## CHAPTER - 4

**EXTRACTION & PHYTOCHEMICAL ANALYSIS**

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The stem part of *Cissus quadrangularis* and the fruit pulp of *Aegle marmelos* were air dried and pulverized into powder. About 25gm of the powdered sample of each medicinal plant were weighed and 100 ml of solvent was added and extracted in a Soxhlet apparatus separately and the process is carried out for 7 days at 40-50˚c. The filtrate was evaporated to dryness at 40˚C in a rotary evaporator. And the above process was repeated for several times, until the sufficient amount of extract is produced. The concentrated extract of each plant was stored at 4˚C until when required for use.

The Phytochemical Investigation of a plant involved in the following

- Extraction of Plant Material
- Identification of the Phytoconstituents
- Separation and Isolation of Phytoconstituents
- Characterization of the Isolated Compounds

4.1. Extraction of Plant Material

![Fig. 4.1: Apparatus used for plant extraction](image)
The plant material was subjected to shade drying, then the shade dried plant material was subjected to pulverization to get coarse powder and it was extracted in a Soxhlet apparatus using various solvents according to their polarity\(^1\).

- Petroleum ether extract
- Ethyl acetate extract
- Chloroform extract
- Acetone extract
- Methanol extract
- Aqueous extract

**Materials Required**

Shadedried coarse powder of *Cissus quadrangularis* and *Aegle marmelos*, Petroleum ether, n-hexane, Chloroform, Acetone, Methanol and 0.25 % Chloroform in water.

### 4. 1. 1. PREPARATION OF EXTRACTS

**Petroleum Ether Extract**

The crude powder was extracted with 2-3 litres of petroleum ether (60°- 80°C) using soxhlet apparatus by continuous hot percolation method. After extraction it was filtered and then the removal of solvent was done under reduced pressure by distillation process. Then the (10gm) extract was stored in a desiccator.

**Ethyl acetate Extract**

After extraction, the marc left out was dried and then it is extracted with 2-3 litres of ethyl acetate (69.0°C) by continuous hot percolation using soxhlet apparatus. After extraction it was filtered and the removal of solvent was done under reduced pressure by distillation process. The (12gm) extract was stored in desiccator.
Chloroform Extract

The residue left after extraction was dried and extracted with 2-3 litres of chloroform (55.5°- 61.5°C) using soxhlet apparatus by continuous hot percolation method. After completion of extraction it was filtered and the solvent was removed by distillation under reduced pressure. The (7gm) extract was stored in desiccator.

Acetone extract

The marc remains after extraction was dried and then it is extracted with 2 - 3 litres of acetone (55.5°C - 56.5°C) using soxhlet apparatus by continuous hot percolation method. After extraction it was filtered and the removal of solvent was done under reduced pressure by distillation process. The (8gm) extract was stored in a desiccator.

Methanolic Extract

The marc remains after acetone extraction was dried and then it is extracted with 2 - 3 litres of alcohol 95 % using soxhlet apparatus by continuous hot percolation method. After extraction, it was filtered and the removal of solvent was done under reduced pressure by distillation process. The (10gm) extract was stored in a desiccator.

Aqueous Extract

The marc remains after extraction was dried and then it is extracted with 2 - 3 litres of chloroform water (0.25 %) using soxhlet apparatus by continuous hot percolation method. After extraction it was filtered and the removal of solvent was done under reduced pressure by distillation process. The extract was stored in a desiccator. The results are shown in Table 4.1.
Table 4.1: Successive Solvent Extraction of Combined Extracts (*Cissus quadrangularis* and *Aegle marmelos*)

