MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1 Materials used

In the present study three groups of seven plants were viz. Citraka (Plumbago indica and Plumbago zeylanica, belonging to the family Plumbaginaceae), Jivanti (Holostemma ada-kodien and Leptadenia reticulata belonging to the family Asclepiadaceae) and Rasna (Alpinia galanga, Alpinia calcarata belonging to the family Zingiberaceae and Pluchea lanceolata belonging to the family Asteraceae) were selected. All the plant materials were collected from natural habitat and also from Herb Garden, Arya Vaidya Sala, Kottakkal. Raw drugs were also collected from different raw drugs markets, wholesalers / retailers, raw drug collectors from 13 Districts of Kerala. The useful parts were fixed in Formalin Acetic acid Alcohol mixture for further study. The plant materials were also collected for the phytochemical studies. The details are given below.

3.1.1 CITRAKA

P. indica & P. zeylanica were collected from the Herb garden, Arya Vaidya Sala Kottakkal. Raw drugs were also collected from different markets like markets of Kuttyadi, Thrissur, Vadakara, Kollam, Kottayam, Idukki, Alappuzha, Trivandrum, Payyannur, Eranakulam, Pathanamthitta.

3.1.1.1 Plumbago indica L. is a pretty subscandent shrub growing to more than six feet high with several erect branching herbaceous to
semi-woody terete strigate stems and flexible branches arising from a system of perennial lateral tuberous roots and bearing simple, alternate, exstipulate, short petiolate oblong leaves. The leaves are entire, wavy, membraneous, smooth, ovate-oblong, narrowed and slightly obtuse at apex, base narrowed abruptly passing on to the very short petiole. Flowers are scarlet or bright red colour, and about one and half inches long, in long loose twiggy spikes that are terminal as well as axillary from upper leaves. The spikes continue to elongate even after flowering, reaching a length of one or two feet. Rachis of spikes glabrous or smooth. Bracts and bracteoles nearly equal ovate, cuspidate, much shorter than the calyx. The bracteoles are often connate at base between the flower and axis. Calyx reddish, subsessile, short, cylindric tubular gibbous shortly and acutely five toothed, five ribbed, covered with small persistent stipulate bifarious gummy glands all along the five ribs. Corolla scarlet, gamopetalous, hypocrateriform, tube of corolla slender, much longer than the calyx, limb rotate, five lobed or parted. Stamens five, hypogynous with their filaments united at the base in to a lobed nectariferous disc surrounding the ovary. Anthers linear, two cleft at the base. Ovary, ovate or oblong. Style filiform hairy at base with five filiform stigmatic branches whose inner faces are covered with many rows of glands. The fruit one seeded and it is enclosed with in the persistant calyx. Ripe fruits break at top. Seed ovate or oblong. Root is the officinal part.
Other names:

Eng. - Rose coloured leadwort, Fire plant
Hind. - Chitra, Rakta chitra, Lal chitra, Lale chitrak
Kan. - Kempucitramula
Mal. - Schettikodiveli, Chuvanna kudiveli, Kodiveli
Tam. - Chen kodiveli, Cithiramulam
Tel. - Yerracitramulam

3.1.1.2 *Plumbago zeylanica* L. is a much branched diffuse rambling or subscandent undershrub, two to four feet high with several nearly uniform spreading perennial roots, from which seemingly arise a number of smooth narrow terete striated, jointed, somewhat declining stems and tender flexuous branches about the size of a quill bearing simple alternate short stalked, entire, ovate or ovate-oblong, wavy membraneous leaves, one and a half to three inches long and slightly less than an inch to two inches broad, ovate or ovate-oblong, the base shortly and abruptly attenuated in to the short petiole, acute at apex, entire, wavy, thin or membraneous and smooth. The petiole is channeled or grooved above, and sometimes slightly winged, with a number of very sticky glands at its upper part and its base often auricled and clasping the stem. Flowers pure white, subsessile with bract and bracteoles borne on simple or branched terminal and axillary spikes half to one foot long. The rachis of inflorescence is covered with very short glutinous hairs or glands. The bracts are considerably larger and about ten times
longer than the bracteoles. They are also covered with sticky glandular hairs. Calyx tubular, gibbous or inflated, one third to half an inch long and about a tenth of an inch in thickness and shortly five toothed at its apex. It is persistent and covered all over with slender stalked glutinous glands that aid in the dispersal of the fruit. Corolla white, gamopetalous with a very long and slender tube three quarters to one inch long, and a rotate five lobed limb with the lobes roundish about one third inch long and patent or spread out. Stamines five hypogynous, nearly free, the filaments linear about as long as the tube of the corolla dilated and connate at base in to a lobed nectar secreting disc. Anthers exserted beyond the throat, dorsifixed, oblong or linear, two cleft at base. Ovary ovate or oblong, narrowed at apex. Style slender glabrous at base, divided in to five filiform stigmatic branches which are densely covered with many rows of glands on the inner side and stigmatose nearly throughout their length. Fruit enclosed within the persistent calyx. It is one-seeded membraneous capsule with the pericarp thin below and thick and hardened above, its dehiscence, at first irregularly circumcisal or breaking at base, afterwards valvately splitting from the base to the middle. Seed solitary, ovate or oblong.

