CHAPTER 4

ABSTRACT

The subject of phytochemistry has developed in recent years as a distinct discipline, somewhere in between natural product organic chemistry and plant biophysics. Phytochemical methods are needed for the extraction, separation, purification and identification of different constituents present in plants. The method to be adopted for the extraction of the compound or a group of a compound should ensure that the complete extraction of the desired compounds take place without their undergoing decomposition, isomerisation or polymerisation. The biocompounds extracted from the particular plant materials were used for the inhibition of cholesterol, which is needed in these days for the control of coronary heart diseases. The general methods adopted are elucidated in this chapter.

4.1 Introduction

The number of discrete compounds present in plants is huge. Phytochemistry deals with the chemical structures of these substances, their biosynthesis, metabolism and functions. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants. Phytochemical methods are needed for the extraction, separation, purification and identification of the many different constituents present in plants.

4.2 Phytochemical Methods

Phytochemical methodology has been aided by the development of rapid and accurate methods of screening plants for particular chemicals and separating them by chromatographic techniques\(^1\). The chemical constituents of plants or phytochemicals can be classified in a number of different ways; one classification is based on biosynthetic origin, solubility properties and the presence of certain key functional groups. They include phenolic compounds, terpinoids, organic acids, lipids, polysaccharides, glycosides etc. The phenolic compounds are substances which are readily recognized by their hydrophilic nature and by their common origin. Terpinoids are the compounds which share lipid properties and a biosynthetic origin from isopentyl pyrophosphate. The procedures to separate and identify the constituents of plant materials have shown that many substances thought to be rather rare in occurrence are almost universal distribution in the plant kingdom. This shows the importance of studying plants for biologically active substances.

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4.3. Extraction methods

Extraction methods are as important as analytical methods in the study of the plant chemistry. The method to be adopted for the extraction of the compound or a group of a compound should ensure that the complete extraction of the desired compounds take place without their undergoing decomposition, isomerisation or polymerization. The precise mode of extraction depends on the texture and water content of the plant material being extracted and on the type of substance that is isolated. The classical procedure for obtaining organic constituents from dried plant tissue is to continuously extract the powdered material in a Soxhlet apparatus with a range of solvents.

4.3.1 Cold Extraction

Cold extraction is recommended for thermo labile compounds like volatile oils, alcohols and proteins. In this process the finely powered plant material is kept covered with the solvent with occasional stirrings for a long range of time, from 30 min to 72 hr. The solvent is then decanted or filtered and the residue is extracted further with a fresh solvent. The material may be extracted repeatedly till all the compounds are extracted. The extracts are pooled and distilled in vacuum to get a concentrated solution.

4.3.2 Hot Extraction

Hot Extraction is more efficient, where the plant material is boiled in a solvent for a stipulated period. When low boiling and inflammable organic solvent are used refluxing is done in which the boiling is filleted with an air/ water
container. The vapors of the boiling solvent get condensed in the container and fall back into the extraction liquid. This prevents loss of solutions and accidental fire due to the escaping vapors. Once the boiling is stopped, the flask is cooled, condenser is removed and the contents filtered. Boiling with fresh solvent is repeated if the extraction is not completed. Soxhelet’s extractor is the ideal choice for hot extraction where with minimum solvent complete extraction is effected.

4.4. **Methods of Separation of the Plant Extract.**

The separation and purification of phytocompounds in the extract prepared is mainly carried out using one or other, or a combination of three chromatographic techniques: Paper Chromatography (PC), Thin Layer Chromatography (TLC) and Gas Liquid Chromatography (GLC). The choice of the technique depends largely on the solubility proportion and volatilities of the compounds to be separated.

