) were obtained from ETOAC – EtOH 9:1 mixture was subjected to X-ray diffraction analysis carried out on an Enrafnonius (CAD) – 4 diffractometer with the $\theta$/2$\theta$ scan mode $\lambda$ ($\mu$K$\alpha$) = 0.71069 Å.

3.2 ANTIMICROBIAL STUDIES

The various solvent extracts of the plant such as hexane, chloroform and methanol were tested for antibacterial, antifungal and antidermatophytic activities.

3.2.1 Test Microorganisms

Bacterial Strains

The bacterial strains employed in the biological assays were gram-positive bacteria such as Bacillus cereus (MTCC 430), Bacillus subtilis (MTCC 441), Staphylococcus aureus (Staph. aureus, MTCC 96), Staphylococcus epidermidis (MTCC 435) and gram-negative bacteria such as Aeromonas hydrophila (MTCC 646), Enterobacter aerogenes (MTCC 111), Escherichia coli (MTCC 724), Klebsiella pneumoniae (MTCC 432), Proteus mirabilis (MTCC 425), Proteus vulgaris (MTCC 426), Pseudomonas aureoginosa (MTCC 741), Salmonella paratyphi (MTCC 735), Salmonella typhi (MTCC 733), Vibrio cholerae, Vibrio parahaemolyticus (MTCC 451) and Vibrio vulnificus (MTCC 1146).
Fungal Strains

*Aspergillus flavus* (MTCC 277), *Aspergillus fumigatus* (MTCC 343), *Aspergillus niger* (MTCC 1344) and *Candida albicans* (MTCC 227).

Dermatophytes


*Vibrio cholerae* was procured from the Department of Environmental Sciences, Bharathiar University, Coimbatore 641 046, Tamil Nadu, *Epidermophyton floccosum* and *Trichophyton mentagrophytes* were obtained from the Department of Dermatology, Sri Ramachandra Medical College and Research Institute, Porur, Tamil Nadu, and the remaining strains were procured from Microbial Type culture collection (MTCC) at the Institute of Microbial Technology (IMTECH), Chandigarh.

3.2.2. Media Used

Mueller–Hinton Agar (MHA), Mueller–Hinton Broth (MHB), Nutrient Broth (NB), Sabourad Dextrose Agar (SDA), and Sabourad Dextrose Broth (SDB) were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India, and used for testing the antibacterial and antifungal activities.

3.2.3 Determination of Antibacterial Activity

Concentration of the Test Extracts

Different concentrations of the test extracts were prepared by two-fold dilution method for agar-well diffusion (100 mg/ml - 12.5 mg/ml) and minimal inhibitory concentration (MIC) (20 mg/ml - 1.25 mg/ml) assays.
Preparation of Inoculum

The inoculum size of the test strains were standardized according to the National Committee for Clinical Laboratory Standards (NCCLS, 1993) guidelines.

Each test bacterial strain was inoculated into Mueller-Hinton Broth medium and incubated for 3-6 hours at 35°C in a shaker water bath until the culture attained a turbidity of 0.5 McFarland unit. The final inoculum size was adjusted to $10^8$ CFU/ml.

Agar-well Diffusion Assay

Susceptibility tests were performed following Perez et al. (1990) with modifications according to the present experimental conditions (Okunji et al., 1990; Okeke et al., 2001).

A one ml volume of the standard suspension of test bacterial strain was spread evenly on Mueller-Hinton Agar plate using a sterile glass rod spreader and the plates were allowed to dry at room temperature. Subsequently, 6 mm diameter wells were bored in the agar of each plate. Different concentrations of the solvent extracts were (100 mg/ml – 12.5 mg/ml) added into the wells using micropipettes and allowed for diffusion at room temperature for 2 h. The plates were incubated at 37°C for 24 h. The solvent without extracts served as negative control. Standard antibiotics such as ampicillin – 10 μ/disc, erythromycin 10 μ/disc, kanamycin – 30 μ/disc, methicillin – 5μ/disc, nalidixic acid – 30 μ/disc, rifampicin – 30 μ/disc, tetracycline 10 μ/disc, gentamicin – 10 μ/disc and trimethoprim – 10 μ/disc were used as positive controls. After 24 h of incubation, diameter of the inhibition zone was recorded in mm. The experiment was repeated thrice and the average values were calculated.
**Determination of Minimal Inhibitory Concentration (MIC)**

MICs of the plant test extracts were determined by macro broth dilution assay method (NCCLS, 1993). Two fold serial dilutions of the test extracts (20 mg/ml – 1.25 mg/ml) were prepared in tubes with Mueller-Hinton Broth (MHB) as diluent. Each dilution was seeded with test organism to the standard concentration (10⁸ CFU/ml). The tubes were incubated at 37°C for 24h. MIC was taken as the lowest concentration of extract that completely inhibited the bacterial growth, indicated by the lack of visual turbidity.

