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EXTRACTION, PHYTOCHEMICAL SCREENING AND IDENTIFICATION OF NATURAL PIGMENTS FROM BETA VULGARIS BY THIN LAYER CHROMATOGRAPHY

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ABSTRACT

The present study was designed to extract, identify the Phyto-chemically active constituents in Beta vulgaris by using TLC method. On the basis of the results obtained, the following salient findings have emerged. Preliminary qualitative phytochemical analysis made for the root of Beta vulgaris revealed that the presence of glycosides, carbohydrates, sterols, and saponins so these species are expected to have many medicinal uses. TLC offers several benefits over the other chromatographic separation. The biggest advantage offered to the chromatography is time saving when compared to other chromatographic techniques. The non-volatile compounds can be separated by this method, where in gas chromatography and other chromatographic techniques only volatile compounds can be separated. All components of UV Light are achieved to visualize microlite `quantity of sample can also be separated in TLC. TLC proved to be successful method of identifying unknown compounds and determining the polarity of the substance. The manual error is accepted in TLC up to 1% but also this provides accurate results where as in other or HPTLC Methods the same error may give different results and also damage the equipment. The Rf value helps to identify the different phytochemical constituents present in the chloroform extraction of Beta vulgaris.

KEYWORDS: Thin layer chromatography, Maceration, Beta vulgaris, Extraction.

1. INTRODUCTION

1.1 Pharmaceutical analysis: The pharmaceutical analysis is a branch of chemistry, which involves the series of process for the identification, determination, quantization, and purification. This is mainly used for the separation of the components from the mixture and for the determination of the structure of the compounds.

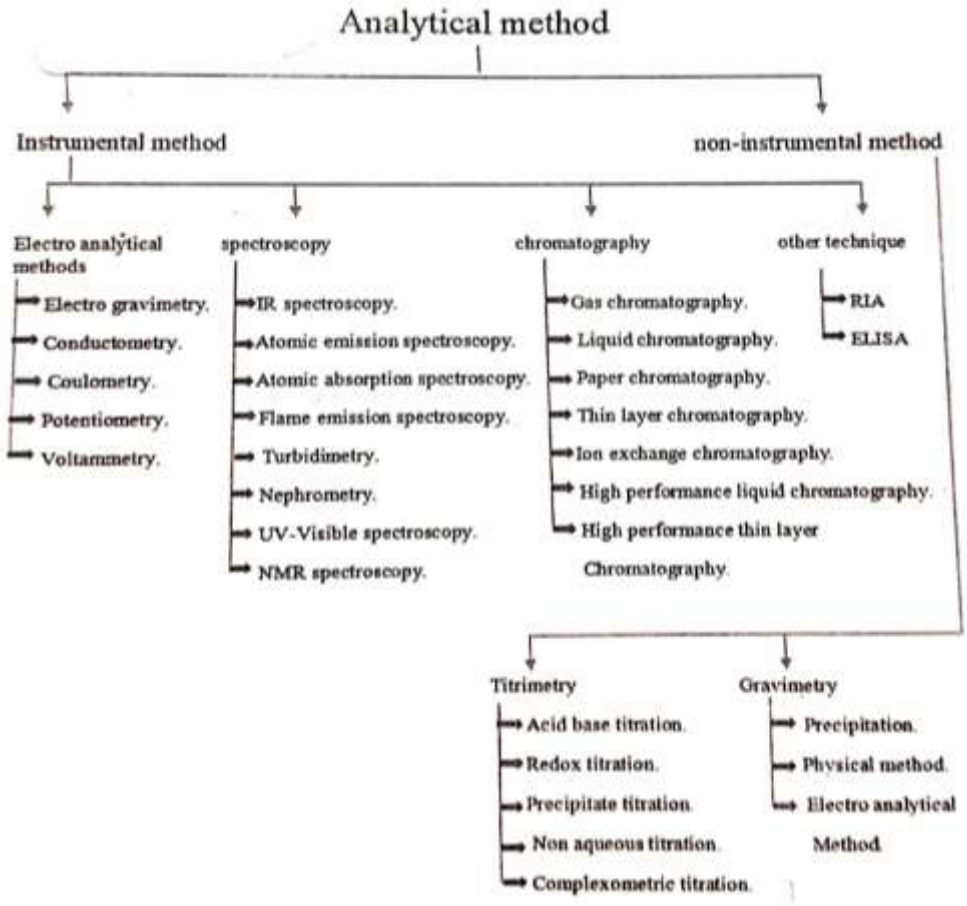
1.2 Analytical techniques: Analytical techniques are the methods used for the qualitative and quantitative determination of concentration of a compound by using various techniques like titrations, spectroscopy's, chromatography,[1] and gravimetric analysis. A qualitative method is used to identify the atomic and molecular species or functional groups in sample. A quantitative method gives an numerical information in the relative amount of one or more of this components.