All types of chromatography are based on a very simple principle. The sample to be examined (called the solute) is allowed to interact with two physically distinct entities—a mobile phase and a stationary phase. The mobile phase, which may be a gas or liquid moves the sample through a region containing the solid or liquid stationary phase called the sorbent. The sorbent varies from one chromatographic technique to another and may be considered as having the ability to “bind” some types of solutes. The sample, which may contain one or many molecular components, comes into contact with the stationary phase. The components distribute themselves between the mobile and stationary phases. Molecules that show weak affinity for stationary phase spend more time with the mobile phase and are more rapidly removed or eluted from the system.
Basically all chromatographic systems consists of a stationary phase, which may be a solid, gel, liquid or a solid/liquid mixture that is immobilized and the immobile phase which may be liquid or gas and which flows over through the stationary phase. The choice of a stationary and mobile phase is made so that the compounds to be separated have different distribution coefficients. This may be attributed by setting up:

- An adsorption equilibrium between a stationary solid phase and a mobile liquid phase (adsorption chromatography)
- A partition equilibrium between a stationary liquid phase and a mobile liquid or gas phase (paper chromatography, gas chromatography)
- An ion- exchange equilibrium between a stationary ion exchange and a mobile electrolyte phase (ion-exchange chromatography)
- An equilibrium between a liquid phase trapped inside the pore of a stationary porous structure and a mobile liquid phase
- An equilibrium between a stationary immobilized liquid and a mobile liquid phase (affinity chromatography)

### 4.5 Modes of chromatography

#### 4.5.1 Adsorption Chromatography

This is the classic form of chromatography first introduced at the beginning of the century. It is based upon the principle that certain solid materials collectively known as adsorption have the ability to hold molecule at their surface. Thin adsorption process occurs at specific adsorptive sites which are occupied by molecules of the eluting or by the analyses present in the mixture in proportion
depending upon the relative strength of thin interaction. As elute is constantly passed down the column differences in these binding strengths eventually lead to the separation of the analytic.

4.5.2. Ion-exchange Chromatography

This form of chromatography relies on the attraction between oppositely charged particles. Many biological materials, for example amino acids and proteins, have ionisable groups and the fact that they made carry a net negative or positive charge can be utilized in separating mixture of such compounds.

Chromatographic methods are divided into two types according to how solute molecules bind to or interact with the stationary phase. They are partition chromatography and adsorption chromatography. Partition chromatography is the distribution of a solute between two liquid phases. Adsorption chromatography refers to the use of a stationary phase that has a finite number of relatively specific binding sites for solute molecules. Partition processes, as used in the case of PC, TLC and GLC, are most effective for the separation of small molecules. It has been widely used for the separation and identification of amino acids, carbohydrates and fatty acids. Adsorption techniques, represented by ion-exchange chromatography, are most effective when applied to the separation of macromolecules including proteins and nucleic acids.

Partition Chromatography is particularly applicable to water soluble plant constituents, namely carbohydrate, amino acids, nucleic acid bases, organic acids and phenolic compounds. TLC is the method of choice for separating all lipid soluble components, i.e. the lipids, steroids, carotenoids, simple quinones and chlorophylls. The third technique GLC finds its main application with volatile
compounds fatty acids hydrocarbon, sulfur compounds and mono and sesquiterpenes. It should be pointed out that there is a considerable overlap in the use of the above technique and often in a combination of PC and TLC or TLC. GLC may be the best approach for separating a particular class of plant compound.

The basic principle behind all the different types of chromatography is however the same. Chromatographic separation takes advantage of the fact that the sample distributes or partitions itself to different extents in two different immiscible phases. This property is described by the partition or distribution coefficient $K_d$. If we consider the immiscible phases as A and B,

$$K_d = \frac{\text{Concentration of the sample in phase A}}{\text{Concentration in phase B}}$$

The effective distribution is then defined as the total amount of the substance present in phase A divided by the total amount of substance present in phase B. The immiscible phase could be a solid and a liquid or a gas and a liquid or a liquid and another liquid. Generally one of the two phases is a stationary and does not move. The other is a mobile phase and moves with respect to the first.

