MATERIALS AND METHODS
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Collection of plant materials

The plant materials for the current analyses were collected from Maruthamalai forests, Coimbatore, Western Ghates, Tamil Nadu, India. *Euphorbia mouritianica* was been propagated from stem cuttings in the botanical garden of the University. The terminal parts of the plants with leaves and stems were collected from shrubs and climbers while the entire above ground plant material was taken from herbs. The collected plant materials were belonging to twelve families. Description of the plant species has been namated separately. Plant materials were chopped into small pieces and dried at sheltered shade conditions with the room temperature \( \sim 30^\circ\text{C} \) except cacti material. Fleshy material were oven dried at \( 50^\circ\text{C} \) for 48 h and all the plant material were ground in a Wiley Mill and passed through a 1 mm screen. Milled samples were then stored at ambient temperature in sealed plastic bags. In plant collection, preference was given to perennial, weedy, aggressive species, and to those taxa showing some evidence of accumulation of potentially useful products such as gums, oils, resins, tars and waxes. Grasses (Poaceae, Gramineae) were given a low priority for collection and screening because this family has been known to be generally low in yields of extractable compounds of interest (Buchanan, et al., 1979). The plant species were identified with the herbarium available in the Department of Botany, Bharathiar University,
Coimbatore 641 046 and with certain extend identifications were made with the help of Botanical Survey of India, Southern circle, Coimbatore.

The laticiferous plant samples were collected from the wild during June-Aug. 1993; Jan.-June, 1994; May-Dec. 1995.

During June-Aug. 1993 the following plant samples were collected

- *Euphorbia hirta*
- *E. lactai*
- *E. vajravelvii*
- *Synadenium grantii*

During Jan.-June, 1994 the following plant samples were collected

- *Ipomaea staphila*
- *Hemidesmus indicus*
- *Cryptolepis buchananii*

During May-Dec. 1995 the following plant samples were collected

- *Sarcostemma brunonianum*
- *Euphorbia antiquorum*
- *E. mouritianica*
- *Pergularia daemia*
- *Carissa carandes*
- *Ceropegia juncea*
- *Nerium indicum*
- *Secamone emetica*
Species known to be capable of producing natural rubber (NR) were collected preferentially. Several species thus were collected whose hydrocarbon content had not previously been reported.

**Extraction of Hydrocarbon, oil and polyphenol fraction**

Milled samples were extracted for 48 h in a soxhlet apparatus following Buchanan et al., (1978a,b) and Cull (1983) to determine weight contents of fractions referred to as oil, polyphenol and hydrocarbon. A scheme was employed for partitioning whole plant samples into major fractions by solvent extraction (Fig.5). The acetone and subsequent benzene-hexane extractions were exhaustive, requiring 48 h using soxhlet apparatus. The solvent for extraction of hydrocarbon fraction was a 2:3 volume ratio mixture of benzene : hexane to match natural rubber (NR) in solubility parameter. Acetone extractives were freed from solvent, then partitioned between hexane and 9:1 methanol: water in separator funnels to give oil and polyphenol fractions respectively. This fractionation procedure had the advantage over more refined analytical methods, such as gas-liquid chromatography. The results were expressed as percentage of dry sample weight. Oil fractions were saponified by conventional procedures and their constituents were partitioned between 50% aqueous ethanol and hexane to obtain sodium salts of organic acids in aqueous ethanol and unsaponifiable matter in hexane. The aqueous – alcohol portions were acidified and extracted
with hexane to obtain the free acids. Free acids and unsaponifiables were oven
dried and weighed and yields were calculated (Buchanan et al., 1978a; Marimuthu et al. 1989).

Rating of plant species

Plant species were rated according to their probable utility as
sources of fiber and protein and according to their oil fraction and hydrocarbon
fractions as shown in the table 1.

Each species was rated independently in each of the four
categories i.e. in two classifications in fiber utility, two classifications in protein
production and four classifications each for oil and hydrocarbon production
respectively. Thus scoring 8 are considered possibilities and those scoring 8 or
less are of definite interest (Buchanan et al., 1978; Cull, 1983) (Table 1).

