Chapter 3

MATERIALS AND METHODS

3.1 AREA OF STUDY – KERALA

3.2 COLLECTION AND PRESERVATION OF PLANT MATERIALS

3.3 ETHNOBOTANICAL STUDIES

3.4 PHYTOCHEMICAL STUDIES

3.5 PHARMACOGNOSTIC STUDIES
Chapter 3

MATERIALS AND METHODS

The present study was carried out to investigate the ethnobotanical, phytochemical and pharmacognostic significance of the genus *Hydnocarpus* in Kerala.

3.1 AREA OF STUDY - KERALA

Kerala is a state in the south-west part of India which is spread over 38,863 square kilometres. It lies between north latitudes 8°18' and 12°48' and east longitudes 74°52' and 77°22' (“About Kerala.”) and is bordered by the state of Karnataka to the north east, Tamil Nadu to the east and the Arabian Sea to the west. The state is divided into 3 distinct geographical and climatic regions; the western low land or coastal plain, the central mid-land and eastern highland (“Kerala at a Glance.”). The Western Ghats, whose major portion lies in Kerala is one of the world’s 34 biodiversity hotspots (Mittermeier et al., 2004). These peculiarities make Kerala a place with exceptional biodiversity and endemism.

3.2 COLLECTION AND PRESERVATION OF PLANT MATERIALS

Fresh and uninfected specimens were collected from specified locations during the study period from 01.04.2011 to 31.05.2013 (Table 1). Necessary permission was obtained from the Department of Forest and Wild life, Government of Kerala (Appendix 1). Photographs were taken using Canon EOS 350D digital SLR camera. The herbarium sheets were prepared according to the standard herbarium techniques (Fosberg & Sachet, 1965) and deposited in C.M.S Herbarium, C.M.S College, Kottayam. Fresh leaf and bark materials were collected. The leaf material for
pharmacognostic studies were fixed in F.A.A. Leaf and bark materials for phytochemical studies were washed, cleaned, oven/shade dried, powdered (20 mm mesh size) and sealed in air tight glass bottles for further phytochemical investigations.

**Table 1**: Localities selected for the collection of plant materials.

<table>
<thead>
<tr>
<th>Si. No.</th>
<th>Species</th>
<th>Parts collected</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Hydnocarpus alpina</em></td>
<td>Leaf and Stem bark</td>
<td>Thenmala RF, Kollam&lt;br&gt; Senthuruni WLS, Kollam&lt;br&gt; Angamoozhi, Pathanamthitta&lt;br&gt; Silent Valley NP, Palakkad</td>
</tr>
<tr>
<td>2</td>
<td><em>Hydnocarpus macrocarpa</em></td>
<td>Leaf and Stem bark</td>
<td>Ponmudi RF, Thiruvananthapuram&lt;br&gt; Adimali RF, Idukki&lt;br&gt; Sholayar RF, Thrissur</td>
</tr>
<tr>
<td>3</td>
<td><em>Hydnocarpus pendulus</em></td>
<td>Leaf and Stem bark</td>
<td>Silent Valley NP, Palakkad</td>
</tr>
<tr>
<td>4</td>
<td><em>Hydnocarpus pentandra</em></td>
<td>Leaf and Stem bark</td>
<td>Ponmudi RF, Thiruvananthapuram&lt;br&gt; Karunagappally, Kollam&lt;br&gt; Mavelikkara, Alapuzha&lt;br&gt; Vadavathoor, Kottayam&lt;br&gt; Angamoozhi, Pathanamthitta&lt;br&gt; Sholayar RF, Thrissur&lt;br&gt; Silent Valley NP, Palakkad&lt;br&gt; Aaralam WLS, Kannur</td>
</tr>
</tbody>
</table>

**3.3 ETHNOBOTANICAL STUDIES**

The methodology adopted for ethnobotanical studies include field survey, data collection and analysis of relevant literature. The data collection was carried out according to the methodology suggested by Cotton (1996), Jain (2001) and George et al. (2003). General conversations, discussions and open-ended semi-structured interviews were conducted among traditional vaidyans, tribals (Mannan, Kani, Ulladan, Paniyar and Muthuvan), forest guards, medicinal plant merchants and local
people with traditional botanical knowledge (Plate 2 - 4) to collect the ethnobotanical data. The latter include vernacular names of the genus, its distribution and the parts of the genus used. It also includes the identification of the medicinal, agricultural, economic, religious and environmental values of the genus. The informants were selected according to their understanding of traditional knowledge of medicinal plants. A model of the interview schedule and the form for the consent of informants are given in Appendix 2 and 3. The data collected from the informants were documented.