In Column chromatography the stationary phase attached to a suitable matrix (an inert, insoluble support) is packed in to a glass of metal column and the mobile phase passed through the column either by gravity feed or by use of a pumping system or applied gas pressure.

Thin layer chromatography in which the stationary phase attached to a suitable matrix, is coated thinly on to a glass, plastic of layer plate. The mobile liquid phase passes across the thin layer plate. This mode of chromatography has
the practice advantage over column chromatography that a large number of samples can be studied simultaneously.

4.5.3 Paper chromatography

Paper chromatography (PC) is a form of partition chromatography in which a stationary liquid phase supported by the cellulose fibers of a paper sheet. One of the main advantages of paper chromatography is the convenience of carrying out separations simply on sheets of filter paper, which serve both as the medium for separation and as the support. Another advantage is the considerable reproducibility of $R_f$ value determined on paper. The $R_f$ value is an important means of describing and distinguishing different pigments.

In two dimensional chromatography the same chromatogram is developed in two directions using two solvent systems of differing properties. The compounds of a mixture can be effectively separated by a judicious selection of two solvent-systems. Normally one solvent system is lipophilic while the second is hydrophilic. Some of the less hydrophilic compounds in a mixture get separated in the first solvent system whereas the more hydrophilic compounds, which may stick together during the first run, get separated in the polar solvent system. For 2-D chromatography square papers are used where the sample is spotted near one corner (leaving a space of about 2cm from both the sides). The spotted paper is developed in the first solvent system and the compounds move in a line parallel to one margin. When the run is over the chromatogram is taken out, dried and placed in the solvent system with the side containing the compounds dipping in the solvent. After the second development the paper is dried and the chromatogram is

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examined as usual. BAW (n-Butanol –acetic acid-water, 4:1:5-organic layer) in the first direction and 15% acetate acid in the second direction from a pair of solvent systems used in the separation of flavonoids. Benzene –acetic acid-water (6:7:3-upper layer) and sodium formate-formic acid-water (10:1:200) are another pair of solvent systems used widely to separate phenolic acid.

The solvent used for chromatography should be very pure lest the impurities present in the solvent may change the R_f values, polarity, react with the sample or interfere with the detection reagent in various ways. The selection of the solvent system plays an important role in providing good separations. The compounds to be separated and the solvent to be used should be somewhat similar in polarity or hydrophilic properties. If they differ greatly in these characters the substances may remain together at the start or move with the solvent front. The literature survey may always lead to a number of solvent systems of which the best suited may be selected. For totally hydrophilic materials (which cannot be extracted from water by other solvents such as butanol) a system with high water content. Butanol saturated with water. BAW (n- butanol: acetic acid: water, 4:1:5, upper layer) or 15% acetic acid give better separations. Compounds, which can be extracted from water into ethyl acetate/ether/chloroform, but not extractable into benzene/petroleum ether, are considered medium polarity such as chloroform, butyl acetate or isopropyl ether with small amounts of water or any other polar solvent is suitable for such compounds. Benzene, cyclohexane, carbon tetrachloride or toluene is used for substances which are readily soluble in lipophobic solvents but poorly soluble in water. Completely hydrophobic compounds (trapezoids, phenols etc.) are separated best in reversed phase systems.
Paper chromatography works on the process of partition and therefore it is always necessary that the solvents used are to be saturated with water. The paper also should be sufficiently moist. Solvent systems based on butanol contain a good amount of water that the paper can take the necessary amount of water from them during development. In cases where the mobile phase contain less than 10% of water, with the paper has to be supplied with necessary amount of polar stationary phase before the beginning of chromatography. This is done by keeping the paper over water in a humid chamber, steaming the paper by or spraying with a fine spray of stationary phase. In another method, the paper is sprayed with 50% methanol till it gives a wet appearance (care is to be taken not to wet the paper so that the solvent drips down the paper) and keeping the paper to dry until the clouding on the paper disappears and the paper looks dry but feels wet and soft to touch.

