Materials and Methods
3. MATERIALS AND METHODS

Sickle bush (locally known as Vidattalai) grown to a height of 3 - 4 meters, is selected as source plant to collect materials for the study as and when required. Flowered twigs from the same plant are collected and brought to our laboratory for taxonomic studies. The plant is identified as *Dichrostachys Cinerea* (L.)Wight & Arn. (Plate 1) and conformed with the Voucher specimen of Botanical Survey of India, Coimbatore. Certified and certificate enclosed.

3.1 Nomenclature

"Dichrostachys" is derived from the Greek words "dis" meaning twice, "chroa" meaning colour, and "stachys" meaning a spike (Leistner *et al.*, 1963). "Dichrostachys" means two-coloured spike, and "cinerea" refers to the greyish hairs of the typical subspecies, which is confined to India (Venter and Venter, 1996).

The plant is also known with the following synonyms such as

*Dichrostachys glomerata* (Forssk.) Chiov.

*Dichrostachys nutans* (Pers.) Benth.

*Dichrostachys platycarpa* Welw.

*Mimosa cinerea* L.

*Mimosa glomerata* Forssk.

*Cailliea glomerata* (Forssk.) J.F. Macbr.

*Mimosa nutans* Pers.

The plant is a native of Africa, with botanical countries such as Kenya and Tanzania (IBPGR, 1984) of East Tropical Africa region (Plate 2).

3.2 Collection of Plant materials:

*Dichrostachys cinerea* plants have been observed on various sites in and around Coimbatore. A luxuriantly grown plant standing at the foot hills of Western
Dichrostachys cinerea Habit

Branch with inflorescences

PLATE 1
Ghats in Thadakam, Coimbatore, Tamil Nadu, India is selected as the source plant for the study. Always fresh specimens are collected from the same plant for the studies. Plant parts are collected for the Pharmacognostic as well as extraction studies and Phytochemical analysis were collected during the month of July – August.

3.3 Methods

The method proposed by Wallis (2005) is followed for the adequate description of the plant.

3.3.1 Roots and Rhizome

i. Dimension and colour: Small and Brown

ii. Shape: Cylindrical

iii. Branching: Fibrous

iv. Condition: Fresh

v. Rootlets:
   a) Present
   b) Kind: Adventitious
   c) Attached: Thin

vi. Directions of growth: Horizontal

vii. Food storage: Starch

viii. Fracture and texture: Fibrous

3.3.2 Stem

i. Kind: Woody and thorny

ii. Direction: Spreading

iii. Shape: Cylindrical
iv. Surface: Rough

v. Phyllotaxy: Alternate

3.3.3 Barks

i. Shape of pieces: Flat

ii. Colour: Dark red-brown or grey

iii. Outer surface: Rough and fissured

3.3.4 Woods

i. Colour and density: Red Streaked with Black

ii. Annual rings absent

3.3.5 Leaves

i. Dimensions: Leaves bipinnate, 3-10cm long, with 5-15 pairs of pinnae, each one with 12-16 pairs of folioles 3-6mm long.

ii. Colour: Green

iii. Condition: Fresh

iv. Lamina

   a) Composition: Compound

   b) Margin: Entire and ciliate

   c) Apex: Acute

   d) Base: oblique

   e) General outline: Bi-pinnate, the main rachis often downy or softly pubescent leaflets numerous, 10-20 pairs, sessile ant closet, linear or strap-shaped

v. Petiole: Sessile
vi. Leaf base: oblique
   a) Stipules: Lanceolate or subulate
   b) Sheath: Absent
   c) Pulvinus: Absent

vii. Phyllotaxy: Alternate

viii. Peculiarities

   Presence of glands: Small erect gland present between each pair of pinnae

3.3.6 Inflorescence and Flowers

i. Colour: Upper florets yellow, the basal ones pink

ii. Morphological nature: Flowers are cylindric peduncled spikes on Short Axillary branchlets

iii. Inflorescence: Spike

   a) Form of the inflorescence: Pendulous
   b) kind of Axis: Long slender
   c) Bracts: Oblong
   d) Type of inflorescence: Spike
   e) Distribution of flowers: Upper flowers are bisexual and yellow where as lower staminodes appear pink or white

iv. Flowers

   a) General features: Bisexual, Regular
   b) Calyx: Companulate, Shortly toothed
   c) Corolla: Petels five, Valvate, connate bellow
d) Androecium: Stamens 10, free exserted, Anthers ending in stalked glands.

f) Gynaecium: style filiform; stigma terminal, truncate.

g) Ovary: Ovary subsessile, many ovuled.