**Determination of Minimal Bactericidal Concentration (MBC)**

MBC determination was done by aspirating 0.1 ml of the culture medium from each tube (in the Macrobroth MIC assay) showing no apparent growth and sub-culturing it on fresh MHA. The latter was incubated at 37°C for 24 h. The MBC was read as the least concentration showing no visible growth on MHA subculture.

**3.2.4 Determination of Antifungal Activity**

**Agar-well Diffusion Assay**

The antifungal activity was determined by the Agar-well diffusion method following Perez et al. (1990) with modifications according to the present experimental conditions (Okunji et al., 1990; Okeke et al., 2001). All the stock cultures were maintained in sabourad dextrose agar. Inoculums for Candida albicans were prepared by spread plating of 24 h old culture grown in sabourad broth. For Aspergillus flavus, Aspergillus fumigatus, and Aspergillus niger, 10⁴ spore/ml of fungi was uniformly distributed on the surface of SDA plates with the help of sterile cotton swab. For the dermatophytes, inoculation was done by taking a piece of fungal colony on a sterile cotton swab and gently swabbing on the surface uniformly.
The plates were allowed to dry at room temperature. Subsequently, 6 mm diameter wells were bored in the agar of each plate. Different concentrations (100 mg/ml – 12.5 mg/ml) of the solvent extracts were added into the wells using micropipettes and allowed for diffusion, as was done for the antibacterial assay. The plates were incubated at similar temperature of 28°C but different time periods such as 24–48 h for *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger* and *Candida albicans*, and 72–96 h for dermatophytes (and more depending on the incubation time required for a visible growth). The solvent without extracts served as negative control. Standard antibiotics of ketoconzole (20 µg/disc) and chloromphenicol (30 µg/disc) were used as positive controls. After the required incubation period, diameter of the inhibition zone was recorded in mm. The experiment was repeated thrice and the average values were calculated.

**Determination of Minimal Inhibitory Concentration**

The assay was performed by NCCLS (1997) protocol as per Rajarajan *et al.* (2002). Inoculums of each test fungus were standardized adopting spectrophotometric method. All the test strains, which were freshly sub-cultured on sabourad dextrose agar plates, were used for the study. A 100 µl each of the standardized inoculum was added to the respective tubes, including the control which was devoid of extract. The inoculated test tubes with various concentrations of 20 mg/ml–1.25 mg/ml of the test extracts and respective test strains were incubated at 25°C till 48 h in the case of *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger* and *Candida albicans*) and 25°C for 96 h in the case of dermatophytes. Then, the test tubes were observed for the presence or absence of turbidity in comparison to that of the control. The lowest concentration of the extract that inhibited the growth
of each fungus, detected by the lack of visual turbidity, was taken as MIC value of the extract for the test fungus.

**Determination of Minimum Fungicidal Concentration (MFC)**

The MFC was determined using the method of Rotimi *et al.* (1988). The test tubes which showed no visible growth (in the MIC assay) after 96 h of incubation were sub-cultured on extract free SDA plates and incubated at room temperature for 5–7 days. The MFC was regarded as the lowest concentration that prevented the growth of any fungal colony on the solid medium.

### 3.3 PHARMACOLOGY

#### 3.3.1 Plant Material

The various solvent extracts of the plant (hexane, chloroform and methanol) and an isolated compound, bergenin, were used for pharmacological studies.

#### 3.3.2 Preparation of the Drugs

The various solvent extracts of the plant such as hexane, chloroform, methanol and bergenin and other standard drugs such as silymarin, indomethacin and glibenclamide were administered orally in the form of suspension in water with 1% w/v sodium carboxy methyl cellulose (SCMC) as the suspending agent.

#### 3.3.3 Animals

Male Wistar albino rats (150–250 g) and male and female Swiss albino mice (25–35 g) were used depending upon the study. The animals were maintained in the Animal House of Arulmigu Kalasalingam College of Pharmacy (Registration No. 509/01/C/CPCSEA/2002), Department of Pharmacology, Anand Nagar, Krishnan Kovil, 629 190, Tamil Nadu. They were initially acclimatized for the study, housed in polypropylene cages and maintained at 24 ± 2°C with relative humidity of 45-50% and equal 12 hour light and 12 hour dark cycles. The animals were fed *ad libitum*
with standard pellet diet (Lipton India Ltd., Bombay) and had free access to water. The animals before test had 24 h fast but water was given to them *ad libitum* whenever required.

