Chapter-3

Extraction, isolation and purification of crystalline compound from *Eupatorium adenophorum* Spreng. Leaves (Family - Asteraceae).

3.1 Introduction

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3.1 Introduction

Plant natural products have been components of phytomedicines throughout human history. In past hundred years, plants have become an important source for the discovery of novel pharmaceuticals with many blockbuster drugs being directly or indirectly derived from plants (Fransworth, 1988; Cordella, 2000). Plants can easily synthesize chiral specific compounds, which can take longer time to be synthesized in the laboratory. Bioprospecting for plants and other organisms in losing out to high throughput drug discovery, which relies more on combinatorial drug design (Clark & Pickett, 2000).

The phytochemical investigation of a plant involve the following: extraction of the plant material, separation and isolation of the constituents of interest, characterization of the isolated compounds, investigation of the biosynthetic pathways to particular compounds and quantitative evaluations. Ideally, fresh plant tissue should be used for phytochemical analysis and the material should be plunged into boiling alcohol within minutes of its collection. Alternatively, plants may be dried before extraction under controlled conditions or in shade to avoid chemical changes occurring. It should be dried as quickly as possible, without using high temperature, preferably in a good air draft.

The classical mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substance that is being isolated. Dried materials are usually powdered before extraction. The procedure for obtaining drug substances from dried powdered leaves is continuous hot percolation by using soxhlet apparatus or cold percolation with a range of solvents like petroleum ether,
methanol or rarely with diethyl ether. The extract obtained is clarified by filtration through celite on a water pump and is then concentrated in vacuo. If a single component is present, it can be purified by recrystallisation and then the material is available for further analysis. In most cases, mixtures of components will be present and it will then be necessary to separate by chromatography techniques. As a standard precaution against loss of material, concentrated extract should be stored in the refrigerator and used for further investigations (Harborne, 1973).

The isolation and purification of plant constituents is mainly carried out using one or a combination of the most useful chromatographic technique i.e. paper chromatography (PC), Thin layer chromatography (TLC), Column chromatography (CC), Gas liquid chromatography (GLC), High performance liquid chromatography (HPLC). The choice of techniques depends largely on the solubility, properties and volatilities of the compounds to be separated.

3.2 Plant material

The powdered leaves of the plant were used in this experiment described under chapter-2.

3.3 Extraction of powdered leaves

1 kg of the powdered leaves were extracted with 2500 ml of methanol in a Soxhlet extraction apparatus. The extraction was carried out for the 50 cycles. The solvent was recovered by distillation under reduced pressure in a steam bath. The extract was concentrated and a greenish brown coloured semisolid mass was obtained (yield
11.6%w/w with respect to dry starting powdered materials). This extract was used for different pharmacological and microbiological experiments and subjected to further separation of active principles. After methanolic extraction the marc was dried and subsequently extracted starting from petroleum ether, chloroform, acetone etc. In each step the marc was dried completely before subsequent extraction. The solvent was recovered by distillation under reduced pressure.

3.4 Chemical group test

The preliminary phytochemical studies were performed by testing different chemical groups present in different extract of the bark with different tests performed for the chemical groups were as follows (Trease and Evans, 1983; Wallis, 1985; Plummer, 1985; Kokate et al, 1990).

In each test 10%w/v solution of extract was taken unless otherwise mentioned in individual test.

3.4.1 Test for alkaloids:

3.4.1.1. 2 ml solution of the extract was taken in a test tube. 0.1 ml of dilute hydrochloric acid and 0.1 ml of Mayer’s reagent were added. No development of yellowish buff coloured precipitate indicated the negative test for alkaloids.

3.4.1.2. 2 ml solution of the extract was treated with 0.1 ml of diluted hydrochloric acid and 0.1 ml of Dragendorff’s reagent in a test tube. No development of orange brown precipitate indicated the absence of alkaloids.
3.4.1.3. 2 ml of the extract solution was treated with 0.2 ml of diluted hydrochloric acid and 0.1 ml Wagner's reagent. No development of reddish brown precipitate suggested the absence of alkaloids.

3.4.1.4 2 ml of solution of the extract was allowed to react with 0.2 ml diluted hydrochloric acid and 0.1 ml Hager's reagent. No development of yellowish precipitate indicated the absence of alkaloids.

3.4.2. Test for reducing sugars:

3.4.2.1. 5 ml of solution of the extract was taken in a test tube, then 5 ml mixture of Fehling solution (A and B) was added, boiled for five minutes. Formation of a brick red colour precipitate gave the positive test for reducing sugars.

3.4.2.2. 5 ml of extract solution and 5 ml of Benedict's solution were mixed in a test tube and heated for few minutes. Formation of a brick red precipitate confirmed the presence of reducing sugars.