3.3.7 Fruits

i. Kind: Pod liner

ii. Dimensions: Up to 100 x 15 mm, in dense, stalked, intertwined clusters

iii. Shape: Flat Coiled

iv. Dehiscence: Indehiscence or opening from apex

vi. Seeds: Seeds 4 to 6, ovoid, Compressed

3.4 Pharmacognosy Studies

3.4.1 Anatomy:

The required samples of different organs were cut and removed from the plant and fixed in FAA (Fomalin-5ml + Acetic acid-5ml + 70% Ethyl Alcohol-90ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary-Butyl alcohol as per the schedule given by Sass, (1940). Infiltration of the specimens was carried by gradual addition of paraffin wax (Melting point 58-60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

3.4.2 Sectioning:

The paraffin embedded specimens were sectioned with the help of rotary Microtome. The thickness of the sections was 10-12 µm. De waxing of the sections was by customary procedure (Johansen,1940). The sections were stained with Toluidine Blue as per the method published by O’Brien et al. (1964). Since Toluidine blue is a Polychromatic stain. The staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the
mucilage, blue to the protein bodies etc. wherever necessary sections were also stained with safranin and Fast-green and IKI (for starch).

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid (Sass, 1940) were prepared. Glycerin mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerine medium after staining. Different cell component were studied and measured.

3.4.3 Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Labphoto 2 microscopic unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, Polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard anatomy books (Easu, 1964).

3.4.4 Leaf constants

The important identifying characteristic of leaf constants like Stomatal Number, Stomatal Index, Vein-islet number, Vein termination number were found out and tabulated.

3.4.4.1 Stomatal number

It is the average number of stomata per square mm of the epidermis of the leaf.
Clear the piece of the leaf (middle part) by boiling with chloral hydrate solution or alternatively with chlorinated soda. Peel out upper and lower epidermis separately by means of forceps. Keep it on slide and mount in glycerine. Arrange a camera lucida and drawing board for making the drawings to scale. Draw a square of 1mm by means of stage micrometer. Place the slide with cleared leaf (epidermis) on the stage. Trace the epidermal cell and stomata. Count the number of stomata present in the area of 1 sq. mm. Include the cell if at least half of its area lies within the square. Record the result for each of the ten fields and calculate the average number of stomata per sq. mm.

3.4.4.2 Stomatal index

Stomatal index is the percentage which the number of stomata forms to the total number of epidermal cells, each stomata being counted as one cell. Stomatal index can be calculated by using following equation.

\[
S.I = \frac{S \times 100}{E + S}
\]

\[
S.I = \text{Stomatal index},
\]

\[
S = \text{No. of stomata per unit area},
\]

\[
E = \text{No. of epidermal cells in the same unit area}.
\]

Clear the piece of the leaf (middle part) by boiling with chloral hydrate solution or alternatively with chlorinated soda. Peel out upper and lower epidermis separately by means of forceps. Keep it on slide and mount in glycerin water. Arrange a camera lucida and drawing board for making the drawings to scale. Draw a square of 1mm by means of stage micrometer. Place the slide with cleared leaf (epidermis) on the stage. Trace the epidermis cell and stomata. Count the number of stomata, also the number of epidermal cells in each field. Calculate the stomatal index using the above formula. Determine the values for upper and lower surface (epidermis) separately.

3.4.4.3 Vein-islet number

Vein-islet is the small area of green tissue surrounded by the vein-lets. The vein-islet number is the average number of vein-islets per square millimetre of a
leaf surface. It is determined by counting the number of vein-islets in area of 4 sq. mm. of the central part of the leaf between the midrib and the margin.

Clear a piece of the leaf by boiling in choral hydrate solution for about thirty minutes. Arrange camera lucida and drawing board for making drawings to scale. Place stage micrometer on the microscope and using 16 mm objectives, draw a line equivalent to 1mm as seen through the microscope. Construct a square on this line. Move the paper so that the square is seen in the eye piece, in the centre of the field. Place the slide with the cleared leaf (epidermis on the stage). Trace off the veins which are included within the square, completing the outlines of those islets which overlap two adjacent sides of the square. Count the number of vein islets in the square millimeter. Where the islets are intersected by the sides of the square, include those on two adjacent sides and exclude those islets on the other sides. (To obtain a critical result for a leaf, 4 sq. mm. should be used, preferably in one large area of 4 sq. mm.). Find the average number of vein islets from the four adjoining squares, to get the values for one sq. mm.

