CHAPTER 4

ANALYSIS OF MEDICINAL PLANTS AND THEIR COMBINATION USING THIN LAYER CHROMATOGRAPHIC TECHNIQUE (TLC)

4.1 INTRODUCTION
There are many substances present in the either in mixture or in a compound which do not combine with each other chemically. Therefore they may be easily separated from each other by using different type of processes of separation. The chromatography is one such technique used for separation of mixtures which is based on the amount of solute present in a mobile phase, i.e. mobile fluid stream and stationery phase i.e. non mobile stream. Chromatography can be defined as the study of separation of molecules based on their differences in their composition and structure. It is a laboratory technique.

Chromatography can be divided into two main types
A-Stationery Phase
B-Mobile Phase

A. Stationery phase
In this type of chromatography the mixture gets separated on the solid stationery phase. The molecules which have tighter interaction with stationary phase move slowly as compared to the molecules which are loosely bound. Thus the loosely bound molecules run down leaving behind the slow moving molecules on the stationary phase. Stationery phase type of chromatography can be divided into three categories:
1. Paper chromatography
2. Column chromatography
3. TLC

1. Paper chromatography
In this technique the mixture is used for separation is applied on small piece of filter paper and dipped in water or solvent. The liquid solvent moves in upward direction by capillary action as the edges of paper are deep into the solvent. The compounds get separated by moving with solvent into constituent colours. The type of separated compounds are determined by
calculating the ratio of distance of solute to solvent. This factor is known as retention factor \( (R_f) \). This is the most commonly used technique of separation of amino acids RNF finger printing. It is commonly used to separate colored compounds and is widely used in artificial and natural pigment research.

2. Column chromatography

It is another common separation technique. This technique is based on same principle as TLC but it requires more amount as compare to paper chromatography and thin layer chromatography. In this technique powdered adsorbent is used as a stationary phase and it is kept in a vertical glass column which contains the mixture to be analysed. The mobile phase solvent is poured from the top and allowed to move which helps in separating the constituents due to difference in their velocity of flow. Different spots are obtained at different points on the glass column.

Difference between paper chromatography and thin layer chromatography

1. Principle: Paper chromatography is based on partition chromatography while thin layer chromatography is based on adsorption separation.
2. Amount of substance: Paper chromatography requires very less amount of substance for separation as compared to thin layer chromatography more amount of substance.
3. Time required: Paper chromatography requires around 2 to 3 hours for separation while thin layer chromatography requires 15-20 min for separation.
4. Paper cannot be heated in an Oven while TLC can be heated in an oven for long time.
5. Separation: Paper chromatography does not show distinct separation of a mixture while thin layer chromatography shows sharp separation of mixture.
6. Physical strength: Paper chromatography lacks physical strength therefore descending technique is used while TLC has high physical strength therefore ascending technique is used.
7. For paper chromatography whatmann filter-paper is used whereas silica gel, alumina is used as coating on glass plate of thin layer chromatography.
8. For TLC strong reagents can be used for identification of components as TLC plate is able to with stand strong solvents and colour forming agents which is not possible in case of paper chromatography as the paper reacts and gets destroyed.
9. In paper chromatography spots easily get diffused hence it is less sensitive while in case of TLC the fractions on plates get less diffused and therefore it is highly sensitive.

10. Paper chromatography cannot be examined under UV while in case of TLC it can be examine under UV.

These are some of the points which shows that paper chromatography is not suitable for the separation of medicinal plant. Therefore for the separation of medicinal plants we selected TLC technique as these medicinal plants are highly complex in structure.

Difference between Column chromatography and thin layer chromatography

1. Column chromatography takes lots of time for separation as compared to TLC which takes very less time for separation.

2. TLC is very simple technique it does not require different preparation while chromatography requires lots of preparations.

3. The main disadvantage of column chromatography is that it is not reproducible.

4. Column chromatography runs under gravity while TLC runs against gravity.

5. Column chromatography is preparatory technique whereas TLC is an analytical technique.

6. Column chromatography requires less polar solvent as compared to TLC to resolve the mixture.

These are some reasons why do not uses paper chromatography and column chromatography for separation of medicinal plants.

Chromatography is a technique of separation and identification of compounds in a mixture. The fluid in which mixture is dissolved is known as mobile phase. Stationery phase is defined as the medium to carry mobile phase. The different travelling velocities of various constituents of the mixture cause them to separate. The separation is predicated on differential partitioning between the mobile and stationary phases. Refined variations in a very compound's partition constant lead to differential retention on the stationary part and so have an effect on the separation.

Thin layer chromatography may be a sort of chromatographically technique that is employed to separate the non-volatile mixtures. Thin-layer chromatography (TLC) may be a terribly unremarkably used technique
in synthetic chemistry for distinguishing compounds, decisive their purity and following the progress of a reaction. It additionally permits the improvement of the solvent system for a given separation drawback. This helps to separate quite 2 parts present in any mixture. This system has been designated owing to following reasons: It is a very sensitive and simple technique.