<table>
<thead>
<tr>
<th>Plant Names</th>
<th>Parts Used</th>
<th>Method of Extraction</th>
<th>Solvents</th>
<th>Colour &amp; Consistency</th>
<th>Average Extractive Value (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined extracts of <em>Cissus quadrangularis</em> and <em>Aegle marmelos</em></td>
<td>Stem and fruit</td>
<td>Continuous hot Percolation by Soxhlet Apparatus</td>
<td>Petroleum ether</td>
<td>Dark green</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ethyl acetate</td>
<td>Green &amp; sticky with oil mass</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chloroform</td>
<td>Light green</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetone</td>
<td>Yellowish green</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Alcohol</td>
<td>Brownish green</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cold Maceration</td>
<td>Aqueous</td>
<td>Brown</td>
</tr>
</tbody>
</table>
The above extracts were used for

- Identification of Constituents by Phytochemical tests
- Separation and Isolation of Plant constituents by Chromatographic Method
- Pharmacological Studies

4. 2. PHYTOCHEMICAL EVALUATION

4. 2. 1. Identification of Constituents by Phytochemical Test

The extracts were subjected to qualitative tests for detection of phytoconstituents present in it viz. alkaloids, carbohydrates, glycosides, phytosterols, fixed oils & fats, phenolic compounds & tannins, proteins and free amino acids, gums & mucilages, flavanoids, lignins and saponins.

Test of Alkaloids

A little fraction of the solvent free petroleum ether, hexane, alcohol and aqueous extracts were mixed individually with a small amount of drops of dilute hydrochloric acid and it is filtered. The filtrate was evaluated carefully with different alkaloidal reagents such as,

a. Mayer’s reagent - Cream precipitate
b. Dragendorff’s reagent - Orange brown precipitate
c. Hager’s reagent - Yellow precipitate
d. Wagner’s reagent - Reddish brown precipitate

Test for Carbohydrates & Glycosides

The least amount of extracts was dissolved in 5ml of distilled water and it is filtered. The filtrate was subjected to analysis for the presence of carbohydrates and glycosides.
a. Molisch’s Test

The filtrate was mixed with 2-3 drops of 1% alcoholic alpha naphthol and along the sides of the test tube; 2ml of concentrated sulphuric acid was added and appearance of purple colour ring at the junction of two liquids.

b. Fehling’s Test

The filtrate was heated with 1ml of Fehling’s A solution. Orange precipitate was obtained indicates the presence of carbohydrates.

Another portion of the extracts was hydrolysed with hydrochloric acid for few hours on a water bath and the hydrolysate was subjected to Legals, Borntrager’s test to detect the presence of different glycosides.

c. Legal’s Test

The Hydrolysate was mixed with chloroform and then the chloroform layer was separated. To this identical quantity of dilute ammonia solution was added. Purple colour in ammoniacal layer was observed.

Test for Phytosterol (Libermann Burchard Test)

One gram of the extract was dissolved in few drops of dry acetic acid, to this 3 ml of acetic anhydride and few drops of concentrated sulphuric acid was added. Bluish green colour appears which shows the presence of phytosterol.

Test for Fixed oils and Fats

Small quantity of different extracts was separately hard-pressed between two filter papers. Oil stain appears on the paper which indicates the presence of fixed oil.
Small quantity of different extracts along with a drop of phenolphthalein was added to few drops of 0.5N alcoholic potassium hydroxide were. Heat the mixture on a water bath for 1-2 hrs. Soap formation or partial neutralization of alkali shows the presence of fixed oil and fats.

Test for Tannins and Phenolic Compounds

Small quantity of extracts were dissolved individually in water and tested for the presence of phenolic compounds and tannins with

i. Dilute Ferric chloride solution 5% - Violet colour
ii. 1% solution of gelatin containing 10% NaCl - White precipitate
iii. 10% Lead acetate solution - White precipitate

Test for Proteins and Free Amino Acids

Small quantity of extracts were dissolved separately in a few ml of water and treated with:

i. Million’s reagent - Appearance of red colour shows the presence of protein and free amino acids.
ii. Ninhydrin reagent - Appearance of purple colour shows the presence of protein and free amino acids.
iii. Biuret test - Equal volume of 5% solution of sodium hydroxide and 1% solution of copper sulphate were added. Appearance of pink colour shows the presence of proteins and free amino acids.