Other names:

Assam. - Agiyachit, Agnachit
Beng. - Chita
3.1.2 JIVANTI

*Holostemma ada-kodien* was collected from the Herb garden, Arya Vaidya Sala Kottakkal and *Leptadenia reticulata* was collected from Attappady regions. Raw drugs were also collected from different markets like markets of Calicut, Kuttyadi, Thrissur, Vadakara, Kollam, Kottayam, Idukki, Palakkad, Alappuzha, Eranakulam, Wynad and Malappuram.

3.1.2.1 *Holostemma ada-kodien* Schult. is a fairly large much branched nearly glabrous to faintly downy or puberous lacticiferous perennial climbing shrub with shiny glabrous sometimes slightly reddish or reddish yellow twining stems bearing simple petiolate opposite ovate or ovate-oblong deeply cordate, entire membraneous leaves. The base of leaves deeply cordate the basal lobes rounded spreading or incurved and meeting, quite glabrous above and faintly down or puberulus beneath especially on the nerves, 7.5 to 15 cm long and 5 to 7.5 cm broad. Midrib prominent with a few small
glands at its base above. 4.5 to 6.5 cm or more long, stout, glabrous, glandular at the base of the midrib above. Inflorescence sublateral few flowered, pedunculate sub-racemose or umbelliform cymes the peduncle 2.5 to 5 cm, long arising close to the base of the petiole outside the leaf axil. Flowers pedicellate, bisexual, subrotate, subglobose in bud frosted white to pale pink outside reddish purple or purplish-crimson inside, fragrant and edible. Calyx deeply divided to the base in to five segments. The segments or lobes glandular, broadly ovate obtuse and veined, about 4mm long and 3mm wide. Corolla gamopetalous, deeply five lobed to nearly 2/3rd downwards, subrotate, 2.5 to 3.8 cm in diameter, white or pinkish outside and purplish within, tube short, the lobes thick about 1.2 cm long and 9 mm to 1 cm wide ovate-oblong subacute or obtuse, incurved, white on the back and margins and overlapping to the right. Stamens five, adnate to the base of the corolla tube, the filaments cohering in a ten winged column, anthers very large oblong with long stiff wings horny and shining with small membraneous tips inflexed over the column. Pistil bi-carpellary, the ovaries free but with a common slender style ending in an oblong, five winged included stigma, each ovary containing numerous ovules on thick swollen placenta. Fruit consists of two short broad, thick, smooth, cylindric linear-oblong or lanceolate follicular mericarps. Seeds 6 mm long, ovoid or oblong very thin, much flattened and winged.
Other names:

Hind. - Chirvel, Charivel
Mal. - Atapatiyan, Atapotiyan, Atakotiyan
Tam. - Palaikkirai
Tel. - Palagurugu

3.1.2.2 *Leptadenia reticulata* (Retz.) Wight & Arn is a twining shrub, stems with corky deeply cracked bark; branches numerous, the younger ones terete, glabrous or hoary-puberulous. Leaves thinly coriaceous, 3.8-7.5 by 2-4.5 cm, ovate, acute, glabrous above, more or less finely pubescent (especially on the nerves) beneath, base rounded or subcordate (rarely subacute); petioles 6-20 mm long, puberulous. Flowers greenish-yellow, in lateral or subaxillary, many-flowered hoary-puberulous globose cymes; peduncles arising from between the leaves or subaxillary, sometimes in pairs, puberulous; pedicels 3-4 mm long, puberulous. Calyx pubescent outside, divided to about the middle; segments 1.25 mm long, ovate-oblong, subacute. Corolla 5 mm long (about 8 mm across when expanded), tube very short, glabrous; lobes of the limb thick, 2.5 mm long, ovate-oblong, sub obtuse, with revolute margins, pubecent on both surfaces, often with a small hairy process (absent in Deccan specimens) on the inner face near the tip; corolline corona of 5 quadrate truncate fleshy lobes at the sinuses; staminal corona minute, annular, close to the staminal column. Anthers without membranous appendages, incumbent on the style-apex; pollen-
masses ovoid, large, waxy, pellucid at the apex, attached to the
minute pollen-carriers by moderately long caudicles. Follicles
subwoody, 6.3-9 cm long, turgid, tapering into an obtuse shortly
curved beak. Seeds 6 mm, long, narrowly ovate-oblong, acute; coma
3.2-3.8 cm long.

Other names:

Hind. - Dori
Guj - Dodi, Nahanidodi, Khirkhodi
Mar. - Dodi, Dodhi, Pala-kuda
Tam. - Palaikkodi, Palakuda
Tel. - Kalasa, Mukkutummudu, Palatig

3.1.3 RASNA

*Alpinia galanga* & *Alpinia calcarata* were collected from the Herb
Garden, Arya Vaidya Sala Kottakkal and *Pluchea lanceolata* was
collected from the market of Calicut. Raw drugs were also collected
from different markets like markets of Calicut, Kuttyadi, Thrissur,
Vadakara, Kollam, Kottayam, Idukki, Palakkad, Alappuzha,
Trivandrum, Payyannur, Eranakulam, and Pathanamthitta.