1. It requires less time for separation of the mixture.
2. The instruments required to carry out the process are easily available and handy.
3. Very small amount of mixture is required for the analysis.
4. It gives accurate results in short span of time.
5. Chemical required for carrying out the process are easily available in the laboratories.
6. It helps within the visualisation of separated compound simply.
7. Most of the compounds may be simply isolated by this method.
8. The separation method is quicker and property of compounds is higher (even tiny variations in their velocities clearly exhibit the separated compound).
9. The purity standards of given sample may be assessed simply.

4.1.1 PRINCIPLE:
it's supported the principle of separation.
1. The attraction of compound between stationary and mobile phase is the main cause of separation.
2. The compound below the influence of mobile phase (driven by capillary action) travels over thy surface of the stationary phase. The attraction of compound to stationery phase will travel at lower rate while other compounds will travel fast so separation of compound within the mixture is done.
3. Once separation done, each element is located on the plate at a various level in a spot shape. Their nature and character are known by suggests that of appropriate detection techniques.

4.1.2. TLC system components consist of
1. TLC chamber:
   This chamber is employed to develop TLC plate. The chamber should be uniform surroundings within for correct development of spots.
It additionally prevents the evaporation of solvents, and keeps the method dirt free.

Diagram 4.1 TLC chambers in different size.

2. TLC plate:
Now a day’s TLC plates are obtained commercially by accurate size of particles. They are prepared by calcium sulphate i.e. gypsum and water, silica gel with binder which act as adsorbent on glass plate or aluminium or plastic plate. This plate is dried by heating in oven for 30 minute temperature is maintained up to 110 degree Celsius. 0.1 to 0.25 mm is standard thickness of TLC plate. A skinny layer of stationary part is applied on their surface layer. The stationary layer on the plate is of uniform thickness and is during a fine particle size.
Diagram 4.2 TLC plates.

3. Stationary phase:
   It is a paste of alumina or similar material layered on glass plastic or metal frame which is about 20 x 20 cm or in different size. The stationery phase should be very thin layered i.e. about 0.25 mm. For the stickiness gypsum is added to the mixture of stationery phase. Sometimes the fluorescent powder is mixed to visualise the spot under UV light. A binder like gypsum is additionally mixed into the stationary phase to create it stick higher to the slide. In several cases, a fluorescent powder is mixed into the stationary phase to change the visual image later underneath ultraviolet light. The separation of sample mixture depends on the polarity of sample. Some modified silica is also used in certain purposes e.g.

   Silica gel G: Silica gel with average particle size 15µm containing 13% Calcium sulfate binding agent.
   Silica gel G₂₅₄: in this Silica gel G with fluorescence added.
   Cellulose: The Cellulose powder of less than 30µm particle size is used as stationary phase.
Diagram 4.3: TLC of two compound mixtures

1. Mobile phase: It contains a solvent or mixture of solvents. The mobile phase used needs to be particulate free and of the very best purity for correct development of TLC spots. The solvents used for this purpose ought to be with chemicals inert with the sample. The ability of mobile phase to manoeuvre up relies on the polarity itself. Volatile organic solvents are ideally used as mobile phase.

2. Filter paper: The paper is moistened within the mobile phase and is placed within the chamber. This helps to develop a standardized rise in mobile part over the length of stationary phase.

3. Capillary Spotter:
   A fine capillary is molten in the middle by placing it in the blue Bunsen flame. As soon as it softens and snag develops, capillary is pulled from both the ends to about 2-3 times of its original length. It is cooled and broken from the middle such that one end of each part remains closed. These parts are used as spotters.
4.4: Capillary spotter.

4. Spotting the plate:
Thinnest side of the capillary i.e. spotter is poured in the mixture to be analysed. Start-up line is marked on the TLC plate and the plate is touched with the solvent. The solvent is allowed to air dry. The plotted 4-5 times by keeping distance between them to enable to move the components of the mixture smoothly.

Diagram 4.5 Spotting.

4.1.3 Apparatus:
1. Glass chamber or jar
2. TLC plate
3. Lid or cover
4. Developing solvent
Diagram 4.6 TLC apparatus.

4.2 Materials and methodology:
This technique has been used for the separation of the components of medicinal plants from their extracts. The selected medicinal plants were washed with tap water and dried. The large quantity of dried and crushed medicinal plants were dissolved in suitable solvent i.e. methanol and shaken thoroughly for some time to get a uniform solution. It was then filtered and used for the TLC analysis.

2.2.1 Thin layer chromatographic technique:
TLC was performed on a by exploitation pre-coated colloid aluminium plate (10 x 10 cm, silica gel 60 F, Merck) for the action separation of the extracted stuff.

By exploitation soft pencil, baseline was created at the bottom of the plate and therefore the sample spots were applied by exploitation capillary spotter. Sample solutions were applied on the spots marked on the line in equal distances.

The mobile phase i.e. combination of ether and ethyl acetate was poured into tlc chamber many cm on top of bottom line. A paper moistened with mobile phase was placed within the inner walls of the chamber to maintain humidness.