All the pharmacological and toxicological experimental protocols were approved by the Institutional Animal Ethics committee (IAEC) for the purpose of control and supervision on experimentation on Animals (CPCSEA), New Delhi, vide sanction on 12th March 2007.

### 3.3.4 Chemicals

Fine chemicals were purchased from Sigma-Aldrich, St. Louis, MO 63103, and S.D. Fine Chemicals, Mumbai, India, and other chemicals from SISCO Research Laboratories Pvt. Ltd., Mumbai, India.

### 3.3.5 Acute Toxicity Studies

The oral acute toxic class method (Roll *et al.*, 1986) was performed as per the Organization for Economic Co-operation and Development (OECD) 423 guidelines. Swiss albino female mice weighing 15-25 g were fasted overnight, provided with water *ad libitum* and divided into groups of six animals each. The test extracts such as hexane, chloroform, methanol and bergenin were administered orally at the initial dose of 5 mg/kg body weight by intragastric tube and observed for 1 week. The animals were observed continuously for 2 h and then at half hourly interval for the next 6 h, 24 h, for observing changes in gross general behavior and daily for 1 week for any possible drug-induced mortality. Since, there was no mortality with 5 mg/kg for 1 week the procedure was repeated for next higher doses such as 50, 500 and 2000 mg/kg for all the test extracts such as hexane, chloroform, methanol and bergenin.
3.3.6 Analgesic Activity

3.3.6.1 Hot-Plate Method (Eddy and Leimback, 1953; Turner, 1965)

Wistar albino mice (25–30 g) were divided into eight groups, each consists of six animals. Group 1 animals served as vehicle control and received only 1% w/v SCMC (10 ml/kg, p.o.). Group 2 animals received MO (5 mg/kg, S.C.). Groups 3 and 4 animals received hexane extract 100 & 200 mg/kg, p.o., respectively. Groups 5 and 6 animals received chloroform extract 100 and 200 mg/kg/p.o., respectively. Groups 7 and 8 animals received methanol extract 100 and 200 mg/kg, p.o., respectively. Morphine (MO) injection was used as the standard drug and the equipment was Eddy’s hot plate (Techno).

The animals were placed on the hot plate which maintained at constant temperature 55°C ± 5°C. Basal reaction time was recorded by observing hind paw licking or jump response (whichever appeared first) in animals. Normally, the response was obtained within 6 – 8 seconds. A cut off period of 15 seconds was observed to avoid damage to the paws. The time of reaction to pain stimulus (interval between placing the mice in the hot plate and the lick or jump response) of the mice was recorded at every 15, 30, 60 and 120 min after drug administration. The percentage increase in reaction time was taken as the index of analgesia.

3.3.6.2 Acetic acid-induced Writhing in Mice (Kulkarni, 1999)

Wistar albino mice (25 – 30g) were divided into eight groups, each consists of six animals. Group 1 animals received only 1% w/v SCMC (10 ml/kg, p.o.). Group 2 animals received indomethacin 100 mg/kg, p.o. Groups 3 to 8 animals received the test extracts (as like that of as mentioned in) hot plate method.

All the extracts and the standard drug were administered orally using intragastric tube, 15 min prior to the administration of acetic acid injection. All the
animals received intraperitoneal injection (i.p.) of 3% v/v of acetic acid (1 ml/100 g), 30 min after the administration of the test drugs and the standard drug indomethacin.

The number of writhings (the constriction of abdominal muscle together with stretching of the hind limbs) produced by each animal was observed individually under a glass jar for a period of 20 min and the same was counted.

A significant reduction in the number of writhes when compared with vehicle treated animals was considered as antinociceptive response. The % protection of analgesic activity was calculated using the formula C-T/C X 100 where C is the number of writhings in control group and T is the number of writhings in the treated group.

3.3.7 Anti-inflammatory Activity

Carrageenan–induced Paw Oedema Method (Winter et al., 1962)

The anti-inflammatory effect of the various solvent extracts was studied using carrageenan-induced hind paw oedema model. The most widely used primary test to screen new anti-inflammatory agents measures the ability of a compound to reduce local oedema-induced in rat paw by injection of an irritant – carrageenan. Carrageenan is a sulphated polysaccharide obtained from seaweed (Rhodophyceae) and causes the release of histamine, 5–HT, bradykinin and prostaglandins it thus produces inflammation and oedema.