3.1.3.1 *Alpinia galanga* (L.) Sw. rootstock perennial, tuberous,
slightly aromatic. Leaves 23-45 by 3.8-11.5 cm oblong-lanceolate,
acute, glabrous, green above, paler beneath, with slightly callous
white margins; sheaths long, glabrous; ligule reaching 10 mm long,
but usually shorter, rounded. Flowers greenish white, in dense-
flowered panicles 15-30 cm long; branches short; rachis pubescent; pedicels 3-4 mm long, bracts 10 mm long ovate-lanceolate. Calyx 10 mm long, tubular, irregularly 3-toothed. Corolla 3.2 cm long; tube 13 mm long; lobes oblong, obtuse, subequal, 6 mm broad. Lip 2.2 cm long; claw green, 6 by 2.5 mm, blade white striated with red rather more than 13 mm long, broadly elliptic, shortly 2-lobed at the apex, with a pair of subulate glands at the base of the claw. Stamen 2 cm long. Fruit the size of a small cherry, orange-red.

**Other names:**

- **Assam.** - Khulanjaana
- **Beng.** - Kulanjan, Kurachi Vach
- **Eng.** - Greater galangal, Javagalangal
- **Guj.** - Kulinjal jaanu, Kolinjan
- **Hind.** - Kulanjan, Kulinjan
- **Kan.** - Doddarasagadde, Dhoomraasmi
- **Mal.** - Aratta, Cittaratta
- **Mar.** - Kulinlain, Koshta Kulinjan, Mothe Kolanjan
- **Tam.** - Arattai, Sittarattai
- **Tel.** - Dumparaastramu

**3.1.3.2 Alpinia calcarata** Rosc. is a perennial rhizomatous herb, with leafy stem. Leaves linear, lanceolate acuminate, glabrous. Flowers white with red lip in terminal panicle. Calyx white tubular, lobed at apex; corolla tube as long as calyx. Ovary densely pilose, ovules many on axile placenta, stigma subglobose.
Other names:

**Mal.** - Chittaratha

**Tam.** - Arattai, Sittarattai

### 3.1.3.3 *Pluchea lanceolata* (DC.) C.B. Clarke

*Pluchea lanceolata* is an erect undershrub, 30-60 cm high, stem & branches terete, ashy & pubescent. Leaves sessile very coriaceous, 2.5-5.7 by 0.6-1.6 cm oblong or lanceolate, obtuse apiculate, narrowed at the base, finely ashy, pubescent on both sides, entire or toothed round the apex, main nerves prominent. Inflorescence heads in compound corymbs. Involucre contracted at the mouth. Outer bracts 2-3 seriate; oblong, very obtuse, pubescent, usually tinged with purple; the innermost bracts linear, sub- acute, few pappus hairs distinctly connected at the base.

Other names:

**Assam.** - Rasna pat

**Beng.** - Rasna

**Eng.** - Sena, Indian sena, Elecampane

**Guj.** - Rasna, Rashana

**Hind.** - Rayasan, Rayasana, Rasna, Rashana

**Kan.** - Rasna, Dumme- rasna

**Mar.** - Rasna, Rayasana, Rashana

**Tel.** - Sanna Rashtramu
3.1.4 Plants for Taxonomical identification studies

The genuine source plants/substitute/ adulterants were collected from natural habitat, Herb Garden, Arya Vaidya Sala, Kottakkal and also from various markets were subjected to taxonomic identification. Herbarium of the plants were also prepared and deposited in CMPR herbarium.

3.1.5 Plant materials for Micro- morphological studies.

The officinal parts like root and rhizome of genuine plant sources / substitutes/ adulterants were subjected to micro morphological studies to recorded the identity and variation between plants.

3.1.6 Plant materials for Histological and histochemical studies.

The useful parts of the genuine plants/ substitutes/ adulterants and the market samples were fixed in Formalin Acetic acid Alcohol mixture. Cellular details of genuine plant and their substitutes/ adulterants were studied by taking sections using microtomes and double staining with appropriate staining procedure and also using histochemical techniques.