Test for Gums and Mucilages
About 10ml of extract were added individually to 25ml of absolute alcohol with continuous stirring and it is filtered. The precipitate was air dried and evaluated for its swelling properties and for the presence of carbohydrates.

**Test for Flavonoids**

a. When mixed with aqueous sodium hydroxide solution, Colour changes from blue to violet colour (Anthocyanins), yellow colour (Flavones), yellow to orange (Flavonones).

b. When mixed with concentrated sulphuric acid, yellowish orange colour (Anthocyanins), yellow to orange colour (Flavones), orange to crimson (Flavonones).

c. Shinoda’s Test

The various extracts were dissolved individually in alcohol, and then mix a portion of magnesium along with conc. hydrochloric acid drop wise. Magenta colour appears which indicates the presence of flavonoids.

**Test for lignins**

With alcoholic solution, phloroglucinol and conc. hydrochloric acid, Red colour appears which indicates the presence of lignin. The results of chemical tests of whole plant powder and extracts were shown in Table-4.2.
Table 4.2: The Preliminary Phytochemical Screening of Combined Extracts of *Cissus Quadrangularis* and *Aegle Marmelos*

<table>
<thead>
<tr>
<th>Phyto constituents</th>
<th>Petroleum ether extract</th>
<th>Ethyl acetate extract</th>
<th>Chloroform extract</th>
<th>Acetone extract</th>
<th>Alcoholic extract</th>
<th>Aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Glycosides</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Fixed oils and Fats</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Saponins</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Phenolic and Tannins</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Lignins</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Proteins, Amino Acids</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Gums and Mucilage</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

(+): Presence  
(-): Absence
4.3. Separation and Isolation of Plant Constituents by Chromatographic techniques

4.3.1. Thin Layer Chromatography

Thin layer chromatography (TLC) is a chromatographical method which is employed to separate mixtures. It is performed on a glass sheet, aluminium or plastic foil, which is covered with a slim layer of adsorbent substance, generally silica gel, aluminium oxide, or cellulose (blotter paper). This film of adsorbent is identified as the stationary silica phase. After the sample has been filled on the plate, a solvent or solvent mixture (mobile phase) is drained up the plate via capillary action. Because dissimilar analytes rise in the TLC plate at different rates, finally the mixture is separated.

Plate Preparation

TLC plates are generally commercially obtainable, with usual particle size ranges to develop reproducibility. They are prepared by adding the adsorbent, such as silica gel, with a least quantity of inert binder like calcium sulphate (gypsum) and water. Thick slurry was prepared by spreading the mixture on a sheet, generally made up of thick aluminum foil, glass, or plastic. Heat the resulting plate for thirty minutes at 110 °C for drying and activation. The width of the adsorbent layer is usually around 0.1 – 0.25 mm for investigative purposes and about 0.5 – 2.0 mm for preparative TLC.

Analysis

If the chemicals which are separated are colourless, several methods are available to visualize the spots.

- Repeatedly a little quantity of a fluorescent compound, generally manganese activated zinc silicate, is mixed with the adsorbent that allows the visibility of spots under a black light at 254 nm. The adsorbent layer will appear in fluorescence light green by self; however this fluorescence is
quenched by the spots of analyte. Iodine vapors are a common unspecific colour reagent. The TLC plate is dipped into the specific colour reagents or which are sprinkled on top of the plate, Potassium permanganate – oxidation, Iodine

Once visible, the $R_f$ value of each spot can be calculated by dividing the distance travelled by the product by the total distance travelled by the solvent. These values which are calculated depend on the nature of solvent used and the nature of TLC plate and will not come under physical constants. The thin layer contains the eluent at the top of the plate.