Based upon the determination type, there are mainly two types of analytical methods.[13] They are as follows:

1. **Qualitative analysis:** This method is used for the identification of the chemical compounds.
2. **Quantitative analysis:** This method is used for the determination of the amount of the sample.

1.2.1 Importance of analytical method

Quality is important in every product or service, but it is vital in medicine as it involves life. Unlike other consumer goods, there can be and there is no second quality. Therefore, analytical methods which of a measure of quality of the drugs play a very comprehensive role in drug development and follow up activities, to assure that a drug product meets the established standard, is a stable and will continue to meet purported quality throughout its shelf life. This method should be selective and sensitive to monitor the known and unknown impurities. Have to be written in a format such that they can be produced over a period of time and from laboratory to laboratory. i.e., these methods should be validated.



1.3 Electro analytical method[12]

1.3.1 Potentiometry: A potentiometer is an instrument for measuring voltage or potential difference& by comparison of an unknown voltage with a known reference voltage. Potentiometry passively measures the potential of a solution between two electrodes, affecting the solution very little in the process. One electrode is called the reference electrode and has a constant potential, while the other one is an indicator electrode whose potential changes with the composition of the sample. The difference of potential between the two electrodes gives an assessment of the composition of the sample. Both electrodes are connected to the potentiometer and are immersed in solution the potential difference between the two electrodes is recorded and referred to as the electromotive force. Potentiometry is mainly used to determine the potential or electromotive force of sample solution. The potential is directly proportional to the concentration of the ions.

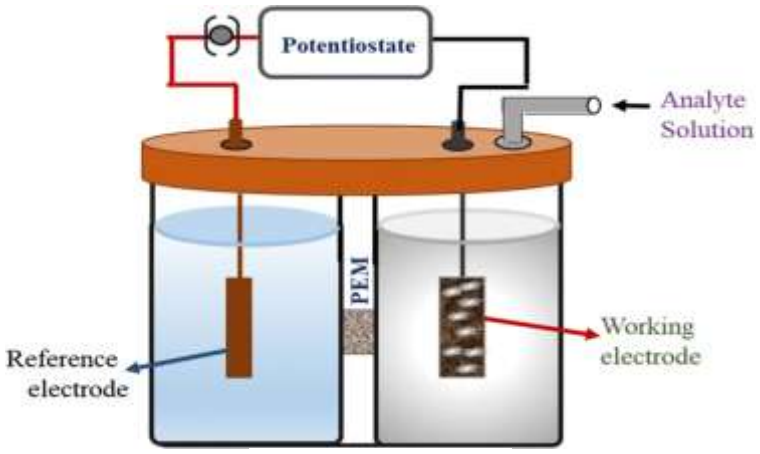


Fig. 01: Potentiometry.

1.3.2 Conductometry:

It is an electro chemical method of analysis used for the determination or measurement of the electrical conductance of an electrolyte solution by means of a conductometer.