3.1.6.1 Fixatives for Anatomical studies

Fixatives: Combination of Formalin, Acetic acid and Alcohol are widely used for fixation in plant microtechnique. This fluid is ideal for anatomical studies, stable and has got hardening action, rapid penetration and materials can be stored.
FAA- standard proportion (Johanson, 1940)

70% Ethyl alcohol - 90 ml
Acetic acid - 5 ml
Formalin - 5 ml

3.1.6.2 Stains and reagents for Anatomical studies

1. Saffranin: Dissolve 1gm saffranin in 100 ml distilled water
2. Fast green: Dissolve 1gm Fast green in 100 ml clove oil

3.1.6.3 Stains for histochemical studies

- **Ferric chloride S:** Dissolve 5 g of ferric chloride in 100 ml of water.
- **Glycerol S:** Mix equal amounts of glycerol and water.
- **Hydrochloric acid:** a suitable commercially available reagent.
- **Iodine S:** Dissolve 2.6 g of iodine and 3 g of potassium iodide in sufficient water to produce 100 ml.
- **Nitric acid S:** Concentrated nitric acid.
- **Phloroglucinol:** dissolve 1 g of phloroglucinol in 100 ml of ethanol.
- **Potassium hydroxide:** dissolve 56 g of Potassium hydroxide in 1000 ml of water
- **Ruthenium red S:** In 10 ml of 10% lead acetate solution dissolve 0.008 g of ruthenium red.
- **Sudan red S:** Dissolve 0.5 g of sudan red in 100 ml of glacial acetic acid AR
3.1.7 Plant materials for the Maceration studies.
Maceration carried out by standard techniques (Jefferey’s and Schultze’s methods)

3.1.8 Plant materials for the raw drug powder studies
Microscopic studies of the powdered drug with proper staining

3.1.9 Plant materials for polarization microscopic studies
Polarization microscopic studies of the useful parts

3.1.10 Plant materials fluorescent microscopic studies
Fluorescent microscopic studies of the useful parts

3.1.11 Plant material for phytochemical studies

3.1.11.1 Physicochemical characters

1. Moisture content:
Materials used to determine the moisture content was Moisture Analyser MJ 33 Apparatus.

2. Water soluble extractive:
Materials used to determine the water soluble extractive were powdered drug, conical flask, water bath and oven.

3. Alcohol soluble extractive:
Materials used to determine the alcohol soluble extractive were powdered drug, round bottomed flask, water bath and oven.

4. Ash value:
Materials used to determine the ash value were powdered drug, muffle furnace, gooch crucible and ashless filter paper.
5. **Acid-insoluble ash:**

Materials used to determine the acid insoluble ash were weighing balance, 2M HCL, Gooch crucible, ashless filter paper and desiccators.

3.1.11.2 **Total Phenolic Assay:**

Materials used to determine the total phenolic assay were Folin-Ciocalteu assay, Gallic acid, volumetric flask, Folin-Ciocalteu phenol reagent, Na₂CO₃ solution and spectrophotometer.

3.1.11.3 **Total Flavonoid Assay:**

Materials used to determine the total flavonoid content were measured by aluminium chloride colorimetric assay, quercetin, volumetric flask, NaNO₂, AlCl₃, 1M NaOH and spectrophotometer.

3.1.11.4 **Estimation of Total Tannins:**

Materials used to determine the total tannin content were measured by using tannic acid, volumetric flask, Folin-Denis reagent, Na₂CO₃ solution and spectrophotometer.

3.1.11.5 **TLC Identity test**

**General method of extraction**

The first step in the phytochemical evaluation of the plant material was the extraction. The choice of extraction method depends on the nature of the plant material and the compounds to be isolated. Dried material was usually powdered before extraction. Extraction was
carried out by cold maceration or by the methods involving heating of
the drug with suitable solvent. Some methods are:

1. **Maceration**

   For maceration the powdered drug was taken in a stoppered
   container with the solvent and allowed to stand for a period of 24 to
   48 hrs with frequent agitation. Then the mixture was filtered and the
   marc was macerated again with fresh solvent.

2. **Extraction by hot maceration technique**

   Refluxed 5 g powdered drug with methanol for 6 hrs. Filtered the
   combined extract and remove the solvent under reduced pressure.
   Dissolved the residue in 10 ml of methanol.

3. **Extraction of volatile oil/essential oil using Clevenger’s apparatus**

   Volatile oil from the drug was extracted by distilling the drug (fresh
   or dried) with water collecting the distillate in the graduated tube of
   Clevenger’s apparatus, in which the aqueous portion of the distillate
   was automatically separated and returned to the distilling flask.
   Measure the volume of the essential oil. In specific cases where the
   volatile oil is miscible with water, it was collected in a definite volume
   of xylene or n-hexane placed above the water column in the
   graduated tube.

**Preparation of spray reagents**

1. **Anisaldehyde sulphuric acid reagent**

   Mixed 0.5 ml anisaldehyde with 10 ml glacial acetic acid then add 85
ml methanol and 5 ml conc. Sulphuric acid. Sprayed the plate and heated at 100° for 5-10 min. use freshly prepared reagent.

**Thin layer Chromatography (TLC) studies**

Thin Layer Chromatographic (TLC) studies conducted using pre-coated plates of silica gel 60 F<sub>254</sub> (E. Merck) of uniform thickness of 0.2mm.