Application of Extracts for Separation

The various diluted extracts spotted on a TLC plate 2cm above its bottom using capillary tube. Most solutions for application were between 0.1 - 1% strength. The starting point was equally sized as far as possible and spots had diameter ranging from 2-5mm.

Method

The combined extracts of *Cissus quadrangularis* and *Aegle marmelos* of petroleumether, n-hexane, chloroform, acetone, alcohol and aqueous extracts were subjected to Thin Layer Chromatography using different solvent systems and observed for characteristic spots under UV light and Iodine chamber.

The different solvent systems used were

1. Benzene : Ethanol (9 : 1)
2. n–Butanol : Glacial acetic acid : Water (4 : 1 : 5)
3. Methanol : Water (8 : 2)
4. Petroleum ether : Ethyl acetate (2 : 1)
5. Ethyl acetate : Methanol : Water (100 : 16.5 : 13.5)
Petroleum ether, Chloroform, Acetone, Methanolic and Aqueous extracts of *Cissus quadrangularis* and *Aegle marmelos* showed characteristic spots with the different solvent systems. The results were shown in table 4.3.

**Table 4.3: Data Showing the Thin Layer Chromatography of Alcoholic combined Extracts of *Cissus quadrangularis* and *Aegle marmelos***

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Stationary phase</th>
<th>Mobile phase</th>
<th>No. of spots</th>
<th>Rₜ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td></td>
<td>Benzene:ethanol (9:1)</td>
<td>5</td>
<td>0.4, 0.66, 0.73, 0.83, 0.86</td>
</tr>
<tr>
<td>Glycosides</td>
<td></td>
<td>Ethyl acetate: nbutanol: water (4:4:3)</td>
<td>5</td>
<td>0.26, 0.46, 0.6, 0.73, 0.86</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Silica gel G</td>
<td>Petroleum ether:ethyl Acetate (2:1)</td>
<td>4</td>
<td>0.13, 0.2, 0.33, 0.26</td>
</tr>
<tr>
<td>Steroids</td>
<td></td>
<td>Chloroform; ethanol (96:4)</td>
<td>7</td>
<td>0.13, 0.2, 0.33, 0.4, 0.46, 0.53, 0.6</td>
</tr>
<tr>
<td>Essential oils</td>
<td></td>
<td>Pure chloroform</td>
<td>2</td>
<td>0.66, 0.4</td>
</tr>
</tbody>
</table>

Fig. 4.2: Thin Layer Chromatography of Combined Alcoholic Extract of Cissus quadrangularis and Aegle marmelos

4.4. COLUMN CHROMATOGRAPHY

4.4.1. Principle

Every compound in a mixture will have a specific solubility in the solvent and a specific affinity to be adsorbed by the solid adsorbent. No two compounds typically behave precisely alike in these respects. This principle is used in column chromatography.6,7

4.4.2. Preparation of Column

Materials Used

Column of size 90 cm X 2.5 cm

Silica gel 100-200 mesh as the adsorbent
Silica gel 100-200 mesh was made into a homogenous suspension by shaking with petroleum ether (first eluent). The bottom of the column was plugged with little cotton to prevent the adsorbent pass out, and then the silica gel suspension was poured into the column, set aside for 10 minutes and used.

**Fig. 4.3 Column Chromatography**

The Alcoholic combined Extracts of *Cissus quadrangularis* and *Aegle marmelos* were subjected to column chromatography over silica gel (100-200 mesh). The column was eluted with solvents of increasing polarity. They are

1. Petroleum ether 100%
2. Petroleum ether 80% and chloroform 20%
3. Petroleum ether 60% and chloroform 40%
4. Petroleum ether 40% and chloroform 60%
5. Petroleum ether 20% and chloroform 80%
6. Chloroform 100%
7. Chloroform 80% and acetone 20%
8. Chloroform 60% and acetone 40%
9. Chloroform 40% and acetone 60%
10. Chloroform 20% and acetone 80%
11. Acetone 100%
12. Acetone 80% and Ethyl acetate 20%
13. Acetone 60% and Ethyl acetate 40%
14. Acetone 40% and Ethyl acetate 60%
15. Acetone 20% and Ethyl acetate 80%
16. Ethyl acetate 100%
17. Ethyl acetate 80% Methanol 20%
18. Ethyl acetate 60% Methanol 40%
19. Ethyl acetate 40% Methanol 60%
20. Ethyl acetate 20% Methanol 80%
21. Methanol 100%.