Electric conductivity of an electrolyte solution depends on

- Type of ions
- Concentration of ions
- Temperature
- Mobility of ions

Instrumentation:

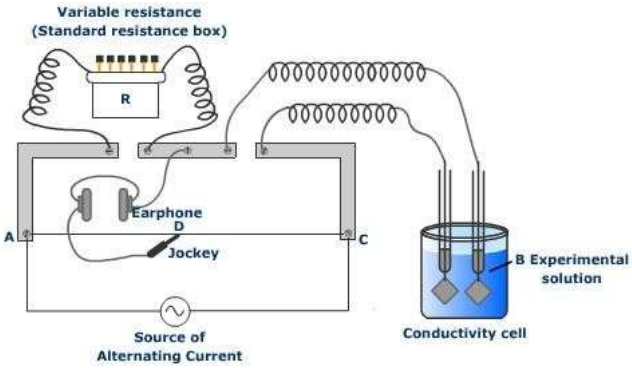


Fig. 02: Conductometry instrumentation.

Fig. 03: Conductometry.

1.4 Spectroscopy[16]

Spectroscopy is the general field of study that measures and interprets the electromagnetic spectra that result from the interaction between electromagnetic radiation and matter as a function of the wavelength or frequency of the radiation. Spectral measurement devices are referred to as spectrometers, spectrophotometers, spectrographs or spectral analyzers'. The central theory of spectroscopy is that light is made of different wavelengths and that each wavelength corresponds to a different frequency. The importance of spectroscopy is centered on the fact that every different element in the periodic table has a unique light spectrum described by the frequencies of light it emits or absorbs consistently appearing in the same part of the electromagnetic spectrum when that light is diffracted.

1.4.1 Ultraviolet-Visible spectroscopy

Ultraviolet (UV) spectroscopy is a physical technique of the optical spectroscopy that uses light in the visible, ultraviolet, and near-infrared ranges and it is based on Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and path length. The method of analysis is based on measuring the absorption of a monochromatic light by colorless compounds in the near ultraviolet path of a spectrum (200-400nm). To determine the "identity, strength, quality and purity" of such compounds.

Principle of UV-Vis Spectroscopy: the absorption of light by a sample in the ultraviolet or visible region is accompanied by a change in the electronic state of the molecules in the sample. The energy supplied by the light will promote electrons from their ground state orbital to higher energy, excited state orbital or anti-bonding orbital. Thus the following electronic transitions can occur by the absorption of ultraviolet and visible light.

σ to σ^*
n to σ^*
n to π^*
π to π^*

Fig. 4: UV Visible spectroscopy.

Instrumentation

Sources of visible radiation: The tungsten filament lamp is commonly employed as a source of visible light. This type of lamp is used in the wavelength range of 350 - 2500 nm.

Monochromator: A monochromator can use either the phenomenon of optical dispersion in a prism, or that of diffraction using a diffraction grating, to spatially separate the colors of light. A prism monochromator is a 1mechanism that emits monochromatic light from a light source.

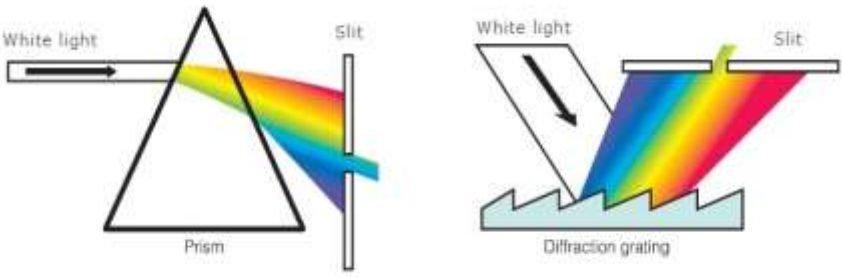


Fig. 05: Monochromators.

Sample cell: The containers for the sample and reference solution must be transparent to the radiation which will pass through them. Quartz or fused silica cuvettes are required for spectroscopy in the UV region.

Detectors: A detector converts a lights signal into an electrical signal. It should give a linear response over a wide range of low noise and high sensitivity.