### 3.1.11.6 Assay/ Analytical Methods

**High Pressure Liquid Chromatography (HPLC)**

HPLC is one of the widely used methods for separation and quantitative estimation of marker compounds present in herbal drugs. Liquid chromatographic system uses narrow columns (~5 mm in diameter), pumping systems operating at pressures up to 200 atm and suitable detectors. Reversed phase silica columns were used. A guard column was used before the column to prevent the entry of highly retard unwanted compounds of the sample solution in to the column. Sample introduction was done by syringe and a loop injector may be fitted with a fixed volume loop between 1-200µl to facilitate accurate sample injection. Detection of the compound of interest was by retention time, UV absorbance fluorescence and electrical conditions. For majority of analyses, variable wavelength UV orphotodiode array UV and RI detectors were used.

**High Pressure Thin Layer Chromatography (HPTLC)**

Chromatographic analyses were performed on silica gel 60 F 254 TLC
plates (20x10 cm; Merck, Darmstadt, Germany). Samples were applied to the plates by means of CAMAG Linomat V sample applicator. Detection and quantification of the developed plate was performed with a CAMAG TLC Scanner 3 at 559 nm.

**Gas Chromatography (GC) analysis**

Studies conducted using Agilent 6890N Network GC having HP-5 (5 percent phenyl methyl siloxane) capillary column (30 m x 320 μm x 0.25 μm) with Flame ionization detector.

**GCMS analysis**

Gas chromatography-mass spectrometry was carried out on an Agilent GC-MS 6850 under electron impact ionization (70 eV). The interface temperature was 230°C, and the MS scan range was 50-800 atomic mass units (AMU). The chromatographic column for the analysis was done by HP5 - MS capillary column (30 m x 0.25 mm internal diameter). The carrier gas used was helium at a flow rate of 1 ml/min. The oven temperature was 60°C to 250°C with a constant increase of 10°C. The injection was performed in split mode at 250°C.

**3.2 METHOD OF STUDY**

**3.2.1 Taxonomical profiling**

For the study, the genuine source plants and substitutes were collected from natural habitats and also from various markets, were subjected to taxonomic identification and herbarium preparation. The correctly identified herbarium of each drug and useful parts
were kept in the Herbarium and raw drug museum of AVS- CMPR for further use. Raw drugs were also collected from different market, wholesalers / retailers, raw drug collectors from 13 Districts of Kerala. The useful parts were fixed in Formalin Acetic acid Alcohol mixture for further study. The plant materials were dried for the phytochemical studies.

**3.2.2 Micro morphological studies**

The genuine plant sources / substitutes/ adulterants and the market samples were subjected to micro-morphological studies and recorded the most identifying features of dermal morphology, texture, colour and cut surface. Organoleptic characters like smell, taste and fracture were also studied.

**3.2.3 Histological studies**

Histological studies of the useful parts were carried out to study the type of cells, shape of stem in transection, nature of epidermal hairs, epidermal cell shape, nature of cortex, type of phloem fibres, crystals and cambium, nature of xylem vessel, secondary wood, pith, type of anatomical growth- normal or abnormalities if any. TS & LS were taken using sledge and rotary microtome and double stained with appropriate staining procedure for the preparation of permanent slide. All the anatomical characters were observed under Carl Zeis Axiostar plus microscope with a G3 Canon camera attached to a computer system.
3.2.3.1 Stains and reagents for Anatomical studies

1. **Safranin**: stains lignified, cutinized and suberised structures

2. **Fast green**: the stains act on non-lignified tissues. It is a good counter stain for safranin.

3.2.3.2 Double staining

Specimens were double stained with safranin and fast green as per the procedure given by Johansen (1940).

**Procedure for double staining**

**Safranin - Fast sgreen**

Double staining was carried out using safranin and fast green according to Johansen (1940). The following steps were used for double staining.

1. Kept the sections in watch glass containing safranin prepared in water for 15-30 min and then washed

2. Dehydrated through graded series of alcohol ie, 50%, 60%, 70%, 80%, 90% and 100% taken in cavity blocks covered with lids. The interval in each series was 1-3 minutes

3. Counter stained the section with fast green dissolved in clove oil for less than 15 seconds

4. Washed excess stain with clove oil

5. Cleaned the section for a few seconds in a mixture of clove oil and xylene followed by 3 changes in xylene and mount in DPX.
**3.2.4 Histochemical studies**

Histochemical characterization of raw drugs were carried out to find the presence of starch, tannin; presence, nature and position of laticifers, resin ducts/ oil ducts; identification of depositions like cutin, lignin, suberin, wax; inclusions like calcium oxalate, calcium phosphate, cystolyth etc. using appropriate histochemical techniques.

- **Starch**
  For examine the presence of starch the specimen was stained with Iodine. The blue colour indicates the presence of starch content.

- **Aleurone grains**
  For examining the presence of aleurone grains prepared a specimen in iodine S; aleurone grains get stained yellow.

- **Fixed oil**
  For examining the presence of fixed oil, prepared a specimen in a solution of sudan red; droplet of fixed oil are coloured orange pink.

- **Mucilage**
  For examining the presence of mucilage, prepared the specimen in ruthenium red S and examine it under a low power microscope. Mucilage appears as pinkish red or yellow coloured mass.

