MATERIALS AND METHODS
MATERIALS

Chemicals

Inorganic compounds, chemicals and the acids used were of Analytical Reagent (AR) grade of Merck (India) Ltd. and the reference chemicals were purchased from Sigma (India).

AR grade solvents used for the extraction and isolation of compounds and High Performance Liquid Chromatography (HPLC) grade solvents used for the separation of compounds in HPTLC, were procured from the Merck (India) Limited. High Performance Thin Layer Chromatography (HPTLC) Silica gel G 60 F<sub>254</sub> purchased from Merck (India) Limited. Ethyl alcohol (having <4% moisture) was obtained from the Distillery Division of Sakthi Sugars, Sakthi Nagar, Tamilnadu. The water used for the various analyses was collected from Elix-3 (Millipore) water purification system. It had a maximum conductivity limit of 18mΩ.

Nutrient broth for microbial investigation was purchased from the Hi Media. The broth containing 2% agar was used for microbiological analysis. Wherever necessary, soft agar containing 0.7% agar was used.

Glass wares

All the glass wares used were of the “Borosil” grade. Standard volumetric flasks and the pipettes were of ‘A’ grade quality.
INSTRUMENTS

High Performance Thin Layer Chromatography (HPTLC)

Camag Linomat 5 is a semiautomatic sample applicator used for qualitative and quantitative analyses as well as for preparative separations. The key features are: operation in stand-alone mode or under winCATS software; sample application as narrow bands using the spray-on technique; application of solutions on to any planar medium and semi-automatic. The samples are changed (cleaning, filling and replacing the syringe) manually.

Linomat 5 is controlled by winCATS Planar Chromatography Manager. Plate dimensions, and distance of tracks, names of samples and volumes to be applied on to each track are conveniently programmed and saved in winCATS. All information is then available for later densitometry evaluation. The Camag Linomat 5 can be Installation Qualification (IQ) / Operation Qualification (OQ) qualified, used in a Good Manufacturing Practices (GMP)/ Good Laboratory Practices (GLP) environment.

Chromatogram development

Planar chromatography differs from all other chromatographic techniques in the fact that a gas phase in addition to stationary and mobile phase is used and it significantly increases the efficiency of separation of compounds. Systematic optimization of developing conditions in HPTLC, optimization of the developing
solvent, after uniform layer preconditioning were standardized according to the conditions required. Camag twin trough chamber offers several ways to improve the results of TLC/HPTLC developing techniques. Low solvent consumption, reproducible pre-equilibration with solvent vapor and gas phase conditioning with any conditioning liquid or volatile reagent are some of the features.

**Camag TLC Scanner 3**

Camag TLC Scanner 3 is incorporated in the novel integrated software concept “winCATS – Planar Chromatography Manager” via the corresponding Equilink. winCATS controls all mechanical and electronic functions of the scanner and acquires all data for processing result computation and reporting. WinCATS also hosts and organizes all scanner related software options.

**Camag Reprostar 3**

The Camag Reprostar 3 – Camag Digistore Documentation System with digital camera, fulfilled documentation and evaluation of planar chromatograms with image processing. Camag immersion device consists of a plate holder, with battery operated, uniform vertical speed, immersion time selector.

**Laminar airflow chamber**

Laminar airflow chamber manufactured by Atlantis, Laminar air flow systems, New Delhi, consists of a High Efficiency Particulated Air (HEPA) filter, through
which air is filtered (<0.45 micron) and sent out. The ultra violet lamp fixed in this instrument was used for sterilizing the work-bench area.

**Moisture Meter**

Manufactured by Advance Research Instruments Company, India (Model M38 Deluxe) was used for measuring moisture content in plant samples.

**High Vacuum Flash Evaporator with Pump**

Flash evaporator comprising of a Thermostat control water bath, SuperFit glass condenser attached with vacuum pump and a speed regulator manufactured by SuperFit, India was used for evaporation of solvents from the extracts of the plants under reduced temperature and pressure (50°C and -730mm Hg).

**Capsule Filling Machine and Accessories**

A manual capsule filling machine manufactured by Gowtham Pharma Distributors, India (Model: 1184) was used for filling 300 capsules of '0' size at a time. The drugs prepared for the clinical trial was filled in capsules using this machine, then placed in a clean, dry, sterilized blisters and manually sealed with Pharma grade aluminum foil.

**Plant species used**

The following plant species were committed in the present investigation:
(a) dried flower buds and flowers of *Bombax malabaricum* DC (Silk cotton tree),
(b) dried leaves of *Bryophyllum pinnatum* (Lam) Oken.Kurz (Sprout leaf plant),
(c) dried flowers of *Butea monosperma* (Lam) Taub. (Flame of the forest) (d) dried plant of *Euphorbia microphylla* Sprengel. and (e) dry fruits of *Tribulus terrestris* L. (Puncture vine Clatrap).