1. Photomultiplier tube detector
2. Photodiode detector

1.4.2 NMR SPECTROSCOPY[28]

Nuclear magnetic resonance is a spectroscopic technique to observe local magnetic field around atomic nuclei. The sample is placed in a magnetic field and the NMR signal is produced by excitation of the nuclei sample with the radio waves into nuclear magnetic resonance, which is detected with sensitive radio receivers. The intra molecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of the molecules and its functional group

Instrumentation

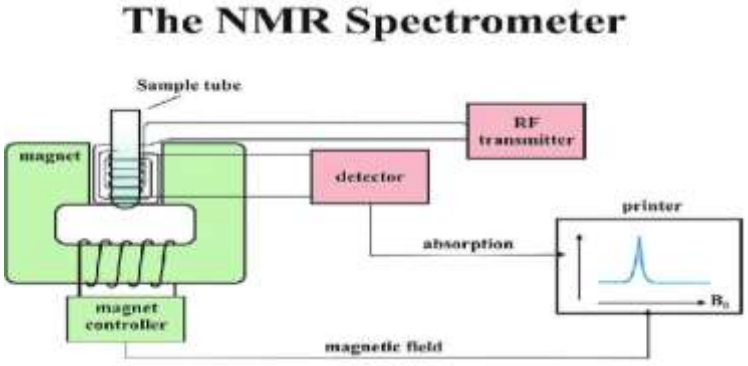


Fig. 06: NMR Spectroscopy.

Sample holder: Glass tube with 8.5 cm long, 0.3 cm in diameter.
Permanent magnet: It provides a homogeneous magnetic field at 60-100 MHZ
Magnetic coils: These coils induce a magnetic field when current flows through them
Sweep generator: To produce an equal amount of magnetic field pass through the sample
Radio frequency transmitter: A radio transmitter coil transmitter that produces a short powerful pulse of radio waves.
Radio frequency receiver: A radio receiver coil that detects radio frequencies emitted as nuclei relax to a lower energy level

Read out systems: A computer that analyses and records the data.

1.4.3 Infrared spectroscopy

Infrared spectroscopy or vibration spectroscopy is the measurement of the interaction of infrared radiation with matter by absorption, emission, or reflection. IR spectroscopy [15] works on the principle that molecules absorb specific frequencies that are characteristic of their structure. It is used to study and identify chemical substance or functional groups in solid, liquid, or gaseous forms. It can be used to characterize new materials or identify and verify known an un known samples.

Instrumentation:

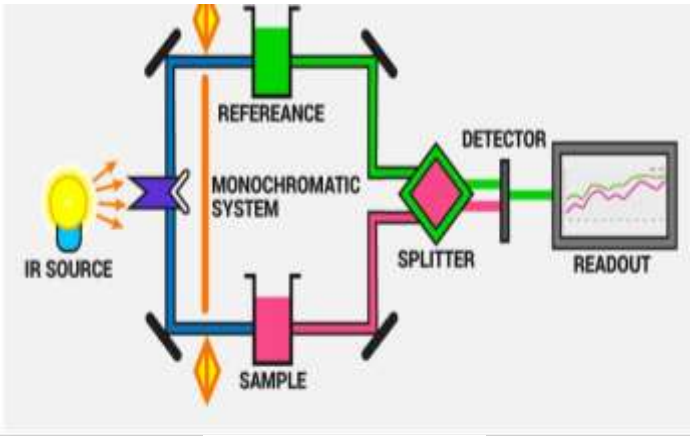


Fig. 07: IR spectroscopy.

1.4.4 Turbidimetry

The measurement of the reduction in light transmission caused by particle formation is termed Turbidimetry. Light transmitted in the forward direction is detected. The amount of light absorbed by a suspension of particles depends on the specimen concentration and on the particle size [17]

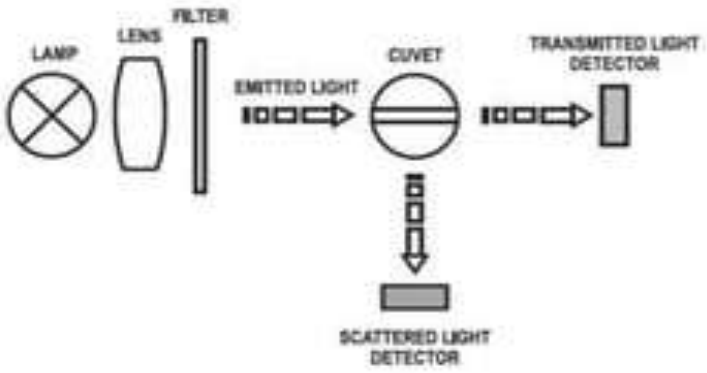


Fig. 08: Turbidimetry.