- **Tannin**
  For examining the presence of tannins, prepared a specimen in ferric chloride S; bluish black or grayish black colouration indicates the presence of tannins.
• **Anthraquinone derivatives**

For examining the anthraquinone derivatives, prepared a specimen in potassium hydroxide; anthraquinone give blood red colour.

• **Lignified cells**

For examining the lignified cells or cell walls, the specimen was stained in phloroglucinol and allowed to drying. Added one or two drops of hydrochloric acid. The presence of lignin indicated by the pink or cherry red colour.

### 3.2.5 Maceration studies

Using routine maceration technique (Jeffrey's method) a detail cellular study of xylem elements like vessel, tracheids and fibers were carried out, which was specific for certain family members.

**Jefferey's methods**

The maceration solution prepared as follows:-

1. 10% Nitric acid
2. 10% Chromic acid

Took the maceration solution (1:1 ratio) in a test tube and added the pieces of plant material in to it. Heated this solution and separated the material in small pieces by piercing a needle in to the material, stopped heating, the material becomes soft and pulp like. Then transferred this pulpy material to a watch glass. Drained out all the maceration fluid and washed the material several times with water,
so that the acid traces are removed completely. Then stained the material with aqueous safranin and mounted in glycerine. Length, width and wall thickness of vessels and fibres were observed by maceration studies.

3.2.6 Raw drug powder studies

For examining the cell structure in powder form, material were powdered and sieved and mounted under glycerol and safranin to study the nature and identification of particles.

3.2.7 Microscopic studies

All the anatomical characters were observed under Carl Zeis Axiostar plus microscope with a G3 Canon camera attached to a computer system.

3.2.8 Polarization microscopic studies

To locate and distinguish the types of crystals and minerals present in the useful parts polarization microscopy was highly useful. The characters were observed under Motic BA 400 polarization microscope.

3.2.9 Fluorescent microscopic studies

Fluorescent microscopic studies of the useful parts were done with the help of UV light. Observations were done under Leica DM 1000 LED fluorescent microscope and photographs were taken with the help of a digital camera.
3.2.10 Comparative analysis of histological details

Comparative analysis of cellular details of root and rhizome and macerated sample were calculated. Data were statistically analysed.

3.2.11 Comparative phytochemical characterization

3.2.11.1 Determination of physicochemical characters

1. Determination of Moisture content

Moisture content was determined using the Moisture Analyser MJ 33 apparatus.

2. Determination of Water soluble extract

Added 2 gm of powdered drug to 70 ml water in a conical flask, boiled for 15 minutes, cooled and then filter in to a weighed beaker. Repeated this process thrice, evaporate on a water bath, drying for 30 min in an oven at 110°C, cooled and weighed the residue. Calculated the percentage of water-soluble extractive with reference to the drug.

3. Determination of Alcohol soluble extractive

Added 2 gm of powdered drug in a Round Bottomed Flask, added 30 ml methanol and filtered into a weighed dish. The process was repeated three times, and then evaporated on a water bath. Dried the residue at 110°C for 30 min and weighed. Calculated the percentage of alcohol soluble extractive with reference to the drug.
4. Determination of Ash value

Weighed accurately 2 g of the air-dried powdered drug in a Gooch crucible and incinerate at a temperature not exceeding 450° until free from carbon, cooled and weighed. If a carbon free ash cannot be obtained in this way, exhausted the charred mass with hot water, collected the residue on an ashless filter paper, incinerated the residue and filter paper until the ash is white or nearly so, add the filtrate, evaporated to dryness and ignited at a temperature not exceeding 450°. Calculated the percentage of ash with reference to the air dried drug.

5. Determination of Acid- insoluble ash

Boiled the ash obtained from the procedure mentioned above (total ash determination) with 25 ml of 2 M hydrochloric acid for 5 min, collected the insoluble matter in a Gooch crucible or on an ashless filter paper, washed with hot water, ignited, cooled in a desiccator and weighed. Calculated the percentage of acid-insoluble ash with reference to the air dried drug.

3.2.11.2 Total Phenolic assay

The total phenolics content were determined by using the Folin-Ciocalteu assay. (Singleton et al., 1965). An aliquot (1 ml) of extracts or standard solution of Gallic acid (20, 40, 60, 80 and 100μg/ml) was added to 25 ml of volumetric flask, containing 9 ml of distilled water. A reagent blank using distilled water was prepared. 1 ml of
Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 minutes 10 ml of 7% Na₂CO₃ solution was added to the mixture. The volume was then made up to the mark. After incubation for 90 minutes at room temperature, the absorbance against the reagent blank was determined at 550 nm with an UV-Visible spectrophotometer (Shimadzu 1700, Japan). Total phenolics content was expressed as mg Gallic acid Equivalents (GAE)

**3.2.11.3 Total Flavonoid Assay**

Total flavonoid content was measured by the aluminium chloride colorimetric assay. (Zhishen et al., 1999). An aliquot (1 ml) of extracts or standard solutions of quercetin (20, 40, 60, 80 and 100 µg/ml) was added to 10 ml volumetric flask containing 4 ml of distilled water. To the flask added 0.30 ml 5% NaNO₂, after five minutes 0.3 ml 10% AlCl₃ was added. After five minutes, 2 ml 1M NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm with an UV-Visible spectrophotometer (Shimadzu 1700, Japan). The total flavonoid content was expressed as mg quercetin equivalents (QE).