Relevant literature from various flora (van Steenis, 1958; Gamble, 1915; Kanjilal and Das, 1982; Manilal, 1988; Vajravelu, 1990; Mitra, R.L., 1993; Subramanian, 1995; Sivarajan and Mathew, 1997; Sivarajan and Sasidharan, 1996; Mohanan and Sivadasan, 2002; Balakrishnan, 2005; Nayar et al, 2006), journals and books (Manilal et al., 1983; Nambiar et al, 1985; Nair, 2000; Sasidharan, 2004) were collected and documented. The data gathered through field survey, data collection and literature survey were subjected to analysis and the results are given in Chapter 4.

3.4 PHYTOCHEMICAL STUDIES

Leaves and stem bark of different species of *Hydnocarpus* in Kerala were subjected to phytochemical studies. The methodology adopted for phytochemical studies includes extraction, preliminary phytochemical screening and HPTLC profiling for major phytochemical groups. It also includes identification of generic and specific chemical markers. Standard procedures suggested by Harborne (1973), Kokate (1999), Evans (1996), Sadasivam & Manickam (2005), Reich and Schibili (2007), Wagner & Bladt ( 1996 ) and Shah & Seth (2010) were used for conducting the present study.
3.4.1 EXTRACTION OF PHYTOCHEMICALS

20 g of the powdered leaf and bark samples were subjected to cold extraction in 200 ml 100% methanol for 2 weeks to extract major phytochemicals except alkaloids. For the extraction of alkaloids, 20 g of the samples were subjected to cold extraction in 200 ml 10% ammoniacal methanol. A serial extraction using petroleum ether, chloroform and methanol was also carried out. The extracts were filtered and concentrated using Rotary vacuum evaporator.

3.4.2 PRELIMINARY PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening for alkaloids, steroids, triterpenes, flavonoids, phenolics, tannins and saponins were conducted using standardised protocols

3.4.2.1 TEST FOR ALKALOIDS

a. Dragendorff’s test

Plant extract was mixed with Dragendorff’s reagent (Potassium Bismuth Iodide). Formation of orangish-red colour indicates the presence of Alkaloids.

b. Mayer’s test

Plant extract was mixed with few drops of Mayer’s reagent (Potassium mercuric iodide). Formation of creamy-white precipitate indicates the presence of Alkaloids.

c. Hager’s test

Plant extract was mixed with few drops of Hager’s reagent (Saturated aqueous solution of Picric acid). Formation of crystalline yellow precipitate indicates the presence of Alkaloids.
d. Wagner’s test

Plant extract was mixed with few drops of Wagner’s reagent (dilute Iodine solution). Formation of reddish-brown precipitate indicates the presence of Alkaloids.

3.4.2.2 TEST FOR STEROIDS

a. Salkowski Test

To 1 ml of the plant extract equal volume of chloroform is added and subjected with few drops of concentrated H$_2$SO$_4$. Appearance of brown ring indicates the presence of steroids.

3.4.2.3 TEST FOR TRITERPENES

a. Liebermann – Burchard Test

2 ml of extract was dissolved in acetic anhydride in a test tube, heated to boiling and cooled. 1 ml of concentrated sulphuric acid was then added along the sides of the tube. Formation of a pinkish red colour indicates the presence of triterpenes.

b. Salkowski Test

2 ml of the extract was shaken with chloroform in a test tube and sulphuric acid was added to the chloroform layer through the sides. Formation of yellow colour indicates the presence of triterpenes.

c. Trichloro acetic acid test

2 ml of extract was treated with saturated solution of trichloroacetic acid. Formation of a coloured precipitate indicates the presence of triterpenes.
3.4.2.4 TEST FOR FLAVONOIDS

a. Shinoda Test

Four pieces of magnesium fillings (ribbon) was added to the methanolic extract followed by few drops of concentrated hydrochloric acid. The presence of flavonoids was indicated by pink or red colour.

b. Zinc hydrochloride test

To the extract, a mixture of zinc dust and concentrated hydrochloride acid was added. Red colour indicates the presence of flavonoids.

c. Sulphuric acid test

Extract was treated with few drops of sulphuric acid. Presence of deep yellow to red colour indicates the presence of flavonoids.

d. Lead acetate test

Few drops of Lead acetate were added to 1-2 ml of the filtered extract. Formation of yellow precipitate indicates the presence of flavonoids.