1.4.5 Nephelometry

Nephelometry is defined as the detection of light energy scattered or reflected toward a detector that is not in the direct path of the transmitted light. A nephelometer is an instrument for measuring the concentration of suspended particulates in a liquid or gas colloid.[17]

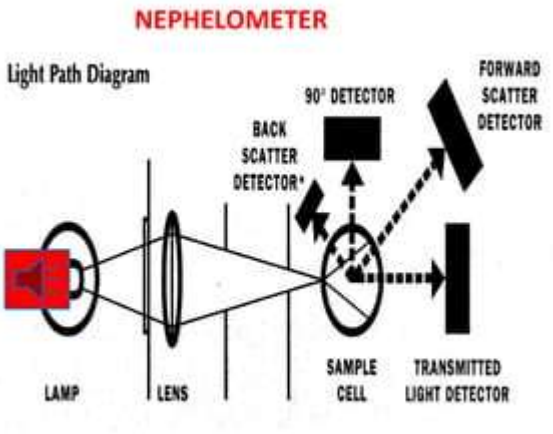


Fig. 09: Nephelometry.

1.5 Chromatography

Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.

Principle of chromatography: Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights.[39]

Three components form the basis of the chromatography technique:

- **Stationary phase:** This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support".
- **Mobile phase:** This phase is always composed of "liquid" or a "gaseous component."
- Separated molecules.

1.5.1 GAS Chromatography

In gas chromatography, the components of a sample are dissolved in a solvent and vaporized in order to separate the analyzes by distributing the sample between two phases: a stationary phase and a mobile phase. The mobile phase is a chemically inert gas such as helium, nitrogen etc. Gas chromatography is one of the unique forms of chromatography that does not need the mobile phase for interacting with the analyte. The stationary phase is either a solid adsorbent, termed gas-solid chromatography (GSC), or a liquid on an inert support, termed gas-liquid chromatography (GLC).The criteria for the compounds to be analyzed in GC is volatility & thermo stability.

Instrumentation:

1) **Sample injection system:** A sample port is necessary for introducing the sample at the head of the column. The vaporization chamber is typically heated 50 °C above the lowest boiling point of the sample and subsequently mixed with the carrier gas to transport the sample into the column.
2) **Carrier gas:** Helium, Nitrogen, argon & hydrogen gases are used as carrier gas depending upon the desired peformance & detector being used**.**
3) **Separation column:** Open tubular columns or capillary columns & packed columns are used in GC.

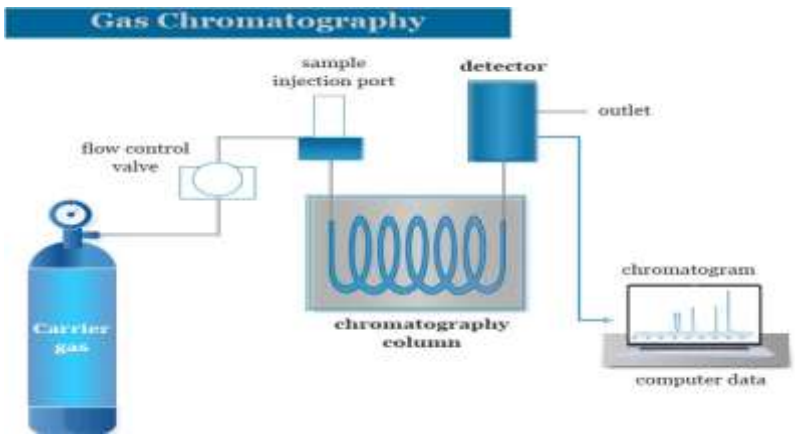


Fig. 10: Gas chromatography.