3.5 Phytochemical Studies (Evans, 2002)

3.5.1 Preparation of plant extracts for preliminary phytochemical studies:

The powdered leaves were successively extracted using 500 ml of ethanol using the Soxhelt extractor for 8-10 hrs. The extract was filtered through Whatmann No.1 filter paper to remove all undissolved matter including cellular materials and other constitutions that are insoluble in the extraction solvent.

Fresh leaves of *D. cinerea* were dried and milled to a coarse powder. The powder was extracted with both cold and hot water. For the cold extraction, 50 grams of the powder material was soaked and left at room temperature with occasional agitation for about 24 hours. The hot extraction was obtained by a modification of the method of Evans (2002). Precisely 50 gms of the herb soaked in 450 ml of distilled water was boiled for 3 hours and then left to stand at room temperature overnight. Each preparation was filtered through a Whatman No.1 filter
paper and the filtrate was stored in sterile containers at room temperature for subsequent analysis.

3.5.2 Qualitative Phytochemical Studies:

The extract was subjected to preliminary phytochemical tests to determine the group of secondary metabolites present in the plant material as follows.

3.5.2.1 Test for Alkaloids

To 1ml of extract in two separate test tubes, 2-3 drops of dragendorff’s and Mayers reagents were separately added. An orange red precipitate turbidity with dragendorffs reagent or white precipitate with Mayers reagent would indicates the presence of alkaloids.

3.5.2.2 Test for Flavanoids

To 4ml of the extract a piece of magnesium ribbon was added followed by concentrated HCL drop wise a colour ranging from crimson to magenta indicated the presence of flavanoids.

3.5.2.3 Test for Glycosides

Keller Kilimi test: To the 2 ml of extract 1ml of glacial acetic acid with ferric chloride and concentrated H₂SO₄ is added. The appearance of blue colour indicated the presence of glycosides.

3.5.2.4 Test for Saponins

One ml of extract was taken in a test tube and 5 ml of distilled water was added and vigorously shaken. A persistent froth that lasted for atleast 5 minutes indicated the presence of saponins.

3.5.2.5 Test for Tannins

Two ml of the extract was diluted with distilled water in separate test tube and 2-3 drops of 5% Ferric chloride (FeCl₃) solution was added. A Green-black (or) Blue black colour indicated the presence of tannins.
3.5.2.6 Test for Steroids

Two ml of the extract was taken in separate test tubes and extracted to dryness. The residues were dissolved in of acetic anhydride then chloroform was added. Conc.H$_2$SO$_4$ was added by the side of the test tube. Formation of brown ring at the interphase of the two liquids and the appearance of the violet colour in the supernatant layer indicate the presence of steroids.

3.5.2.7 Test for Terpenoides

Five ml of extract was mixed with 2ml of chloroform and con.H$_2$SO$_4$ to form a layer. A reddish brown coloration of the interphase showed the presence of terpenoides.

3.5.2.8 Test for Phenols

Five ml of conc. extract was taken and 2ml of neutral Ferric chloride solution was added appearance of violet colour indicates presence of phenol.

3.6 Analytical Studies:

3.6.1 Thin layer chromatography (TLC)

Thin layer chromatography is a solid – liquid partitioning technique.

3.6.1.1 Stationary phase:

The clean rectangular glass plates should be washed with soap solution and, rinse with water and then allowed to dry thoroughly on paper towels. The required amount of silica gel G powder was mixed with distilled water to make slurry. The slurry is coated with glass plate and form a thin layer about 0.25mm thickness. Further, the plated are allowed to dry about 100°C for 1 hour.

3.6.1.2 Mobile phase :

Toluene : Chloroform : Ethanol : Water

28.5 : 25.5 : 45.5 : 1
3.6.1.3 Sample preparation

About 5 g of the powdered leaves was extracted with ethanol by percolation method for 48 hour.

3.6.1.4 Spot loading:

A few drops of the sample were applied to the plate at about 2 cm. the position is just above the level of mobile solvent in the chromatographic chamber on one end of the thin layer chromatography plate.

3.6.1.5 Developing (or) running

The end of the plate that contains the sample spots is dipped into a solvent system contained in a jar. The jar is closed airtight and kept undisturbed. The solvent moves upwards through the thin layer of the absorbent by capillary action.