**3.2.11.4 Estimation of Total Tannins**

**Preparation of standard**

100 mg of tannic acid was dissolved in 100 ml of distilled water. 1 ml of this solution was diluted into 100 ml with distilled water to give 10
μg/ml tannic acid solution.

**Method**

A series of calibrated 10 ml volumetric flask were taken and working standards of 5-45 μg solutions were taken. To each flask 0.5 ml Folin-Denis reagent and 1 ml sodium carbonate solution were added, the volume was made up to 10 ml with distilled water. The solution without tannic acid was used as blank. The blue colored complex thus produced was measured at 775 nm with an UV-Visible spectrophotometer (Shimadzu 1700, Japan).

1 ml of sample was made up to 10 ml in similar manner. From the calibration curve the corresponding concentration of tannins were calculated (Burns & Cope, 1974). It was expressed as milli gram equivalent of Tannic acid (mg ET).

**3.2.11.5 TLC Identity test**

Chromatography refers to the separation of chemical compounds by partitioning them between a stationary phase and a mobile phase. TLC techniques involved application of sample (dissolved in suitable solvent) at one end of the pre-coated plate, development of the plate in the solvent system in a closed chamber to the specified height and visualization of the plate for the compound in natural light, under UV 254 & 366nm or by derivatization of the plate with suitable reagents. The Rf values and colours of the bands were recorded and
finger print profiles were established. Identification of the chemical marker was done by the comparison of Rf values.

Rf - Distance traveled by the solute / Distance traveled by the solvent

3.2.11.6 Assay/ Analytical Methods

3.2.11.6.1 HPLC estimation of Plumbagin

Test solution

Extracted about 3 g of roots of P. indica and P. zeylanica was mixed with 50 ml ethanol and kept for overnight. This extract was filtered and the solvent was evaporated off using rotary evaporator till dryness. The residue was redissolved in ethanol in order to obtain sample solution containing 100 µg/ml for analysis.

Standard solution

Dissolved 0.0013 g of plumbagin in 10 ml of acetone.

HPLC conditions

Column : Phenomenex Luna C 18 (2) (250 x 4.6mm)
Detection : PDA detector at 266 nm
Mobile phase : Methanol: Sodium dihydrogen phosphate (9:1)
Flow rate : 0.8 ml/min
Injection volume : 20µl

Procedure and Estimation of plumbagin

For calibration, standard plumbagin solutions were prepared at concentrations of 1, 2, 3 ... 20µg/ml in ethanol. The standard
solutions were injected in triplicate and the average detector responses measured. Plant samples were assayed in triplicate and detection was done at 265 nm.

3.2.11.6.2 HPLC estimation of β-sitosterol

Test solution

3g each of the roots of *H. ada-kodien* and *L. reticulata* was refluxed with 50 ml methanol for six hours. The extract was filtered and the solvent was evaporated off using rotary evaporator till dryness. The residue was redissolved in 10 ml methanol.

Standard solution

Dissolved 1mg of β-sitosterol in 10 ml methanol.

HPLC conditions:

- Column : Phenomenex Luna C 18 (2) (250 x 4.6mm)
- Detection : PDA detector at 266 nm
- Mobile phase : Methanol and water (25:75)
- Flow rate : 1.0 ml/min
- Injection volume : 20μl

Procedure and Estimation β sitosterol

The column was equilibrated with the mobile phase for an hour and then pumped at the rate of 1.0 ml/min. The concentration of β Sitosterol was calculated using area under the curve method.
3.2.11.6.3 HPTLC estimation of Plumbagin

TLC plates

Precoated plates of silica gel 60 F254 (E. Merck) of uniform thickness of 0.2 mm

Solvent system

Hexane: Ethyl acetate (8:2)

Scanning

254 & 365nm

Test solution

Extracted about 3 g of roots of *P. indica* and *P. zeylanica* was mixed with 50 ml ethanol and kept for overnight. The extract was filtered and the solvent was evaporated off using rotary evaporator till dryness. The residue was redissolved in ethanol in order to obtain the sample solution containing 100μg/ml for analysis.

Standard solution

Dissolved 0.0013g of plumbagin in 10 ml of acetone.

Calibration curve

Applied 2 to 14 μl of standard solutions corresponding to 0.26 μg to 1.56 μg of plumbagin on a precoated silica gel 60 F254 TLC plate. Developed the plate using the solvent system in a twin trough chamber to a distance of 8 cm and scanned densitometrically at visible, 254nm & 366 nm. Recorded the peak area and prepared the
calibration curve by plotting peak area vs concentration of plumbagin applied.

**Estimation of plumbagin in the drug**

Applied 3, 5µl of the test solution on a precoated silica gel 60 F_{254} TLC plate (E. Merck). Developed the plate using the solvent system and recorded the chromatogram. Calculated the amount of plumbagin present in the sample from the calibration curve.