4) **Column oven or thermostat chambers:** The thermostat oven are there to control the temperature of the column.
5) **Detectors:** Mass Spectrometer , Flame ionization detector (FID), Electron capture detector (ECD), Thermal conductivity detector (TCD),Atomic emission detector (AED), Photo ionization detector (PID).
6) **Amplification and recorder system:** These are meant to record the signals that come from the detector. Amplify the signals so as to display in an understandable graphical format that represents several peaks of the constituents of the sample under analysi.

1.5.2 High performance liquid chromatography:

High-performance liquid chromatography (or) High pressure liquid chromatography (HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules.[24]

Types of HPLC method:

1) **Normal phase chromatography:** NP-HPLC uses a polar stationary phase and a non-polar mobile phase. This method separates analytes based on polarity.
2) **Reversed phase chromatography:** Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions.
3) **Size exclusion chromatography:** Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size.
4) **Ion exchange chromatography:** In Ion exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase

Instrumentation

1) **Injection of the sample:** Septum injectors are available; using which sample solution is injected. Sample can be injected when the mobile phase is flowing or it is stopped. A new advanced rotary valve and loop injector can be used to produce reproducible results.[28]
2) **Detector:** Generally UV spectroscopy is attached, which detect the specific compounds. Many organic compounds absorb UV light of various wavelengths. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.
3) **Interpreting the output from the detector:** The output is recorded as a series of peaks, each one representing a compound in the mixture passing through the detector and absorbing UV light. The area under the peak is proportional to the amount of substance, which is passed through detector, and this area can calculated automatically by the computer linked to the display.

1.5.3 Thin layer chromatography[33]

Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminum oxide, or cellulose (blotter paper). This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action.

Principle of TLC:

1) Thin layer chromatography uses a thin glass plate coated with either aluminum oxide or silica gel as the solid phase. The mobile phase is a solvent chosen according to the properties of the components in the mixture. The principle of TLC is the distribution of a compound between a solid fixed phase (the thin layer) applied to a glass or plastic plate and a liquid mobile phase (eluting solvent) that is moving over the solid phase. A small amount of a compound or mixture is applied to a starting point just above the bottom of TLC plate.

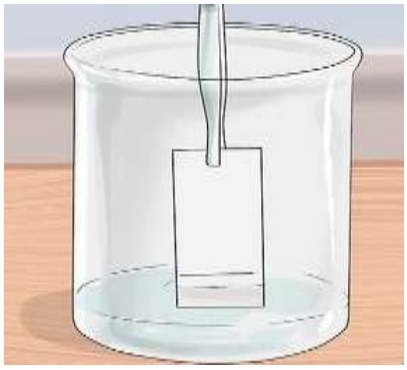


Fig. 11: TLC.

2) The solvent is drawn up through the particles on the plate through the capillary action, and as the solvent moves over the mixture each compound will either remain with the solid phase or dissolve in the solvent and move up the plate.

Instrumentation

1) **Glass plate:** Glass plates which are specific dimension like 20cm×20cm, 20cm×10cm, 20cm×5cm can be used. Microscopic slides can also be used for some applications like monitoring progress of chemical reaction. Glass plates of different dimension can also be used.

Fig. 12: Glass plates.

2) **Stationary phases:** Several adsorbent is used as a stationary phase. Some of the stationary phase, their composition is mixed with the water or other solvents to form a slurry for preparing thin layer chromatography.

3) **Mobile phase:** For silica gel chromatography, the mobile phase is an organic solvent or mixture of organic solvents. As the mobile phase moves pass the surface of the silica gel it transports the analyte pass the particles of the stationary phase. Thus, the fraction of the time that the analyte is bound to the surface of the silica gel relative to the time it spends in solution determines the retention factor of the analyte.