The Results are shown in Table 4.4.
<table>
<thead>
<tr>
<th>S.NO</th>
<th>Eluent</th>
<th>Ratio</th>
<th>Nature of residue</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petroleum Ether</td>
<td>100</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Petroleum Ether : chloroform</td>
<td>80 : 20</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Petroleum Ether : chloroform</td>
<td>60 : 40</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Petroleum Ether : chloroform</td>
<td>40 : 60</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Petroleum Ether : chloroform</td>
<td>20 : 80</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Chloroform</td>
<td>100</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Chloroform : Acetone</td>
<td>80 : 20</td>
<td>Greenish Yellow crystal</td>
<td>CAC1</td>
</tr>
<tr>
<td>8</td>
<td>Chloroform : Acetone</td>
<td>60:40</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Chloroform : Acetone</td>
<td>40:60</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Chloroform : Acetone</td>
<td>20:80</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Acetone</td>
<td>100</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Acetone : Ethyl Acetate</td>
<td>80:20</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Acetone : Ethyl Acetate</td>
<td>60:40</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Acetone : Ethyl Acetate</td>
<td>40:60</td>
<td>No residue</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>Acetone : Ethyl Acetate</td>
<td>20:80</td>
<td>No residue</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4.4: Data showing the column chromatography of alcoholic combined extracts of *Cissus quadrangularis* and *Aegle marmelos*

The Greenish yellow crystalline compound was obtained by column chromatography in the fraction of chloroform extract (Chloroform: acetone – 80:20)

**Description of the Isolated Compound (CAC1)**

<table>
<thead>
<tr>
<th>Nature</th>
<th>Crystalline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Greenish yellow Crystal</td>
</tr>
<tr>
<td>Shape</td>
<td>Needle</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in water and in organic solvents</td>
</tr>
<tr>
<td>Melting Point</td>
<td>136° - 139°C</td>
</tr>
<tr>
<td>TLC</td>
<td>Solvent System Toluene: Ethyl Acetate: Formic acid:</td>
</tr>
<tr>
<td>Methanol (30:30:8.0:2.5)</td>
<td>Rf value : 0.85</td>
</tr>
</tbody>
</table>
Chemical Test

2 ml of acetic anhydride was added with 0.5 g of the extract of both with 2 ml of H$_2$SO$_4$. The colour changes from Violet to blue or green colour in some samples showing the presence of steroids.

4.5. Characterization of Isolated Plant Constituent

Isolated Compound

SITOSTEROL ACETATE

![Chemical Structure of Sitosterol Acetate](image)

Fig. 4.4 Isolated compound

4.5.1. Spectroscopic Methods

The chemical constituents present in the drug possess characteristic features because of which its characterization becomes possible. At every stage of structure determination from isolation and purification of constituents to its final comparison with an authentic sample, the spectral data facilitates the description of structure. Interpretation of molecular spectra is generally based on empirical correlations of spectral data with reasonable assurance to a particular group or arrangement of atoms in the molecule.
Ultra-Violet (UV) spectrum provides limited information about the structure. Infra red (IR) spectrum is generally complicated and out of many peaks relatively a few can be interpreted with assurance. Proton ('HNMR) spectra provide information about the number, nature and environment of the protons and carbon skeleton in the molecule, respectively. Mass spectrum (MS) provides the information about the molecular weight and the fragmentation pattern of the compound\textsuperscript{8, 9}.