The chromatogram may be dried by keeping them below ceiling fan. Poorly volatile, corrosive (phenol) or un-pleasant smelling (pyridine, butanol, amyl alcohol etc.) solvents are vapourised using a hot air oven kept at 60° C. For a quicker drying, the chromatograms may be held over a hot plate.

The detection of colorless compounds is done using chromomeric sprays. If the substances are not soluble in the reagent the chromatogram may be drawn quickly through the reagent solution taken in a trough. Glass sprays (atomizers) are commercially available with the help of which the papers are sprayed uniformly (avoiding dripping of the reagent). Some of the reagent gives colors with the spots immediately while others produce colors only after heating. When heating is required the paper is kept in a drying oven adjusted at the desired temperature or held over a hot plate. A gradual warming over the hot plate gives
an advantage in that the change in colors produced by certain compounds (sugars, steroids etc) can be easily followed. Phenolics may be visualized by observing the papers in a UV light (360 mm) before and after spraying.

The position of a spot in a chromatogram is recorded as its \( R_f \) value. It is often a diagnostic feature of a compound. \( R_f \) is the ratio of the distance traveled by the compound to the distance traveled by the solvent.

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R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}
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These distances are calculated from the start, i.e., the centre of the original spot. The distance traveled by solute is the distance between the centre of this spot and the centre of the migrated spot. The distance traveled by the solvent is the distance between the start and the solvent front.

Chromatography on paper involves both partitions as well as adsorption process. In partitions process, the compounds are partitioned between a largely water – immiscible alcoholic compound. By contrast adsorption forces are one of the main features of PC in aqueous solvents a compound exhibit difference in solutions when dissolved separately in two immiscible or partly miscible liquids depending upon its affinity to the liquid molecule. A mixture of components dissolved in a system of such liquids in distributed between them in depends of the satellites of the individual components in he two phases. The distribution of the components is determined by the distribution coefficient \( K_d \). The compounds get repeated when one of the phase moves over the other phase. To achieve this movement of one liquid relative to the other it is necessary to fix this phase firmly- the stationary phase. The second phase, which moves slowly over the stationary phase, is the mobile phase. Both phases must be mutually saturated to ensure that
no change in concentration takes place in the solvent system during chromatography. The efficiency of separation is proportional to the magnitude of difference of distribution control of the compounds to be repeated.

In paper chromatography (PC) the paper provides the inert support and partition take place between the water held on the paper and the mobile phase. Though cellulose is considered inert it is seen that the solution get bound to the free hydroxyl groups of cellulose through secondary valence forces and / or hydrogen bonds. Amorphous regions of cellulose retain more water than the crystalline regions. Water is also held in the interstitial regions. Cellulose exhibits electrically changed nature when kept in water or polar solutions and due to this reason adsorption is the main phenomenon during chromatography with predominately aqueous solvents.

The chromatography papers are mostly of pure cellulose, the composition as approximately 98-99% α-cellulose, 0.3 - 1% β-cellulose and 0.4 - 0.8% pentasons. Since the solutions migrate on the paper by capillary ascent the density and the strength of the network of paper influence the speed of chromatography run. The denser the paper, the lower will be the capillary ascent. A good paper will be the one in which the capillary ascent is higher. Whatman No.1 paper is ideally suitable for most of the qualitative separations. Thicker papers, Whatman No.3 and No .4, are used when comparatively large amounts of compounds are to be separated.

The choice of apparatus for PC depends to some extend on the amount of laboratory space available. In most labs, PC in carried out by descant in tanks which will accommodate Whatman papers of size 46 x 57 cm. In PC, compounds
are usually deleted as colored or UV Fluorescent spots, after reaction in a chromatographic reagent used either as a spray or on a dip.