**3.2.11.6.4 HPTLC estimation of β sitosterol**

**TLC plates**

Precoated plates of silica gel 60 F_{254} (E. Merck) with uniform thickness of 0.2 mm

**Solvent system**

Toluene: Ethyl acetate (7:3)

**Scanning**

500 nm

**Test solution**

5 g each of roots of *H. ada-kodien* and *L. reticulata* was extracted with 100 ml chloroform in a Soxhlet extractor at 60° C for 2 hrs. The extract was filtered and the solvent was evaporated using rotary evaporator till dryness. The residue was redissolved in chloroform and made up to 10 ml. 3 g each of roots of *H. ada-kodien* and *L. reticulata* was extracted with 50 ml methanol in a Soxhlet extractor.
at 60° C for 2 h. The extract was filtered and the solvent was evaporated to dryness using rotary evaporator. The residue was redissolved in methanol and made up to the mark in a 10 ml volumetric flask. This solution was used for HPTLC profile.

**Standard solution**

Dissolved 1mg of $\beta$- sitosterol in 10 ml of methanol.

**Calibration curve**

Applied 2 to 10 $\mu$l of standard solutions on a precoated silica gel 60 F$_{254}$ TLC plate. Developed the plate using the solvent system in twin trough chamber to a distance of 8 cm and scanned densitometrically at 500nm. Recorded the peak area and prepared the calibration curve by plotting peak area vs concentration of $\beta$- sitosterol applied.

**Estimation of $\beta$ sitosterol in the drug**

Applied 2 to 4 $\mu$l of the test solution on a precoated silica gel 60 F$_{254}$ TLC plate (E. Merk). Developed the plate using the solvent system and recorded the chromatogram. The amount of $\beta$ sitosterol present in the sample was calculated from the calibration curve.

**3.2.11.6.5 Phytochemical characterization using Gas Chromatography**

**Gas Chromatography (GC) conditions for Alpinia galanga, Alpinia calcarata and Pluchea lanceolata**

**Test solution**

5 g each of the powdered samples were kept in 100 ml each of
petroleum ether for overnight. The extract was then filtered and the solvent was evaporated under reduced pressure in a rotary evaporator. The residue was then dissolved in 10 ml of petroleum ether.

**Chromatographic conditions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector</td>
<td>Flame Ionization Detector</td>
</tr>
<tr>
<td>Fuel gas</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>Flow rate of carrier gas</td>
<td>2.0 ml/min</td>
</tr>
<tr>
<td>Split ratio</td>
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</tr>
<tr>
<td>Column</td>
<td>HP-5 (5 percent phenyl methyl siloxane) capillary column (30 m x 320 μm x 0.25 μm)</td>
</tr>
<tr>
<td>Injector temperature</td>
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</tr>
<tr>
<td>Detector temperature</td>
<td>300°</td>
</tr>
<tr>
<td>Temperature programming</td>
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</tr>
<tr>
<td></td>
<td>50 ° C to 250 ° C at 4°/min,</td>
</tr>
<tr>
<td></td>
<td>200° maintained for 0 min</td>
</tr>
<tr>
<td>Samples</td>
<td>1 μl each of the Petroleum ether extract</td>
</tr>
<tr>
<td>Total run time</td>
<td>50 Minutes</td>
</tr>
</tbody>
</table>
3.2.11.6.6 Phytochemical characterization using GCMS Analysis

**Extraction**

Fresh rhizomes were chopped, crushed and kept in water for 24 hrs with continuous shaking for 12 hrs. It was then filtered and passed through anhydrous sodium sulphate for removing aqueous matter if any. The oil was used for GCMS studies.

**GCMS conditions**

In *A. galanga* and *A. calcarata*, Gas chromatography-mass spectrometry was carried out on an Agilent GC-MS 6850 under electron impact ionization (70 eV). The interface temperature was 230°C, and the MS scan range was 50-800 atomic mass units (AMU). The chromatographic column for the analysis was done by HP5 - MS capillary column (30 m x 0.25 mm internal diameter). The carrier gas used was helium at a flow rate of 1 ml/min. The oven temperature was 60°C to 250°C with a constant increase of 10°C. The injection was performed in split mode at 250°C.

In *P. lanceolata*, Gas chromatography-mass spectrometry was carried out on an Agilent GC-MS 6850 under electron impact ionization (70 eV). The interface temperature was 230°C, and the MS scan range was 50-800 atomic mass units (AMU). The chromatographic column for the analysis was done by HP5 - MS capillary column (30 m x 0.25 mm internal diameter). The carrier gas used was helium at a flow rate of 1 ml/min. The oven temperature was 60°C to 250°C with a
constant increase of 5°C. The injection was performed in split mode at 250°C.

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

GCMS Analysis for the water extract of *Alpinia galanga*, *Alpinia calcarata* and *Pluchea lanceolata* were carried out and compounds were detected.

3.2.12 Preparation of thesis

Compilation of all data available from the studies carried out with necessary photographs for the preparation of final report.