4.5.2. Ultraviolet-Visible Spectrum

The absorption of light energy by organic compound in the UV (200 – 400\(\mu\)) range and visible (400 – 800\(\mu\)) range involves promotion of electrons from the ground state to higher energy states. The compounds with chromospheres, auxochrome and conjugated system are dissolved in suitable solvent. The UV spectrum obtained shows absorption bands which gives valuable information regarding the nature of compound.

The crystals obtained from an alcoholic combined Extracts of \textit{Cissus quadrangularis} and \textit{Aegle marmelos} were subjected to ultra violet spectral analysis using perkin – elmer spectrophotometer – Lambda 35 model. The \(\lambda\) max of the isolated compound was found to be 205.12 nm the spectrum is shown in figure. 4.4.
4.5.3. Infrared Spectrum

The constantly vibrating molecules stretch and bend their bonds with respect to one another, by absorbing infrared light. IR spectrum is highly characteristic to establish the identity of compounds. The region $1430 - 910 \text{ cm}^{-1}$ is called ‘fingerprint’ region where many more bending vibrations of the molecules are found. The identities of two samples that have identical spectra in the fingerprint region give conclusive identification of compounds. The crystals obtained from Alcoholic combined Extracts of *Cissus quadrangularis* and *Aegle marmelos* were subjected to Infrared Spectroscopy and the spectrum is shown in figure 4.5.
IR Spectra of the crystalline compound showed characteristic absorption

Fig. 4.6: IR Spectra of Isolated compound CAC1

<table>
<thead>
<tr>
<th>S.NO</th>
<th>FREQUENCY</th>
<th>MODE OF VIBRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3337</td>
<td>polymeric (O-H) stretching,</td>
</tr>
<tr>
<td>2</td>
<td>2927</td>
<td>aromatic C—H stretching</td>
</tr>
<tr>
<td>3</td>
<td>1708</td>
<td>C=O Stretching (acetate)</td>
</tr>
<tr>
<td>4</td>
<td>925</td>
<td>C=C stretching (unsaturation in the ring)</td>
</tr>
</tbody>
</table>
4.5.4. Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance Spectroscopy deals with the study of spin changes in the presence of magnetic field, at the nuclear level when radio frequency energy is absorbed.

As we are analyzing organic compounds for the nature, type, number and environment of protons (Hydrogen), the solvent used in the NMR spectroscopy should not contain hydrogen atoms. Hence we use solvents like

- Carbon tetrachloride (CCl$_4$)
- Deuterated chloroform (CDCl$_3$)
- Deuterated Water (D$_2$O)
- Deuterated Methanol (CD$_3$OD)
- Deuterated acetic acid (CD$_3$COOD)
- Deuterated dimethyl sulfoxide (DMSO)

Infrared Spectroscopy (IR) is used to identify functional groups, the number and nature of chemical entities in a molecule was identified by using NMR spectrum analysis. On the other hand, NMR provides more information when compared to IR. It can also be used to study mixtures of analytes, to know dynamic effects such as change in temperature and reaction mechanisms and is a helpful tool in understanding protein and nucleic acid structure and function.

The crystals obtained from combined chloroform Extracts of *Cissus quadrangularis* and *Aegle marmelos* were subjected to NMR and the spectrum is shown in Figure. 4.6.
The $^1$HNMR and $^{13}$C NMR spectrum exposed three different patterns of proton resonances.