The tlc plate was the immersed within the tlc chamber at an angel of 45o, with
the sample line facing the mobile phase. The chamber was closed with lid and left for a few time for development of spots. As shown in diagram four.4.

Diagram 4.7 Procedure of TLC techniques

Diagram 4.8 the plate after the solvent has moved about half way

Diagram 4.9 Schematic illustration of the exchange method between gas space solvent system reservoir and TLC plate before and
through development. The plate was removed and dried. The sample spots were then ascertained underneath extremist Violet light. The same procedure was repeated for the analysis of the extracts of medicinal plants by using other solvent combinations in various ratios as mobile phases as shown in the table 4.1.

<table>
<thead>
<tr>
<th>SOLVENT SYSTEM</th>
<th>COMPOSITION</th>
<th>RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ETHER:ETHYL ACETATE</td>
<td>9:1</td>
</tr>
<tr>
<td>2</td>
<td>METHENOL : AMMONIA</td>
<td>9:1</td>
</tr>
<tr>
<td>3</td>
<td>HEXANE : ACETONE</td>
<td>8:2</td>
</tr>
<tr>
<td>4</td>
<td>BENZENE : ACETONITRILE: METHENOL</td>
<td>8:1:1</td>
</tr>
</tbody>
</table>

Table 4.1 Solvent systems (mobile phase)

All these systems were placed in different glass chambers for saturation time of 20 min. and then TLC plates were placed into the jar to run up to solvent front. Each diagram shows red colour circle of the spots run on the plate.

Diagram 4.10 Separation on TLC plate in Ether: Ethyl Acetate (Solvent system 1)
In the above solvent system solute i.e. mobile phase has travelled up to 7.5 cm from the base line. Each plant show separation points except Zingiber and Allium sanctum. The basil and curcuma longa show one separation point while Carica papaya and Azadirachta Indica show two and three separation points respectively. The $R_f$ of each plant separation is discussed in table below.

Diagram 4.11 Separation on TLC plate in Methanol: Ammonia (Solvent system 2)

The second solvent system the plate was run about 7.5 cm from the base line. Here basil shows two separations. AzadirachtaIndica and Carica papaya show three separations. Curcuma longa and Allium sanctum show only one separation while Zingiber do not show any separation.
Diagram 4.12 Separation on TLC plate in Hexane: Acetone
(Solvent system 3)

In this system Allium sanctum shows more separation i.e. it shows 4 separation points. Curcuma longa shows two separation points while Basil, Carica papaya and Zingiber shows single separation point. This plate was also allowed to run about 7.5 cm from the base line.

Diagram: 4.13 Separation on TLC plate in Benzene: Acetonitrile: Methanol
(Solvent system 4)

In the last solvent system Basil, AzadirachtaIndica and Carica papaya shows two separations while Zingiber and Curcuma longa shows single separation. And Allium sanctum does not show separation.
All the TLC plates were run into the solvent systems for 20 minutes and each plate was analysed for the separation of components, as shown in above diagrams. The developed plates were then observed under UV light of wavelength 275 nm in order to know the exact characteristic of spots. The final result of thin layered chromatography is calculated by finding out the $R_f$, i.e. retention factor. Retention or Retardation Factor is the fraction of analyte in mobile phase of chromatographic system. It is defined as the ratio of distance travelled by the centre of spot to the distance travelled by the solvent front.

This can be determined by using formula,

$$R_f = \frac{\text{distance pigment traveled}}{\text{distance solvent traveled}}$$

This formula is used to calculate the $R_f$ value of each spot in various mobile phases as shown in table 4.2 below.

![Diagram 4.14 measuring of $R_f$ value.](image)

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Solvent system</th>
<th>Basil azadirachta Indica</th>
<th>Carica papaya</th>
<th>Allium Sativum</th>
<th>Zingiber</th>
<th>Cur cuma longa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ether : ethyl acetate</td>
<td>0.13 0.06 0.76</td>
<td>0.73 1.33</td>
<td>-</td>
<td>-</td>
<td>0.706</td>
</tr>
</tbody>
</table>
Values shown in the table 4.2 clearly indicate that in first solvent system i.e. ether and ethyl acetate each medicinal plant shows separation except AzadirachtaIndica and zingiber. In second system i.e. methanol and ammonia except zingiber each medicinal plant shows clear separation. In the third solvent system except Allium sativum, each medicinal plant shows clear separation. In this system AzadirachtaIndica shows more enhanced separation i.e. it separates in 4 different spots. In last system i.e. Benzene Acetonitrile and methanol all other medicinal plants shows clear separation except Allium Sativum.

This separation helps us to know the number of components which are present in each medicinal plant. This separation also enables us to study the reaction of medicinal plants with the blood samples of different diseases. Therefore from the analysis of the TLC by using various solvent systems, we conclude that methanol: ammonia is the best solvent system for TLC technique to separate the all medicinal plants above.