**Others**

Hard gelatin capsules of Pharmagel A (pH 4.8 – 5.2 in water) with low disintegration time, having a shell size of ‘O’, that can hold at least 500mg of the drug was purchased from Gowtham Pharma, Chennai, India. Pharma grade plastic blisters with 20 X 6 bubbles per sheet were purchased from Gowtham Pharma, Chennai, India for packing capsules. The sheet was cut into 6 pieces each having 20 bubbles and used for the packing purposes. Aluminum foil with thickness of 0.2mm was used for packing the blisters. Capsule dye to seal, a wooden frame carved to the size to hold the plastic blister sheet with 20 bubbles was used as the capsule sealing dye for blister packing.

**METHODS**

**Collection of Plants**

Fresh flower buds and flowers of *Bombax malabaricum* DC (Silk cotton tree, Bombacaceae), fresh leaves of *Bryophyllum pinnatum*(Lam)Oken.Kurz (Sprout leaf plant, Crassulaceae), fresh flowers of *Butea monosperma* (Lam) Taub.
(Flame of the forest, Fabaceae), fresh whole plants of *Euphorbia microphylla* Sprengel. (Euphorbiaceae) and the fresh fruits of *Tribulus terrestris* L. (Puncture vine Caltrap, Zygophyllaceae) were harvested at three different photoperiods viz. morning (5 AM) noon (12 AM) and night (7 PM).

**Identification of Plants**

All the plants were identified by their morphological characteristics as represented in *FLORA OF THE PRESIDENCY OF MADRAS* (Gamble, 1935) and further authentication was done by comparing with the existing herbarium of THE BOTANICAL SURVEY OF INDIA, Coimbatore - 641 003.

**Drying**

All the plant materials were shade dried at 30°C ± 1°C, RH 70% for 3 days.

**Sizing of Plants**

The plant materials were ground in Wiley mill equipped with 2 mm sieve to obtain uniform size of 2 mm.

**Storing**

The pulverized plant materials were stored in a dark room at room temperature 28 ± 2°C in polythene containers.
**Extraction**

Fresh plants were used for studying chromatographical finger print profiles in different photoperiods and dried plants were used for quality control studies during the manufacturing of the drug. Known individual plant samples (20g) were extracted in a soxhlet apparatus at 55°C for 8h using ethyl alcohol - water (70:30). The hydro alcoholic fraction was cloth filtered subsequently followed by Whatman No.1 filter paper. The wet residue was again re-extracted with 100% ethyl alcohol for another 4h. in the Soxhlet apparatus. Similarly, the alcohol fraction was cloth filtered and Whatman No.1 paper filtered. The hydro alcoholic extract and the alcoholic extracts were pooled and taken for further condensation (Harborne, 1983; Wagner and Bladt, 1996).

**Condensation of the extract**

The pooled extracts of each plant material was concentrated under vacuum at reduced temperature to remove ethyl alcohol and water using a flash evaporator. The pressure and temperature were maintained between - 720 and - 730 mmHg and 50°C respectively for the condensation of ethyl alcohol and water. The concentrated extract having more than 50% TSS (total soluble solids) was stored in a refrigerator below 10°C for the analysis of compounds through chromatography. The extracts were dried completely and used for the preparation of drug. Each plant material extract, thus dried were used in the ratio as given below for the preparation of DCBT5678 formulation.
Table A: Ingredients of DCBT5678

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Plant name</th>
<th>Part(s)</th>
<th>Plant raw material equivalent in mg / capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bombax malabaricum</td>
<td>Flower buds and flowers</td>
<td>330</td>
</tr>
<tr>
<td>2</td>
<td>Bryophyllum pinnatum</td>
<td>Leaves</td>
<td>330</td>
</tr>
<tr>
<td>3</td>
<td>Butea monosperma</td>
<td>Flowers</td>
<td>330</td>
</tr>
<tr>
<td>4</td>
<td>Euphorbia microphylla</td>
<td>Whole plant</td>
<td>330</td>
</tr>
<tr>
<td>5</td>
<td>Tribulus terrestris</td>
<td>Fruits</td>
<td>900</td>
</tr>
</tbody>
</table>

The above table indicated the raw material equivalent of each plant part used in the DCBT5678 formulation where individual hydro-alcoholic (30:70) extracts were blended together, dried under vacuum and used as drug.

**Toxicology Studies**

The formulated drug sample was sent to Shriram Institute for Industrial Research, New Delhi -110 006 for toxicology studies in rats and the acute oral LD$_{50}$ was determined.