The rats were divided into eight groups, each consists of six animals. Group–1 animals served as vehicle control and received only 1% w/v SCMC (10 ml/kg, p.o.). Group 2 animals received indomethacin (10 mg/kg, p.o.). Indomethacin was employed as the standard drug. Groups 3 and 4 animals received hexane extract 100 and 200 mg/kg, p.o., respectively. Groups 5 and 6 animals received chloroform
extract 100 and 200 mg/kg/p.o., respectively. Groups 7 and 8 animals received methanol extract 100 and 200 mg/kg/p.o., respectively.

A mark was made on both the hind paws (right and left) just beyond tibiotarsal junction so that every time the paw was dipped in the mercury column up to the fixed mark to ensure constant paw volume. The initial paw volume (both right and left) of each rat was measured by mercury displacement. After 30 min of drug administration, 0.1 ml of 1% (w/v) carrageenan was injected in the right hind paw, sub-planar region of each rat. The left paw served as reference (non-inflammatory paw) for comparison. The paw volumes of both the legs of control and test extracts (drugs) treated rats were measured at 0 h, 1 h, 3 h, and 5 h after carrageenan administration.

The mean increase in paw volume and the percentage inhibition of inflammatory swelling were calculated. The percentage inhibition of paw volume of the different test extracts were compared with that of control. The percentage inhibition of inflammatory swelling was calculated using the formula C-T/C x 100 where C was the oedema rate of control group and T was treated group.

3.3.8 In vitro Antioxidant Activity

3.3.8.1 DPPH Radical Scavenging Assay by Spectrophotometric Method (Sree Jeyan and Rao, 1996).

Chemicals

1, 1 diphenyl–1–2–picryl hydrazyl (DPPH) was obtained from the Sigma-Aldrich, St. Louis, MO 63103, naphthylene diamine dichloride was obtained from S.D. Fine Chemicals, Mumbai, India. All other reagents used were of analytical grade.
Experimental Protocol

The free radical scavenging activity of 100 µg/ml each of hexane, chloroform and methanol extracts of the plant was examined using DPPH radical.

To an ethanolic solution of DPPH (200 µl), 0.05 ml each of the test extracts (100 µg/ml concentration) was added. An equal amount of ethanol was added to the control. After 30 min, decrease in the absorbance of the test mixture was read at 517 nm. The experiment was performed in triplicate and the percentage inhibition was calculated according to the eq.1.

\[
\text{Inhibition percentage (\%)} = \left( \frac{[\text{AC}(0) - \text{AA}(t)]}{\text{AC}(0)} \right) \times 100 \quad \text{eq.1}
\]

Where AC (0) is an absorbance of control DPPH solution at 0 minute and AA (t) is an absorbance of test sample at 30 min. The antioxidant activity was compared with vitamin C which was used as the standard antioxidant.

3.3.8.2 Nitric oxide Radical Scavenging Assay (Sree Jayan and Rao, 1997)

Chemicals

Sodium nitroprusside, naphthyl ethylene diamine and sulphanilamide were obtained from S.D. Fine Chemicals, Mumbai, India.

Experimental Protocol

Sodium nitroprusside (5 mM) in phosphate-buffered saline was incubated at 25°C for 150 min, with different test extracts of 100 µg/ml each of hexane, chloroform and methanol which were dissolved in standard phosphate buffer. After 150 min, 0.5 ml of incubated solution was removed and diluted with 0.5 ml of griess reagent (prepared by mixing equal volume of 1% w/v sulphanilamide in 2% v/v phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride in water) and
the absorbance of chromophore formed was read at 546 nm. The control experiment was also carried out in similar manner.

The experiment was performed in triplicate and the percentage reduction in absorbance was calculated using the following formula:

\[\frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100\]

The activity was compared with vitamin C, which was used as standard antioxidant.

3.3.9 Wound Healing Activity

3.3.9.1 Excision Wound Model (Udupa et al., 1994a; Saha et al., 1997).

This model was used to monitor wound contraction and formation of epithelization time.

Materials

Simple ointment B.P., 0.2% w/w nitrofurazone ointment (as the reference standard), 0.5 g of (5% w/w) each test extract ointment (where 5 g of each test extract such as hexane, chloroform and methanol extracts were incorporated in 100 g of simple ointment base B.P. (Anonymous, 1993) was applied once daily, till the wound was completely healed.

Five groups of animals (male Wistar albino rats 150 – 180 g) containing six animals in each group were anesthetized with ether. The rats were depilated on the back and a predetermined area of 500 mm² full thickness skin was excised in the dorsal intercapsular region. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. Wounds of rats were left undressed to the open environment.