3.4.3. Test for gums:

3.4.3.1. With 2 ml of 10% extract solution, 2 ml concentrated sulphuric acid was added. Then, it was treated with 15% alcoholic alpha-napthol (Molish's reagent) solution. Formation of red–violet ring at the junction of sulphuric acid layer and extract indicated the positive test for gums (Molish's test).

3.4.4. Tests for flavonoids:

3.4.4.1. 5 ml of extract solution was hydrolysed with 10% sulphuric acid and cooled. Then it was extracted with ether and divided in three separate test tubes. 1 ml of dilute ammonia, dilute sodium carbonate and sodium hydroxide solution was added to the first,
second and third test tubes respectively. In each test tube, formation of yellow colour indicated the presence of flavonoids.

3.4.5. Tests for tannins:

3.4.5.1. 5 ml of the extract was treated with 1 ml of 5% ferric chloride solution. Grenish black colouration showed the presence of tannins.

3.4.5.2. 5 ml solution of the extract was treated with 1 ml of 10% lead acetate solution in water. Formation of yellow coloured precipitate, indicated the presence of tannin.

3.4.5.3. 5 ml extract solution was treated with 1 ml of 10% potassium dichromate solution in water. Formation of yellowish brown precipitate, indicated the presence of tannins.

3.4.6. Test for saponins

3.4.6.1. 1 ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. No formation of stable foam suggested the absence of saponins.

3.4.6.2. 1 ml of the extract solution was treated with 1% lead acetate solution. White precipitate is not formed. It is indicated the absence of saponins.

3.4.7. Test for steroids and triterpenoids:

3.4.7.1. Lieberman - Burchard reaction – 10 mg of the extract was dissolved in 1 ml chloroform and then 1 ml acetic anhydride was added followed by 2 ml concentrated sulphuric acid, a reddish violet colour developed, indicating the presence of steroid and triterpenoid.
3.4.7.1. **Salkowski test:** 10 mg of extract was dissolved in 1 ml of chloroform and 1 ml of concentrated sulphuric acid was added. Reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer indicated the presence of steroid.

3.4.7.3. **Noller test** – 5 mg of the extract dissolved in 2 ml of 0.01% anhydrous stannic chloride in pure thionyl chloride. A purple colour, formed, then changed to deep red after few minutes, indicates the presence of triterpenoids (Noller et al., 1942).

**Table- 9  Chemical group tests of different methanol extract of leaf of Eupatorium adenophorum.**

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Alkaloids</th>
<th>Tannins</th>
<th>Flavonoids</th>
<th>Steroids</th>
<th>Anthraquinone</th>
<th>Terpenoid</th>
<th>Gums</th>
<th>Reducing sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

‘+’ = presence of chemical group, ‘−’ = absence of chemical group.

3.5 **Separation of unsaponifiable constituents:**

The unsaponifiable constituents are not saponifiable by alkali hydroxides and are separated by extraction with an organic solvent from a solution of the saponified substance being examined.

15 gm of the substance (Extracted materials) is taken into a 250 ml flask fitted with a reflusk condenser. 2 gm of KOH in 40 ml ethanol (95%) is added and heat on a water bath for 1 hour. Shaking frequently. Then the contents are transferred to a separating funnel with the aid of 100 ml of hot water and while the liquid is still warm, shake very carefully with three quantities, each of 100ml of peroxide free ether. Combine the ether
extracts in a second separating funnel containing 40 ml of water, swirl gently for a few minutes, allow to separate and reject the lower layer. Wash the ether extract with two quantities, each of 40 ml of water and with three quantities, each of 40 ml a 3% w/v solution of KOH, each treatment being followed by a washing by a washing with 40 ml of water. Finally, wash the ether layer with successive quantities, each of 40 ml, of water until the aqueous layer is not alkaline to Phenolphthalein solution. Transfer the ether layer to a weighed flask, washing out the separating funnel with peroxide free ether.

Distil off the ether and add the residue to 6 ml of acetone. Remove the solvent completely from the flask with the aid of a gentle current of air. Dry at 50 to 60°C for 1 hour and cooled. The process was repeated for five times and total unsaponifiable matters were collected. Then different unsaponifiable compounds were separated by Thin Layer Chromatography and Column Chromatography technic.

3.6. Thin layer Chromatographic study:

3.6.1. Preparation of plate: Glass plates of 5 x 20 cm size were coated with silica gel G (E. Merck and Co.) with the help of spreader to a layer thickness of 0.25 mm. After spreading, the plates were first air dried and then activated at 110°C for 30 minutes. After cooling, the plates were kept in a desiccator until required for further use (Stahl, 1969).