Infra-red spectroscopy studies

Where the yield of hydrocarbon fraction was at least 0.4% of
plants which were examined by Infra-red (IR) spectroscopy to determine
whether they were predominantly natural rubber (NR), gutta and or waxes.

Nuclear Magnetic Resonance (NMR) spectroscopy studies

NMR spectra of hydrocarbon fractions of some of the plants
analysed were obtained in a Perkin Elmer EM 390 90 MHz with CDCl₃ as
solvent.
Analyses of crude protein, crude lipid and crude fiber

The crude protein content was calculated by multiplying the percentage Kjeldahl nitrogen (Humphries, 1956) by the factor 6.25. The contents of crude lipid, crude fibers were estimated by AOAC (1970) methods.

Gross heat value

Gross heat value of the plant samples was determined by using Bomb calorimeter (Anonymous, 1966).

Chromatographical studies

There are several techniques followed to separate chemical compounds. Chromatography is a technique where the plant pigments or chemical substances are separated. In this study column chromatography and thin layer chromatography were applied to separate the compounds from the fraction of polyphenol of the *Ceropegia juncea* belonging to Asclepiadaceae family.

Column filled with petroleum ether (60-80°) and silica gel (60-120 mesh). Then the fraction of polyphenol of the plant *Ceropegia juncea* was added into the column. Elution was commenced with and continued step wise with petroleum ether: benzene (5:1, 2:1 and 1:1) mixtures. Elution was collected in 150 ml fractions and evaporated. TLC spots were observed in
each 150 ml elution. Similarly 100 ml. fractions were collected separately and checked whether any new spots/compounds are crystallized.

Isolation and characterization of the compounds

The first four elutions of the above column were observed inseparable mixtures spots and the fifth one with petroleum ether (1:1) as eluant furnished a product 4 after evaporation of the solvent (yield 150 mg; melting point 213°C). To identify the compounds Infra-red, Nuclear Magnetic Resonance and Mass spectroscopy were applied.

Recrystalization of the product from petrol-benzene furnished it as white needles and it was identified to be lupeol (Halsall and Jones, 1954) melting point 215°C; mass: m/z 426.

Phytochemical compounds screening

The plant samples for these screening were collected during July-Dec. 1997. Twenty five gram of the powdered plant material was extracted with 200 ml ethanol in a soxhlet apparatus for 15 hrs (Shuchitakumar et al., 1990; Alagesaboopathy et al., 1996). About 10 ml of the ethanol extract was kept separately for testing flavonoid and phenolic substances and the remaining portion of the ethanol extract was evaporated to dryness on a water bath. This residue was used for qualitative calorimetric identification tests for flavanoid, alkaloid, saponin, tannin, steroid, and terpenoid and rotenoid.
Steroid and terpenoid

There were two tests followed to screen steroid and terpenoid type compounds.

i. Salkowski Test – In a test tube a small amount of the extracted solid test sample was added with ~5 ml of chloroform and few drops of concentrated sulphuric acid. If gave red colour then confirm the presence of steroid and terpenoid in the sample (Finar, 1982).

ii. Libermann-Burchard Test – In a test tube a small quantity of the test solid sample was taken and added chloroform solution into the sample and this was treated with few drops concentrated $\text{H}_2\text{SO}_4$ and ~2ml acetic anhydride, when a green colour produced it is a positive result for the presence of steroid and terpenoid type compounds (Finar, 1982).

Rotenoid

Durham Test – A small quantity of the extracted test solid sample was added with 5 ml of concentrated nitric acid in a test tube. The mixture turned to red colour thus indicating the presence of rotenoid type compounds in the extract (Prabhakaran, 1996).

Saponins

There were two methods followed to screen saponins in the plant samples

i. Evaporated residue from ethanol extract was dissolved in water. After vigorously shaking honeycomb froth persisted for 30 minutes indicating the presence of saponins.
Another method was that the residue was dissolved in ~5ml of chloroform and filtered. A few drops of Conc. H$_2$SO$_4$ was added and 1 ml of acetic anhydride to 1ml of iced filtrate. The appearance of blue, bluish green or reddish brown colour often accompanied the formation of a pink ring shows the presence of saponin. (Shuchithakumar et al., 1990).