- Name of the species: Rattus rattus albanicus
- Strain of the animals: Wistar
- Number of animals used per dose: 5 male, 5 female
- Age of the animals used: 8 to 10 weeks
- Weight range: 160 to 180gms
Acclimatization period : 7 days
Route of administration : oral
Vehicle used : corn oil

The acute oral LD$_{50}$ in male and female rat was found to be $> 5000$mg/kg body weight. A copy of the summary sheet of the findings from the Shriram Institute for Industrial Research is given.

Blisters

Blisters were washed with mild detergent (Tween 20) followed by rinsing in tap water and further by distilled water. They were allowed to dry in shade for 24 hours. The blisters were thoroughly cleaned with a clean tissue paper.

Irradiation

The cleaned, trimmed blisters were sterilized under ultra-violet irradiation (UV-C 320 nm) for half an hour just before packing.

Capsules

The empty green colored '0' sized gelatin dissolvable capsules were also irradiated using ultra violet rays at 320 nm for 30 min just before filling.

Capsule filling

Using a semi automatic capsule-filling machine, 300 capsules were lined in a stainless steel tray with gloved hands under sterile conditions (Laminar flow hood
chamber). Individual extract of each of the plant part from the DCBT5678 formulation was pooled based on the raw material ratio given in table A and lactose was blended to get the appropriate content. One percent of pharmaceutical grade talcum (Magnesium silicate) was mixed with the above formulation for smooth filling in capsules. Thus, 120g of the drug formulation was filled in the 300 capsules manually (400 mg/capsule) that contain the requisite amount of the plant material extracts.

Sealing and locking

The filled capsules were cleaned thoroughly with a soft sterilized towel and sealed in the blisters using the aluminum foils.

Analytical Procedures

High Performance Thin Layer Chromatography

HPTLC was performed on 10cm X 10cm silica gel 60 F254 HPTLC plates, particle size 5-40µm, and layer thickness 0.2mm, from Merck (Darmstadt, Germany). The chamber was pre-saturated with the respective mobile phase by placing Whatman No. 1 filter paper for 10 minutes in the sides before developing in plates. The extracted samples were collected at 3 different photoperiods viz., 5 am, 12 noon and 7 pm were applied (500µg / 5µL) as narrow 8mm bands by means of Camag Linomat 5 (Muttenz, Switzerland) equipped with a 100µL Hamilton syringe. Plates were then developed at room temperature (25°C ± 2) in
a Camag twin trough chamber containing 10ml (v/v) of the respective mobile phase for a distance of 80mm.

After development, the plate was dried for 2 minutes with hair drier and the spots were scanned by Camag TLC Scanner 3 in absorbance/reflectance mode. The software used for data storage and peak area integration was winCATS version 1.3.4.

**Mobile phases used for different studies**

For the finger print profile of each plant ingredient used in DCBT5678, the following mobile phases were used a) chloroform : glacial acetic acid : methanol : water in the ratio of 8 : 4 : 1.5 : 1, b) chloroform : glacial acetic acid : methanol : water in the ratio of 8 : 4.5 : 1.75 : 1.25 and c) chloroform : glacial acetic acid : methanol : water in the ratio of 10 : 2.2 : 1.1 : 2.6. The F254 plates were then visualized in UV 254 nm and UV 366 nm and the results were noted.

The phytochemical profile of the extract of each plant part collected in different photoperiods (7AM, 12 noon & 7 PM) were screened in the mobile phase, ethyl acetate : methanol : water (in the ratio of 10 : 1.35 : 1) individually in the precoated F254 plates. The results were recorded after illumination of the plates under UV 254 nm and UV 366 nm. The presence of low concentration of certain compounds in each of the plant extract (that could not be seen through naked eye) were also been picked as compounds by the HPTLC scanner and their respective peak area obtained were presented in the tables.
Drug stability study of phytochemicals

The sample capsules of 0 month, 6 months, 1 year, 2 years and 3 years old that were stored in room temperature earlier was used in this study. The drug material from the capsules were extracted with ethyl alcohol:water (70:30) and analysed in HPTLC. The analysis was performed as per the method mentioned above. The plates were developed at room temperature in a Camag twin trough chamber containing 10ml of the mobile phase, EtOAC : MeOH : H₂O (in the ratio of 10 : 1.35 : 1) to a distance of 80mm.

Derivatization and Visualization

With Camag chromatogram immersion device the plate was dipped into anisaldehyde-sulfuric acid reagent solution for 5s, and then dried in pre- heated oven for 5 minutes at 120°C. The chromatogram was recorded with Camag Reprostar 3 at UV 254 nm, UV 366 nm and after derivatization.