The group 1 animals served as control and treated only with simple ointment base B.P. The group 2 animals were served as reference standard and treated with
0.2% w/w nitrofurazone ointment. Animals of groups 3, 4 and 5 were treated with 0.5 g of the hexane, chloroform and methanol extract ointments respectively. The treatment was continued till the wound was completely healed.

The progressive changes in wound area were monitored planimetrically by tracing the wound margin on a graph paper every alternate day. The changes in healing of wound, i.e. the measurement of wound on graph paper, were expressed as unit mm$^2$. Wound contraction was expressed as percentage reduction of original wound size.

3.3.9.2 Incision Wound Model (Udupa et al., 1994 b)

This model was employed to assess the breaking strength of skin in rats.

**Materials**

Black silk surgical thread (No. 000), curved needle (No. 11), simple ointment B.P, 0.2% w/w nitrofurazone ointment (as the reference standard), 0.5 g of 5% w/w test extracts such as hexane, chloroform and methanol extracts as ointments. The ointments were applied to the wound twice daily until complete recovery.

Five groups with six animals (male wistar albino rats 150–180 g) in each group were anesthetized under light ether anesthesia, by the open mask method. Two para vertebral long incisions were made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of the depilated back of the rat. Full aseptic measures were not taken and no local or systemic antimicrobials were used throughout the experiment (Udupa et al., 1994 b) After the incision was made the parted skin was kept together and stitched with black silk surgical thread (No 000) at 0.5 cm intervals using a curved needle (No.11). The continuous threads on both the wound edges were tightened for good adaptation of wound and the wound was left undressed.
All the groups were treated in the same manner as mentioned in the case of the excision wound model such as the group 1 animals (control) were treated only with simple ointment base B.P., group 2 animals were treated with 0.2% w/w nitrofurazon ointment and the animals of the groups 3, 4 and 5 were treated with 5% w/w, 0.5 g of the test extract ointments such as hexane, chloroform and methanol extracts respectively throughout the period twice daily for nine days. When wounds were cured thoroughly the sutures were removed on the ninth day and tensile strength was measured using a tensiometer.

3.3.10 Antidiabetic Activity

**Streptozotocin (STZ)-induced Diabetes** (Elfellah *et al.*, 1984; Sood *et al.*, 2000)

**Materials**

Hexane, chloroform and methanol extracts of the plant were prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals. For inducing diabetes STZ in a citrate buffer (pH 4.5) was employed. Glibenclamide 10 mg/kg was used as a standard drug.

**Experimental Protocol**

**Induction of Diabetes**

Diabetes was induced into the 16 h fasted rats by a single intravenous injection of 50 mg/kg STZ in a citrate buffer with pH 4.5 (Basnet *et al.*, 1994). Diabetes was confirmed by the presence of serum glucose levels higher than 300 mg/dl, 2 days after the STZ treatment (Elfellah *et al.*, 1984; Sood *et al.*, 2000).

**Antihyperglycemic Screening**

The rats were divided into 8 groups of six animals each. Group 1 served as diabetic control and received only 1% w/v SCMC. Group 2 served as positive control and received glibenclamide 10 mg/kg. Groups 3 and 4 received hexane extract 100
mg, 200 mg/kg, p.o., respectively. Groups 5 and 6 received chloroform extract 100 mg, 200 mg/kg, p.o., respectively and Groups 7 and 8 received methanol extract 100, 200 mg/kg, p.o., respectively. The treatment was continued for 8 days by administering the respective extracts/drug or 1% w/v SCMC twice daily.

Collection and Processing of Blood for Estimation of Glucose and Biochemical Parameters

On the ninth day of the therapy, blood samples (1 ml) were collected from the tail vein under mild ether anesthesia in Eppendorf tubes containing 100 µl of anticoagulant (10% tri sodium citrate solution).

Plasma was separated by centrifuging the samples at 5000 rpm for 10 min and stored in a refrigerator until analysis. The plasma was analyzed for glucose, total protein, cholesterol, triglycerides, alkaline phosphatase (ALP), aspartate aminotransferase (ASAT) formerly called glutamic oxaloacetic transaminase (GOT) and alanine aminotransferase (ALAT) formerly called glutamic pyruvic transaminase (GPT) using standard procedures in an autoanalyzer, Microlab 200 using Ecoline kits (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India).

3.3.11 Antiarthritic Activity

3.3.11.1 Complete Freund’s Adjuvant (CFA)-induced Arthritis (Pearson, 1956)

Materials

The various test extracts such as hexane, chloroform, methanol and bergenin, standard drug indomethacin were prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals.