The compounds in the sample get separated by the moving solvent and they occupy distinct positions on the thin layer as separate spots. Remove the plate from the chamber once the mobile solvent nears the other end of the plate.

3.6.1.6 Detection

The plates are allowed to dry. Sprayed with Dragendorff’s reagent for detecting the presence of alkaloid. An orange brown zone appears in visible light after derivetization, which conform the presence of alkaloids.

3.6.2 Column Chromatography

Column chromatography is performed by packing a glass tube with adsorbents. The column is packed with silica gel 120-200 mesh. The sample to be purified is then dissolved in a small amount of toluene: chloroform solvent and added carefully to the top of the column, so as not to disturb the column. Fractionation of the extract in the column is achieved by adding more solvent to the top and collecting the fractions of eluate that come out of the bottom in separate conical flask. The solvents used for the development of the column is given in table 1
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Solvent</th>
<th>Ratio</th>
<th>Flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Toluene : chloroform</td>
<td>28.5 : 58</td>
<td>32 drops/ml</td>
</tr>
<tr>
<td>2.</td>
<td>Chloroform : Acetone</td>
<td>35 : 30</td>
<td>32 drops/ml</td>
</tr>
<tr>
<td>3.</td>
<td>Chloroform : Acetone</td>
<td>50 : 30</td>
<td>32 drops/ml</td>
</tr>
<tr>
<td>4.</td>
<td>Chloroform : Ethanol</td>
<td>58 : 14.5</td>
<td>32 drops/ml</td>
</tr>
</tbody>
</table>

Glass tube is borosil with 18cm height and 1cm diameter. Column packed up to 15cm.

### 3.6.3 HPLC

The HPLC analysis of the samples were carried out on Shimadzu lc 10At model by injection 20µl of extract with Hamilton syringe using c-18 column (Tracer Analiitica, Nuclcosil 100, 25cmx0.4cm diameter, 5µl. with shimaya photo diod array (spo-M10Avp model) detector. The mobile phase Methanol : Potassium dihydrogen ortho phosphate (55 : 45) was pumped carefully at a flow rate of 0.8µl/min An comptothecins were detected by their absorption at 214 nm.

### 3.7 UV-Vis Spectral analysis

For the characterization of alkaloid in sample eluted from column chromatography the U.V Spectrophotometer was used. The UV Spectrophotometer mode used was SHIMADZU UV – 1700. The eluted sample was subjected to spectral analysis to the range of 200-700nm. For dilution of the sample ethanol was used. The same is used as blank also for the baseline correction. Absorption spectrum of the sample was recorded.

### 3.8 LC-MS

Liquid chromatography and mass spectroscopy carried out in SRIPMS.LC.MS is a tool for specific detection and potential identification of phytochemicals from the mixture of other compounds is characterizing the compound used the technique by separation with their m/z value.
3.8.1 Sample preparation

The column purified traction was dissolved thoroughly in petroleum ether and stored in refrigerator.

3.8.2 Liquid chromatography with mass stereoscopy

A mass spectrum was obtained on shimadya-Lc-Ms instrument coupled with an Agliked zoeban C18 column(5cm×2.5mm 3µ)A 20ml of petroleum their dissolved sampled from column purified traction was injected in analytic liquid chromatography. The chromatographic studies were performed using methanol and acetic acid in the ratio of 9:1 as mobile phase for full scan ms analysis the spectrum was recorded in the range of M/z 50 to 600.

3.9 GC-MS

GC analysis was carried out Gstng a Hewletl packard 6890 GC system Hp- Inoowax FSC Column (60 X 0.25 mm inner diameter 0.23 µm film thickness) was used with helium(1.4ml/mramp flow) As carrier gas. The oven temperature was kept at 40°C for 10 min and programmed to 240°C at a rate of 5°C /min. Split flow was adjusted to 12ml/min.

The injector and Field detector temperature were at 240°C Mass spectra were recorded at the mass range was between m/z 40 to 1000.

3.10 FTIR


3.11 NMR

$^1$H and $^{13}$C NMR Measurements at 400 MHzAll NMR measurements were performed on a Bruker Avance 400 Ultrashield spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm SEI probe with Z-gradient coils, using a Bruker Automatic Sample Changer (B-ACS 120). $^1$H NMR spectra were
acquired at 300.0 K without sample rotation. 13C NMR spectra were acquired using a Bruker zgpg30 pulse sequence with 1024 scans and 4 prior dummy scans.