**Table 4.6. Signals in proton NMR and carbon NMR**

<table>
<thead>
<tr>
<th>S.NO.</th>
<th>Signals in proton NMR</th>
<th>Signals in carbon NMR</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>-</td>
<td>10.96</td>
<td>Terminal methyl in ethyl group</td>
</tr>
<tr>
<td>2.</td>
<td>-</td>
<td>14.04</td>
<td>Dimethyl group</td>
</tr>
<tr>
<td>3.</td>
<td>0.818 to 1</td>
<td>23.76 to 29.61</td>
<td>Methyls attached to rings</td>
</tr>
</tbody>
</table>
4.5.5. Mass Spectroscopy

To determine the uniqueness of individual molecules, a mass spectrometer converts them to ions because ions can be moved about and manipulated by external magnetic and electric fields. The three vital functions of a mass spectrometer and the related components are:

1. By loss of an electron, a tiny sample is ionized, usually to cations. - **The Ion Source**
2. According to their mass and charge the ions can be sorted and separated. - **The Mass Analyzer**
3. The ions which are separated, then measured and the reports were displayed on a chart. - **The Detector**

|   | 1.2 to 1.5 | 30.37 to 38.86 | Methylene
|---|------------|----------------|-----------------
| 5 | 2.985      | 22.61          | Acetate methyl group |
The crystals obtained from combined ethanolic extracts of *Cissus quadrangularis* and *Aegle marmelos* were subjected to MASS and the spectrum is shown in figure 4.7. The mass peak of the isolated compound was found to be m/e 444.8 as Parent ion of sitosterol acetate, 402.7 as Acetyl unit.

### 4.6. RESULTS AND DISCUSSION

In consideration of the importance of phytochemical investigation on endemic medicinal plants, the phytochemical analysis of the two endemic medicinal plants of Western Ghats namely
Cissus quadrangularis and Aegle marmelos was carried out in the present work. The results of the study and the discussion pertaining to it are presented below.

The powder of Cissus quadrangularis and Aegle marmelos was extracted with different solvents of increasing polarity and the results were reported in Table No.4.4 and the various extracts were subjected to preliminary phytochemical screening and reported in Table No.4.2. All the extracts showed positive results for steroids, glycosides, phytosterols & lignins.

All the extracts were subjected to thin layer chromatography by using different solvent system and the numbers of spots with its Rf values are presented in Table 4.3.

The extracts were subjected to qualitative chemical tests and steroids, saponins, terpenoids and carbohydrates were found to be present. TLC was run for the confirmation of steroids. Steroids were isolated from ethyl acetate and alcohol extract of Cissus quadrangularis and Aegle marmelos.

The chloroform and Alcohol extract of Cissus quadrangularis and Aegle marmelos was subjected to column chromatography for the separation of compounds. The Chloroform: acetone (80:20) fraction of alcohol extract gave greenish yellow crystalline compound and designated as CAC1.

Greenish yellow crystalline nature, melting point 137-139°C, soluble in water and organic solvents. UV spectra of the crystalline compound CAC1 showed $\lambda_{max}$ of 205.12nm. The IR spectrum shows the frequency peaks at 3337cm$^{-1}$, 1708cm$^{-1}$ shows the presence of phenolic and acetate group. The NMR spectrum was results shown presence of steroid nucleus at signal at $\delta$10.09 (Terminal methyl in ethyl group), 23.76 to 29.61 (methyl attached to the rings), 22.61 (acetate group). The mass peak of the isolated compound was found to be m/e 444.8 as Parent ion of sitosterol acetate, 402.7 as Acetyl unit. The characterized isolated compound was Sitosterol.
4.7. CONCLUSION

The present work is undertaken to produce some pharmacognostical standards and these findings may help to proper identification and ensures the quality of the drug and also help this amazing plant grown on commercial basis for better use in pharmaceutical herbal formulations. In attitude of our interest in the chemical constituents of native medicinal plants, the present phytochemical re-evaluation of the dried leaves of Cissus quadrangularis and Aegle marmelos has now been undertaken. The isolated compound steroids were subjected to spectral studies UV, FT-IR, $^1$H NMR, C$^{13}$ NMR, Mass spectroscopy. The isolated compound steroids were partly confirmed with those observations. Further, that the isolated compound was subjected for LC-MS, HPTLC, X ray crystallaography etc.