Preparation of Complete Freund’s Adjuvant (CFA)

Twenty five mg of heat killed Mycobacterium tuberculosis cells (being killed at 60°C in 5–20 min in an Autoclave) was finely grounded using mortar and pestle
with sufficient amount of liquid paraffin, referred to as complete freund's adjuvant. The liquid paraffin alone was referred in the study as incomplete freund's adjuvant.

**Induction of Arthritis**

Arthritis was induced by a single intra dermal injection of 0.1 ml of complete freund's adjuvant (CFA) containing 1 mg dry heat killed *Mycobacterium tuberculosis* per ml sterile paraffin oil into a foot pad of the left hind paw of male rats (Mizushima *et al.*, 1972). A glass syringe (1 ml) with locking hubs and a 26G needle were used for injection. The rats were anesthetized with ether inhalation prior to and during adjuvant injection as the very viscous nature of the adjuvant exerted difficulty while injecting. The swelling in hind paws was periodically examined in each paw from the ankle using Plethysmography (Winter *et al.*, 1962).

**Experimental Setup**

The animals (male wistar rats 200–250 g) were divided into ten groups of six animals in each group as follows:

- Group 1 animals served as inflamed control group and received only normal saline.
- Group 2 animals served as non-inflamed control group, consisted of rats injected with 0.1 ml of liquid paraffin alone.
- Group 3 animals received 10 mg/kg/p.o. of the standard drug indomethacin.
- Group 4 animals treated with bergenin 50 mg/kg/p.o., Groups 5 and 6 animals treated with the hexane extract 100 and 200 mg/kg/p.o., respectively.
- Groups 7 and 8 animals administered with the chloroform extract 100 and 200 mg/kg/p.o., respectively.
- Groups 9 and 10 animals treated with the methanol extract 100 and 200 mg/kg/p.o., respectively.

The test groups consisted of complete freund's adjuvant-injected rats challenged with doses of the test drugs administered orally 18 h and 2 h before induction of arthritis. The drug administrations were continued daily at the same time of the day for 19 more days.
Assessment of Arthritis

Arthritis was assessed by physical and biochemical measurements as well as by radiographic analysis. Initial and successive body weight changes and paw volume changes were measured and recorded. The body weight changes of the various groups were recorded at periodic intervals up to the day 19. Development of adjuvant-induced swelling in the paws of the injected and non-injected limbs of each rat was monitored daily as the percentage increase in paw volume.

Biochemical Studies

As there was dose-dependent inhibition observed in the hind paw swelling only the animals treated with the higher doses of the test extracts were selected for the biochemical studies such as hexane, chloroform, methanol, 200 mg/kg/p.o. respectively and bergenin 50mg/kg/p.o.

Rats were killed on day 19 and blood was collected from the various groups and serum was separated. The liver, kidney and spleen were dissected out from the body and washed with cold saline and their weights were recorded. Then, they were cut into small pieces and homogenized using buffer (pH 7.4) to prepare a 10% homogenate. This was centrifuged at 12,000 g for 30 min (Latha et al., 1998). The supernatant fluid was used for the assay of various enzymes. Estimations were done such as aminotransferases by King (1965 a) using sodium pyruvate as standard, acid phosphatase by King (1965 b) using disodium phenylphosphate as substrate, and Cathepsin-D by the modified method of Etherington (1972).

Radiographic Analysis

On the last day of experiment, before collecting blood and tissues for biochemical parameters, the rats were placed on a radiographic box at a distance of 90 cm from X-ray source. Radiographic analysis of arthritic hind paws was performed
by X-ray machine (Univet LX 160), Multimage, Cavaria, Italy) with a 40-kw exposition for 0.01S (Rojasa et al., 2003).

3.3.12 Immunomodulatory Properties

Materials

Antigen (SRBC)

Fresh blood of a healthy sheep was collected from the local slaughter house in a mixture of 0.49% ethylene diamine tetraacetic acid (EDTA) and 0.9% sodium chloride solution. It was preserved at a temperature from 2-8°C. On the day of immunization, the blood sample was centrifuged at 5000 rpm for 10 min and then washed thrice to remove plasma with 0.9% sodium chloride solution and adjusted to a concentration of 0.1 ml containing 1 \times 10^8 cells for immunization and challenge.

Culture

_Candida albicans_ was purchased from the IMTECH, Chandigarh, India.

Standard Drug

The standard drug cyclophosphamide was obtained from Sigma-Aldrich St. Louis, MO 63103. The test extracts such as hexane, chloroform and methanol and the standard drug cyclophosphamide were prepared in the form of 1% w/v SCMC suspension.