3.12 Bioactivity Studies

3.12.1 Antimicrobial studies

A vast number of experiment work carried out to show the antimicrobial efficacy of the plant extracts to cure large number of pathogenic diseases. Antimicrobial activity of the crude extract ethanol of *D. cinerea* leaves and column profiled fraction were determined by disc diffusion method (Podschun and Ullman (1998).

3.12.1.1 Collection and maintenance of microorganism:

The following microorganisms were used in this study,

3.12.1.1.1 Bacterial strain

G (+) Ve Bacteria and G (-) Ve Bacteria like *Pseudomonas, Streptococcus, Staphylococcus, Salmonella, E.coli*. are studied

3.12.1.1.2 Fungi species:

*Mucor* and *Penicillium* are used to analyse the antifungal study. These microbes were maintained at 4°C on nutrient agar starts for bacteria and potato dextrose agar strains for fungi and kept in refrigerator prior to subculture.

3.12.1.2 Media used

Frequently prepared nutrient agar Medium and potato dextrose agar medium were used for the culture of bacteria and fungi respectively.

3.12.1.2.1 Composition of nutrient agar medium:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Beet extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>
Distilled Water - 1000 ml
pH - 7.0

3.12.1.2.2 Composition of PDA medium:

Potato - 200 gm
Dextrose - 200 g
Agar - 15 gm
Distilled Water - 1000 ml
pH - 6.2

3.12.1.3 Method

The culture media were prepared and autoclaved at 121°C at 15. psi for 20 minutes and store in refrigerator. The media were melted before the process of inoculated. The clean dry start Petri dishes were poured with nutrient agar medium (for bacteria) and potato dextrose agar medium (for fungi). The 10ml broths were prepared separately for nutrient agar medium and PDA medium in test tubes and plugged with cotton and antibacterial. The test tubes were labelled according to the microbes to be inoculated at 37 ± 0.5°C for 18 hours. After the inoculated the test tubes with bacteria and fungi were smeared on the nutrient agar and PDA Plates respectively using a gentle cotton swab.

The 6mm diameter of disc was placed in the medium labelled properly and 25 and 50 micro liters of crude and eluate samples were loaded on disc. The same volume of extraction solvent was used as control. Plates were felt for some time till the extracts difference in the medium with the lid closed and incubated at 37°C for 24 hours and zone of inhibition was measured with scale and readings were recorded after incubation.
3.12.2 Antioxidant Studies

The Antioxidant activity of crude and eluted extracts was determined by different in vitro methods such as the DPPH free radical Scavenging assay and reducing power method.

3.12.2.1 DPPH radical scavenging activity

DPPH Scavenging activity was carried out by the method of Braca (2001). Crude and eluted extracts of *D. cinerea* leaves were dissolved in DMSO (Dimethyl Sulfoxide) and taken in test tubes in triplicates. Then 5ml of 0.1M DPPH solution (1,1, Diphenyl, 2-picrylhydrazl) was added to each of the test tube and were shaken vigorously. They were then allowed to stand at 37°C for 20 min.

The control was prepared without extracts. Methanol was used for base line corrections in absorbance (OD) of samples measured at 517nm. A radical scavenging activity was expressed as 1% scavenging activity and was calculated by the following formula.

\[
\text{Radical scavenging activity (\%)} = \frac{OD \text{ Control} \times OD \text{ Samples} \times 100}{OD \text{ Control}}
\]

3.12.2.2 Reducing power

Reducing activity was carried out by using the method of Oyaizu (1986). Crude and eluted sample were dissolved in DMSO (Dimethyl Sulfoxide) and taken in test tubes in triplicates. To the test tube 2.5ml of sodium phosphate buffer and 2.5ml of 1% potassium ferric cyanide solution was added. These contents were mixed well and incubated at 50°C for 20min. After incubation 2.5ml of 10% TCA was added and kept for centrifugation 5ml of supernatant were taken and to this 5ml of distilled water was added. To this that 1ml of 1% ferric chloride was added and incubated at 35°C for 20 min. The OD (absorbance) was taken at 700nm at the blank was prepared by using every other solution but without extract and ferric chloride (0.1%) and the control was prepared by adding all other without extract. The reducing power of the extract is overly proportional to the concentration of the sample.