3.6.2. Development of the chromatogram:

The extract was spotted on the plates and chromatogram was developed in chromatographic chamber using different solvent system at a room temperature (28°C) and at an angle of 75°.
In all the cases the solvent system was allowed to a distance of 10 cm from the point of application of the extract in the plates. The time required for development varied from 30-40 minutes. After completion of the run, the plates were removed from chamber and were allowed to dry in air. These plates were observed under UV light for the presence of the spots. The plates were sprayed with different spraying reagents and colour developed was noted, hRf values of the spots were calculated and recorded.

3.6.3 Solvent system used

i) Chloroform : Methanol (10:1)

ii) Chloroform : Methanol (20:1)

iii) Chloroform : Methanol (40:1)

3.6.4 Spray reagent used:

i) Liebermann- Burchard reagent (5ml acetic anhydride was mixed under cooling with 5ml sulphuric acid, this mixture was added to 50 ml absolute ethanol).

ii) Iodine vapour

iv) Vanillin – Phosphoric acid (1 gm vanillin was dissolved in 100 ml of 50% aqueous phosphoric acid)

The TLC studies and chemical group tests clearly indicate the presence of specific steroidal and triterpenoidal compounds in Petroleum ether fraction of methanol extract. So, it is further subjected to column chromatographic separation.
Table –10  Thin Layer Chromatographic study of unsaponifiable fraction

<table>
<thead>
<tr>
<th>No of spot</th>
<th>Colour of the spot Under UV light</th>
<th>Colour of the spot after spraying</th>
<th>hRf values in different solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>1.</td>
<td>Pink</td>
<td>Pink</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Pink</td>
<td>Pink</td>
<td>Pink</td>
</tr>
<tr>
<td>3.</td>
<td>Pink</td>
<td>Pink</td>
<td>pink</td>
</tr>
<tr>
<td>4.</td>
<td>Blue</td>
<td>Blue</td>
<td>Blue</td>
</tr>
</tbody>
</table>

3.7.  Separation of compound by column chromatography

3.7.1.  Column: A glass column 3.0 cm I.D., and 20 cm in length fitted with a stop cock was used.

3.7.2.  Adsorbent: Alumina (Brockmann grade, Neutral, Glaxo India Ltd., Bombay) was activated by heating at 110⁰C for one hour was used as absorbent. Solvent used petroleum ether (60-80⁰C) (S.D. Fine Chem., Bombay).

3.7.3.  Preparation of column:

Activated almina (Neutral grade) was measured in a beaker and it was made into a slurry with Chloroform. The bottom of the column was plugged with glass wool and the alumina slurry was poured into the column. The alumina was allowed to pack until there was no further shrinkage of the column. The final dimension of the alumina column was 3.0 x10 cm. The solvent i.e. chloroform : methanol (40:1) was allowed to drip at the rate of 75 drops per minutes and a level of 4 cm of the solvent was maintained on the top of the alumina layer.

The excess solvent on the top of the column was allowed to flowout and 10 ml of chloroform solution of the extract was carefully layered on the top of the column and the solvent was allowed to flow out slowly till the solution was absorbed on the top of the alumina layer. The rate of elution was adjusted at 30 drops per minute. Then the
following solvent was allowed to pass through the column. Firstly petroleum ether was allowed to pass through the column. The solvent eluting through the alumina column was collected in a separate flask by a fraction collector with a time gap of 15 minutes per fraction. Each fraction was collected for 10 minutes. The eluant was collected in different flasks and were numbered serially 1,2,3 etc. The TLC was carried out for each fraction of the elute collected in the flask separately, using the solvent system chloroform : methanol (40:1). These plates were observed under uv light for the presence of the spots. The plates were sprayed with Lieberman – Burchard reagent (5ml acetic anhydride was mixed under cooling with 5 ml sulphuric acid, this mixture was added to 50 ml absolute ethanol) and seen under U.V. light and colour developed was noted. The hRf value of the major spots were calculated. The eluants having hRf values 50 were then mixed together and concentrated to 1/10th of its volume. The concentrated material again dissolved in small volume of chloroform and passed through freshly prepared alumina column as above. The eluant was evaporated and off white semisolid mass was obtained. Then semisolid mass was taken in a small conical flask and dissolved in minimum chloroform and methanol mixture (2:1) in warm condition and decolourised by boiling with charcoal and filtered and concentrated to a small volume at room temperature. Fine needle shaped colourless shiny crystals were obtained. It was filtered through whatman filter paper no. 1 and the materials were taken into small container and dried in a vacuum desiccator for few hours.