3.4.2.5 TEST FOR PHENOLICS

a. Ferric chloride test

2 ml of distilled water followed by few drops of 10 % ferric chloride was added to 1 ml of the plant extract. Formation of blue or green colour indicates the presence of phenolics.
3.4.2.6 TEST FOR TANNINS

a. Ferric chloride test

About 0.5 mg of dried powdered sample was boiled in 20 ml of water in a test tube and filtered. 2 ml of 0.1 % ferric chloride was added to the plant extract. Formation of dark blue or greenish black precipitate indicates the presence of tannins.

b. Gelatin test

1 % solution of gelatine containing 10 % sodium chloride was added to the extract. Presence of white precipitate indicates the presence of tannins.

3.4.2.7 TEST FOR SAPONINS

a. Foam test

1 ml of the extract was taken in a measuring cylinder and shaken well for 15 minutes with 20 ml distilled water. Development of a layer of foam indicates the presence of saponins.

3.4.3 EXTRACTION OF ALKALOIDS

Acidic phase of chloroform extraction

The ammoniacal methanol extract of the leaves and bark were transferred to water bath in a beaker for few hours. 5 % HCl was added to the dried extract, warmed for few minutes and filtered. The filtrate was transferred to a separating funnel and chloroform was added. The funnel was closed with a stopper and shaken well. This process was repeated thrice. The gas which was produced in the funnel was expelled by opening the stopper of the funnel. The solution was allowed to settle down for few
minutes and the lower layer containing the acidic phase of CHCl₃ extract was collected in a fraction collector.

**Alkaline phase of chloroform extraction**

After collecting the acidic phase of CHCl₃ extract, NH₄OH was added to the solution contained in the separating funnel to make the solution alkaline. After completing the reaction, CHCl₃ was added to the alkaline solution and shaken well as in the case of acidic phase. The lower layer containing the alkaline phase of CHCl₃ extract was then collected in another fraction collector. Both the acidic and alkaline fractions were concentrated and used for HPTLC profiling.

**3.4.4 HPTLC METHOD AND CHROMATOGRAPHIC CONDITIONS**

The HPTLC system (Camag, Muttenz, Switzerland) has Linomat V auto sprayer connected to a nitrogen cylinder, a twin trough chamber (10 x 10 cm) and a derivatization chamber. Pre-coated silica gel 60 F₂₅₄ TLC plates (10 x 10 cm, layer thickness 0.2 mm – E Merck KGaA, Darmstadt, Germany) was used as the stationary phase. TLC plates were prewashed twice with 10 ml of methanol and activated at 80 °C for 5 minutes prior to sample application. Densitometric analysis was carried out using a TLC scanner III with winCATS software.

**3.4.5 SAMPLE APPLICATION**

7 µl of sample was spotted on pre-coated TLC plate in the form of narrow bands (8 mm) with 10 mm from the bottom using Linomat V spotter. Samples were applied under continuous dry stream of nitrogen gas at constant application amount 7 µl.
3.4.6 MOBILE PHASE AND MIGRATION

The spotted plates were developed using different mobile phases to detect the various classes of phytochemicals. The proportion of the chemicals in the mobile phases is as follows:

**Alkaloids** - Toluene: Methanol: Diethyl amine (8:1:1)

**Essential oils** - Toluene: Ethyl acetate (8.5:1.5)

**Steroids** - Toluene: Methanol: Acetone (6:2:2)

**Triterpenes** - Toluene: Chloroform: Ethanol (4:4:1)

**Flavonoids** - Toluene: Ethyl acetate: Formic acid (7:3:0.1)

**Flavonoid glycosides** - Ethyl cete: Acetic acid: Formic acid: Water (10:1.1:1.1:2.6)

**Flavonolignans** - Chloroform: Acetone: Formic acid (7.5:1.65:0.85)

**Phenolics** - THF: Toluene: Formic acid: Water (16:8:2:1)

**Tannins** - Ethyl acetate: Acetic acid: Ether: Hexane (4:2:2:2)

**Saponins** - Chloroform: Acetic acid: Methanol:Water (6.4:3.2:1.2:0.8)

Linear ascending development was carried out in 10 x 10 cm twin trough glass chamber equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 20 minutes at 25 ± 2 °C with a relative humidity of 60 ± 5 %. Ten millilitres of the mobile phase (5 ml in trough containing the plate and 5 ml in other trough) was used for the development and allowed to migrate a distance of 70 mm from the point of sample application. After development, TLC plate was dried and the
chromatogram was viewed at 254 nm and 366 nm to visualise and detect various phytochemical constituents.

3.4.7 DERIVATIZATION

The HPTLC plates were derivatized with the following reagents to detect the various classes of phytochemicals.

Alkaloids - Dragendorff reagent

Essential oils, Saponins and Triterpenes - Anisaldehyde sulphuric acid

Steroids - Vanillin sulphuric acid

Flavonoids, Flavonoid glycosides & Flavonolignans - NP/PEG Reagent

Phenolics and Tannins - Fast blue salt B

3.4.8 DOCUMENTATION

The various conditions for documentation were selected based on the recommendations given in the CAMAG TLC Scanner III manual. The plates were photographed in various conditions under UV 254 nm, UV 366 nm and UV 366 nm after derivatization. The plates were subjected to scanning prior to derivatization. Densitometric scanning was performed on CAMAG TLC scanner III in absorbance mode and operated by winCATS planar chromatography version 1.3.4. The source of radiation utilized was Deuterium lamp. The spots were analysed at a wave length of 218 nm. The slit dimensions used in the analysis were of 6 mm length and 0.30 mm width, with a scanning rate of 20 mm/s. It covers 70 % - 90 % of the application band length. The monochromator band width was set at 20 mm. Concentration of compound chromatographed were determined on the basis of the intensity of diffusely reflected light and evaluated as peak areas against concentration using linear regression equation. A comparison chart was prepared and dendrograms were
constructed using SPSS software for statistical analysis to identify the affinity and evolutionary significance of the different species of *Hydnocarpus* in Kerala.

### 3.5 PHARMACOGNOSTIC STUDIES

Pharmacognostic studies were carried out in collaboration with Dr. D. Narasimhan, Centre for Floristic Research, Department of Plant Biology and Plant Biotechnology, Madras Christian College, Tambaram, Chennai - 600059. Anatomy of leaf and petiole were studied in details using the following techniques. The methodology adopted for pharmacognostic studies included hand and microtome sectioning, maceration, vein clearing, microscopic observation and tabulation.

#### 3.5.1 LEAF AND PETIOLE ANATOMY

Hand and microtome sections were taken from the middle part of the midrib, lamina and the petiole. Microtome sections of the specimens were prepared using rotary microtome at 10μ and stained with toluene blue and mounted in DPX. Characters were observed and recorded using Dewinter Crown microscope. Measurements of the cells were made using an ocular micrometer. All the characters studied were tabulated and comparison charts were prepared.

#### 3.5.2 VEIN CLEARING - VENATION PATTERN

Venation pattern was studied by clearing the veins. Leaf pieces of 0.5 cm² were taken both from the midrib and the blade. Materials were boiled with 70 % alcohol for 10 minutes and it was then transferred to sodium hydroxide solution (10 g of NaOH pellet in 100 ml of distilled water). The materials were kept at 58 °C for 4 - 5 days. Cleared materials were washed in distilled water and stained with safranin. 4 % F.A.A. was used to preserve the cleared material for further use.
3.5.3 EPIDERMAL AND STOMATAL MORPHOLOGY

Jeffrey’s maceration technique was used to record the details of epidermis and stomata. Leaf pieces of 0.5 cm² were taken and heated with Jeffrey’s maceration fluid (10 g of chromic acid dissolved in 100 ml of distilled water + 10 ml of nitric acid dissolved in 100 ml of distilled water) at 58 °C for 24 hours (Sass, 1940; Prasad & Prasad, 1970). The macerated epidermal tissues were washed in distilled water and stained with safranin. 4 % F.A.A. was used to store the material for further investigation.