Animals

Swiss albino mice weighing 18-25 g and albino rats (150 to 200 g) were used.
Methods

3.3.12.1 Delayed Type Hypersensitivity Reaction using SRBC as an Antigen (Gokhale et al., 2003)

Mice were divided into eight groups of six in each group. Group 1 animals served as vehicle control and received 1% w/v of SCMC (10 ml/kg, p.o.). The Group 2 animals treated with the immunosuppressive drug cyclophosphamide 50 mg/kg/p.o.

Groups 3 and 4 animals were administered with the hexane extract 100 and 200 mg/kg/p.o., respectively. Groups 5 and 6 animals were treated with the chloroform extract 100 and 200 mg/kg/p.o., respectively. Groups 7 and 8 animals were received methanol extract 100 and 200 mg/kg/p.o., respectively. In all the groups ranging from 2 to 8, the respective drugs were administered on day 0 and have been lasted till the day of challenge. The mice were primed with 0.1 ml of SRBC suspension containing $1 \times 10^8$ cells, i.p., on day 7 and challenged on day 14 with 0.05 ml of $2 \times 10^8$ SRBC in the right hind foot pad. The contralateral paw received equal volume of saline. The paw volume was determined after injection of SRBC challenges and at after 72 h by Plethysmographic method.

3.3.12.2 Humoral Antibody Response to SRBC (Gokhale et al., 2003)

The mice were divided into eight groups of six in each group. Group 1 animals served as vehicle control and received only 1% w/v of SCMC (10 ml/kg p.o.). Group 2 animals were treated with cyclophosphamide (50 mg/kg/p.o.) was administered 2 days before the experiment.

Groups 3 to 8 animals were treated with the test extracts (as like that of/as mentioned in) the previous test (delayed type hypersensitivity reaction). In all these groups from 3 to 8, the treatment was started on 0th day and has been lasted till the day of experiment. On day 7, the mice were immunized with 0.1 ml of $1 \times 10^8$ SRBC
Blood samples were collected from the orbital plexuses of individual animals on day 14 and the antibody titres were determined. After preparing sera from peripheral blood, aliquots (25 μl) of two-fold diluted sera in saline were challenged with 25 μl of 1% v/v SRBC suspension in microtitre plates. The plates were incubated at 37°C for 1 h and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre.

3.3.12.3 Non-specific Immunity Determined by Survival Rate against Fungal Infection (Thakur et al., 2007)

Wistar albino rats (150 to 200 g) were used. The rats were divided into 5 groups comprising of six animals each. The group 1 animals served as vehicle control and were administered with 1% w/v SCMC only. Group 2 animals received 200 mg/kg hexane extract and Groups 3 and 4 animals received 200 mg/kg of chloroform and methanol extracts, respectively. Treatments of all the four groups started 14 days before challenge. On the day of challenge, all the groups were injected with 5 x 10^7 viable Candida albicans cells and observed daily for mortality for a period of 10 days.

3.3.12.4 Macrophage phagocytosis by carbon clearance method (Jayathiratha and Mishra, 2004)

Swiss albino mice of either sex, weighing 20-25 g were divided into seven groups comprising six animals each. The group 1 animals served as vehicle control and were administered with 1% w/v SCMC only. Groups 2 and 3 animals received methanol extract of 200 and 100 mg/kg/p.o. respectively. Groups 4 and 5 animals received hexane extract of 200 and 100 mg/kg/p.o. respectively and groups 6 and 7 animals treated with chloroform extract of 200 and 100 mg/kg/p.o. respectively.

The treatment was continued for five days. At the end of five days, after 48 h, mice were injected via the tail vein with carbon ink suspension (10 μl/g body weight)
Blood samples were collected (in EDTA solution, 5 µl) from the retro-orbital vein at 0 and 15 min, and 25 µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and its absorbance at 660 nm was determined. The phagocytic index K was calculated using the following equation:

\[
K = \log_{OD1} - \log_{OD2}/15, \quad \text{where OD1 and OD2 were the optical densities at 0 and 15 min, respectively.}
\]

### 3.3.12.5 Cyclophosphamide-induced Myelosuppression Assay (Gokhale et al., 2003; Jayathiratha and Mishra, 2004)

Swiss albino mice were divided into eight groups of six animals in each group. Group 1 animals served as vehicle control and received only 1% w/v of SCMC. Group 2 animals served as cyclophosphamide control. Groups 3 and 4 animals treated with hexane extract of 100 and 200 mg/kg/p.o. respectively. Groups 5 and 6 animals administered with chloroform extract of 100 and 200 mg/kg/p.o. respectively and groups 7 and 8 animals received methanol extract of 100 and 200 mg/kg/p.o. respectively. Animals treated with the test extracts (Groups 3 to 8), administration of extracts started 13 days prior to administration of cyclophosphamide. On 11th, 12th and 13th days, all the animals except group 1 were administered with cyclophosphamide 30 mg/kg/p.o. 1 h after the administration of extract. Blood samples were collected on 14th day from retro-orbital plexuses of individual animals and the total white blood cell (WBC) count was determined.