Antibacterial Studies

Antibacterial activity was assayed by standardized filter-paper disc agar diffusion method as given by Kirby et al., 1966.

Muller Hinton broth gelled by the addition of 2% agar was used.
Ingredients of the medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5gm L⁻¹</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5gm L⁻¹</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5gm L⁻¹</td>
</tr>
<tr>
<td>Yeast extracts</td>
<td>1.5gm L⁻¹</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>7.2 ± 0.2</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in Milli-Q purified water. The prepared medium was sterilized by autoclaving at 15 pound pressure for 15 minutes at 121°C. The petri dishes were sterilized by means of hot air oven at 110°C for 1h. Agar gelled nutrient was poured into separate petri-dishes to a uniform depth of 5mm. The petri dishes were then incubated at 37°C for 10 minutes to dry off the moisture that developed on agar surface.

Two urinary tract infective bacterial cultures obtained from Vedhanayagam Hospitals, Coimbatore, India, identified previously as *Escherichia coli* and *Proteus* spp were used in this study. Individual cultures were grown overnight in the Muller-Hinton broth and 10⁷ cells of each bacteria were plated on the petri-dish containing the agar media using spread plate method, using L-rod. Each dish was divided into equal sectors by drawing lines on the bottom of the each dish. Ten microlitre of 70% hydro-alcoholic extract equivalent to 500mg raw material of each plant part used in DCBT5678 formulation was added to the sterile discs and placed on the above petridishes. Ten microlitre of the 70% alcohol impregnated sterile disc was served as the control. These discs were air
dried before placing them on the petri-plate containing media and the bacterial culture.

The plates were kept in an incubator at 37°C for 24h. for growth of microbial cultures. After the incubation period, the results were analyzed, recorded, and tabulated.

**Heavy metal analysis**

**Sample Digestion**

50mg of shade dried, sieved individual plants of DCBT5678 mixed with 10ml of concentrated HNO₃, 4ml of 60% perchloric acid and 1ml of concentrated H₂SO₄, and the contents were kept undisturbed overnight. It was heated on a hot plate containing concentrated H₂SO₄ in a beaker, until the brown flame ceased coming out and allowed to cool. After cooling it was filtered through Whatman No 42 filter paper and the filtrate was made up to 100ml with glass distilled water.

**Estimation of heavy metals**

Heavy metals like mercury, arsenic, lead and chromium were estimated by collaborative study of wet and dry ashing technique for the elemental analysis of plant tissue by atomic absorption spectrophotometer Perkin Elmer Model-5000 (Issac and Johnson, 1975).
Pesticides Analysis

Sample preparation

25g of individual fresh plant part of DCBT5678 was crushed with warm methanol (45 to 50°C) in a pestle and mortar and extracted thrice. After incubation in methanol for two hours at a temperature of 45-50°C, the extracts were filtered through Whatman No 1 filter paper and made up to a constant volume.

Estimation of organophosphorous pesticides

The following are the conditions of the Gas chromatography analysis of organophosphorous and organochlorine pesticide residues from the sample digested accordingly

US EPA Method 8141 Organophosphorous pesticides

GC Column : Equity™ 5, 30m×0.25mm ID., 0.25mm (28089-U)
Oven temperature : 120°C (3 min), 5°C/min, to 270°C (5min)
Injector temperature : 250°C
Detector temperature : ECD, 260°C
Carrier gas : Helium, 30cm/sec @120°C
Injection volume : 1.0μL splitless (0.3min)
Liner : 4mm I, D., double taper
Standard : 40ng on column of a custom organophosphorus pesticide mix
US EPA Method 8081 Chlorinated pesticides

GC Column : Equity™ 5, 30m x 0.25mm ID., 0.25mm (28089-U)

Oven temperature : 100°C (2min), 15°C increase/min to 160°C and then 5°C increase/min to 300°C and hold at 300°C for 10min

Injector temperature : 225°C

Detector temperature : ECD, 310°C

Carrier gas : Helium, 30cm/sec @100°C

Injection volume : 2.0μL splitless (0.5 min)

Liner : 4mm I.D., double taper

Standard : 50ppb of a 22 component chlorinated pesticide standard (46845).

OPEN CLINICAL TRIALS

Two open clinical trials for kidney stones were conducted for the evaluation of the plant formulation DCBT5678. First trial included 45 kidney stone patients with all types of stones and the 2nd trial included 10 patients with urinary stones only, using the following protocol and case record form. This protocol and the case record form submitted to Ethical Committee of the hospital in which the trial was undertaken and the clearance was obtained.