**Tannin**

After evaporating the ethanol extract of the test sample to dryness, the residue was dissolved in ~5 ml of water and tested with gelatin solution (1%), gelatin salt reagent (1% gelatin and 60% NaCl) and salt solution (10% NaCl). The appearance of a white precipitate with gelatin solution or with gelatin salt reagent indicates the presence of tannin but appearance of precipitate with salt solution reveals negative test for tannin (Harborne, 1973).

**Flavonoid**

In a test tube with 1 ml of ethanol extract of the test sample, a few drops of Conc. HCl and Mg. Turnings were added. The appearance of pink or magenta colour indicates the presence of flavonoid (Prabhakaran, 1996).

**Alkaloid**

Dissolved a little portion of the test sample residue in 5 ml of 1% HCl, filtered, and it was made alkaline with 28% NH$_4$OH and extracted with an
equal volume of chloroform. Then extracted this solution with an equal volume of 1% HCl, separated with the help of separating funnel and tested with Mayer's/Dragendorff's reagent and silicotungstic acid. Any precipitate or turbidity confirms the presence of alkaloid (Shuchithakumar et al., 1990).

**Phenolic compound**

The alcoholic extract of the plant test sample was treated with 1 drop of FeCl₃. Intense blue to violet colour will denote the presence of phenolic compound (Harborne, 1973).

**Anatomical studies of laticifers**

Staining of laticifers is of paramount importance to identify high or low rubber yielding varieties and to evaluate rubber-containing cells in specific parts of the plant. The literature on the techniques for staining of the rubber in plant tissues is meager.

Simple, easy and inexpensive methods have been evolved to detect laticifers in plants with the help of bright-field microscopy using Oil Red O stains (Inamdar et al., 1987).

Localization of laticifers in plant tissue is important for plant scientists because of the increasing use of latex as a source of rubber and hydrocarbon. Moreover, alkaloids and other constituents derived from latex are used in medicine (Watt and Breyer-Brandwijk, 1962). However the
identification and localization of laticifers is a difficult process owing to their high hydrocarbon concentration (Shukla and Krishnamurti, 1971).

Techniques proposed so far for identification and localization of laticifers are either expensive (Wilson et al., 1984) or involve lengthy process, such as fixation, dehydration and microtome sectioning (Bruni and Tosi, 1980). Hence simple, reliable and inexpensive methods for localization of laticifers using bright-field microscopy have been evolved.

Their hand sections were cut from fresh shoot apices of stem segments obtained from laticiferous plants.

i) Sections were treated with 88/12 (v) acetone-water at 50c for 45 min, giving a minimum of three changes of preheated solvent to deracinate.

ii) Extraction of total lipids was carried out at room temperature for 30 min. in a mixture of chloroform and methanol 1:1 (v/v).

iii) Further, lipids adhering to the cell wall and other wood elements along with alkali soluble resins were also removed by agitating the sections with 5% NaOH in 95% ethanol for 30 sec.

iv) Finally the sections were thoroughly washed with 2N HCl followed by distilled water and were subsequently used for localizing laticifers in situ. and microscopically identified for the presence of articulated, non-articulated laticifers and articulated branched and non-articulated branched laticifers.
Table 1. Evaluation of plant species for potential as hydrocarbon producing Crops

<table>
<thead>
<tr>
<th>Species</th>
<th>Fiber</th>
<th>Protein</th>
<th>Oil</th>
<th>Hydrocarbon</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apocynaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nerium oleander</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td><strong>Aclepiadaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ceropegia juncea</em></td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td><em>Pergularia daemia</em></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td><em>Secamone emetica</em></td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td><strong>Euphorbiaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Euphorbia antiquorum</em></td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td><em>E. mouritianica</em></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><em>Synadenium grantii</em></td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

Plant species were rated according to their probable utility as sources of fiber, protein, oil fraction and hydrocarbon fractions. Thus scoring 8 are considered possibilities and those scoring 8 or less are of definite interest (Buchanan et al., 1978; Cull, 1983).
Fig. 5. Scheme for partitioning whole plant samples