The position of a spot in a chromatogram is recorded as its $R_f$ value, which is often a diagnostic feature of a compound. $R_f$ value is the ratio of the distance traveled by the compound to the distance traveled by the solvent. This always appears as a fraction and lies between 0.01 and 0.99. When comparing $R_f$ values of a series of structurally related compounds it is useful to refer to another chromatographic constant, the $R_m$ value. This is related to $R_f$ by the expression

$$R_m = \log \left( \frac{1}{R_f} - 1 \right)$$

Paper Chromatography is less cumbersome than Thin Layer Chromatography in the sense that papers available commercially are used directly. Excepting for the solvent systems containing butanol or any higher alcohol, chromatographic run is over within 2-4 hr with smaller piece of paper say 20-25 cm in length. The chromatogram may be preserved for future reference. The only disadvantage is that corrosive spray or sprays containing higher amounts of Conc. $H_2SO_4$ cannot be used in PC.

### 4.5.4. Thin Layer Chromatography

In thin layer chromatography (TLC), a uniform thin layer of a solid powder (sorbent) held on a glass plate forms the stationary phase and a molecule phase runs over separating the compound of a mixture. Depending on the sorbent, the principle of TLC may be anything from partition, adsorption, ion-exchange or gel filtration. In many cases TLC separations are the result of a combination of separation mechanisms though, due to the selection of proper conditions one process becomes
predominant. For separation of non-polar substance adsorption is the preferred criteria while for polar substances partition is employed. For separating inorganic compounds ion-exchange chromatography is used and for macromolecules gel filtration is resorted. The special advantage of TLC compared to PC includes versatility, speed and sensitivity. Versatility is due to the fact that a number of different adsorbents may be spread on the plate employed for chromatography. Although silica gel is the most widely used, layers may be made up from aluminum oxide, celite, calcium hydroxide, magnesium phosphate, polyamide, sephadex, polyvinylpyrrolidone etc. One method of preparation of TLC plate is shown in figure 4.1. Greater speed of TLC in due to the more compact nature of the adsorbent when spread on a plate. The sensitivity of TLC is such that separation of less than a microgram amount of material can be achieved.

![TLC Plate Preparation Diagram](image)

**Figure 4.1. TLC plate preparation**
A wider range of solvents have been applied to TLC than to PC. Rf value is considerably less reproducible than on paper and it is therefore essential to include one or more reference compounds as markers. TLC is usually carried out by ascenting a tank which is paper-lined so that the atmosphere is saturated in the solvent phase.

Detection of compounds on TLC plates is normally carried out by spraying suitable chemicals on the area of the plate, making this a relatively sample procedure. One advantage over PC is that glass plates may be sprayed with Conc.\(\text{H}_2\text{SO}_4\), a useful detection reagent for steroids and lipids. Compounds which absorb in the near UV can be detected either by adding a fluorescent dye to the solvent or by spraying the developed plate with a dye solution. Preparative TLC is carried out using thick instead of thin layers of adsorbent. The separated constituents are recovered by scraping off the adsorbent at the appropriate places on the plate, eluting the powder with a solvent such as ether and finally centrifuging to remove the adsorbent. The literature on TLC is enormous. The most comprehensive book on the topic is that edited by Stahl.\(^6\) A simple introduction is given in the book of Truter.\(^7\) Other important contribution is the works of Bobbitt\(^8\) and Randerath\(^9\).

4.6. Conclusion

Phytochemical methods find its application in various fields like agriculture, biophysics, food and nutrition, pharmaceutical research etc. Although these methods are essential in chemical and biochemical studies, and their application in more strictly biological spheres has only come within last two decades. Even in disciplines as remote from the chemical laboratory as systematics,
phytochemistry, ecology and paleobotany, phytochemical methods have become
ingredient for solving certain types of problems and will be used with increasing
frequency in the future. In this investigation phytochemical methods are employed
to separate and identify the different compounds present in the plant Cassia fistula
which were used as the inhibitors during the growth of cholesterol crystals.

4.7. Bibliography

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