### 3.3.13 Hepatoprotective Studies

#### 3.3.13.1 In vitro Inactivation of HBsAg

The assay was performed using ETI-MAK-4HBsAg Kit followed by modified version of Venkateswaran et al. method (1987). Serial dilutions of the methanol extract of the plant and bergenin were mixed with an equal volume of sera positive for
HBsAg and the mixture was incubated for 60 min at 37°C. The evaluation was carried out using the procedure as stated in the Kit insert. Briefly anti-HBs (sheep) coated microtitre wells were filled with 100 µl of serum drug suspension and incubated for 60 min at 37°C followed by anti-HBs conjugate addition. Each step was followed by washing and finally chromogen treatment was done. The reaction was stopped by addition of 0.5 M sulphuric acid. The results were read on Micro Elisa reader. The cut off value for each set of screening was calculated using the mean absorbance of negative controls. Samples with an absorbance either equal or greater than the cut off were considered as HBsAg positive. Inactivation of HBsAg activity was expressed as the decrease (in per cent) in the absorption of the test sample compared to that of control.

3.3.13.2 Isoniazid (INH) and Rifampicin (RMP)-induced Hepatic Injury in Rats

(Tasduq et al., 2005 b; Pal et al., 2006)

Materials

The various solvent test extracts such as hexane, chloroform, methanol, bergenin and the standard drug silymarin were prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals. INH and RMP solutions were prepared separately with sterile distilled water. The pH of rifampicin solution was adjusted to 3.0 with 0.1 mol/L HCl (Bahri et al., 1981).

Isoniazid and rifampicin were procured from the Lupin Pharmaceuticals Ltd., Mumbai, and silymarin from the Silybon, Microlabs, Bangalore, was used as standard drug.

The serum marker enzymes and other biochemical markers were estimated such as aspartate transaminase (AST) and alanine transaminase (ALT) by Reitman & Frankel (1957), alkaline phosphatase (ALP) by Kind & King (1954), bilirubin (BILN)
by Malloy & Evelyn (1937), total proteins (TPN) by Lowry et al. (1951), triglycerides (TGL) by Rice (1970) and total cholesterol (CHL) by Pattabiraman (2004).

**Experimental Protocol**

The male Wistar albino rats weighing 150–200 g were randomly divided into 7 groups of six animals in each group. Group 1 served as vehicle control and received only 1% w/v SCMC (10 ml/kg, p.o.) for 9 days. Group 2 served as toxic control and received INH + RMP (50 mg/kg, p.o.) per day for 9 days. Group 3 animals served as standard drug control and received silymarin 100 mg/kg/p.o., along with INH+RMP 50 mg/kg/p.o. for nine days. Group 4 animals received bergenin 200 mg/kg/p.o., along with INH + RMP p.o. for nine days. Groups 5, 6 and 7 animals received 200 mg/kg each of hexane, chloroform and methanol extracts respectively along with the INH + RMP 50 mg/kg/p.o. for nine days respectively.

The test drugs were administered orally half-an-hour before the INH + RMP (50 mg/kg/p.o.) doses in groups 3, 4, 5, 6 and 7.

**Biochemical Analysis**

After the experimental period, the blood samples were collected by sinus orbital puncture using sterilized capillary tube under light ether anesthesia in sterile vials from all the groups. Coagulation was permitted and serum was separated from blood by centrifugation (3000 rpm for 15 min) and then the serum was subjected to the analysis of various biochemical parameters such as AST, ALT, ALP, BILN, TPN, TGL and CHL.

**Histopathological Examination**

On 10th day, after withdrawal of the blood, the rats were sacrificed by the cervical dislocation. The liver was quickly dissected out from the animals and washed separately with normal saline and processed for dehydration, infiltration and
embedding. Initially, the materials were fixed in 10% buffered neutral formalin for 48 h and then with bovine solution for 6 h. They were infiltered and embedded with paraffin. The microtome sections were taken at 5 μ thickness, processed in alcohol–xylene series, stained with alum haematoxylene and eosin and examined under microscope for the evaluation of histopathological changes.

3.3.14 Statistical Analysis

All the data were statistically evaluated with SPSS 10 software for windows. The data were expressed as mean ± standard error for mean (SEM). The difference among means has been analyzed by Student's t-test (Woolson, 1987). A value of P<0.05 was considered statistically significant.