4) **Preparation and activation of TLC plates:** They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulphate (gypsum) and water. This mixture is spread as thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. The resultant plate is dried and activated by heating in an oven for thirty minutes at 110 °C. The thickness of the adsorbent layer is typically around 0.1- 0.25 mm for analytical purposes and around 0.5- 2.0 mm for preparative TLC. Plate with Silica gel.

5) **Methods of preparation of TLC of plates**

- **Pouring method:** the slurry is prepared and poured on to a glass plate which is levelled on a glass plate the slurry is spread uniformly on the surface of the glass plate. After setting, the plates are dried in an oven is used for spotting the samples.

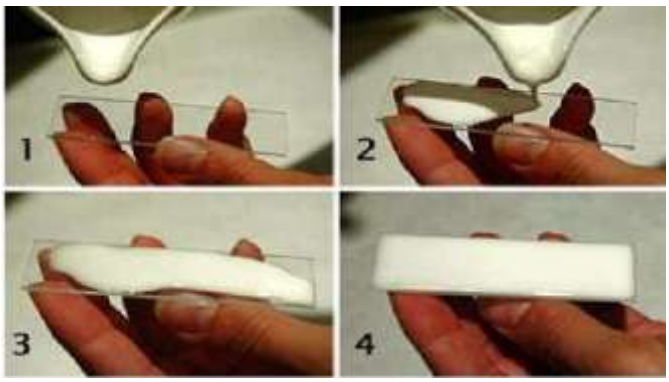


Fig 13: Pouring method.

- **Dipping method:** The two plates are dipped into slurry and are separated after removing from slurry and later dried.
- **Spraying method:** This method by making use of a small paint sprayer for the distribution of the suspension or slurry onto a surface of the glass plates.
- **Spreading method:** It is best technique where a TLC spreader is used. The specific dimension are stacked on a base plate. After preparation of the

slurry is poured inside the reservoir of TLC spreader. The thickness of the adsorbent layer is adjusted by using a knob in the spreader. The plates are allowed for setting (air drying). After setting, the plates are activated by keeping in an oven at 100°c to 120°c for 1 hour.

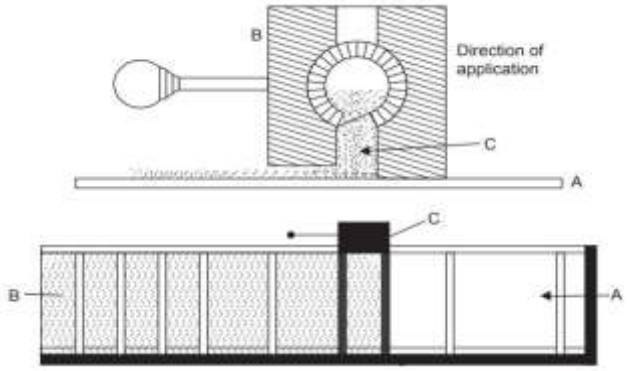


Fig. 14: spreading method.

6) **Capillary spotters:** Place a melting point capillary and in the dark blue part of the Bunsen burner flame. Hold it there until it softens and starts to sag. Allow the capillary to cool down, and then break it in the middle. Make sure to break off the closed end on one of them.

Fig. 15: Capillary spotting.

7) **Spotting the plate:** The thin end of the spotter is placed in the dilute solution; the solution will rise up in the capillary (capillary forces). Touch the plate briefly at the start line. Allow the solvent to evaporate and spot at the same place again. Try to avoid spotting too much material, because this will deteriorate the quality of the separation considerably (‘tailing’). Location of spots: The position of various solutes separated by TLC can be located by various methods. Colored substances can be seen directly when viewed against stationary phase.

8) **Developing methods**

1) **Vertical development or ascending chromatography**

In this common TLC development method, the plate is placed in a suitable TLC developing chamber such that the solvent wets the TLC layer below the starting line. Due to capillary forces, the solvent rises up the layer, transporting the sample mixture. Once the solvent front has reached the predetermined height (10-15 cm for TLC and 3-7 cm for HPTLC), the plate is removed from the chamber, the solvent front is marked with a pencil or spatula, and the plate is dried.

Fig. 16: Ascending method.

2) Horizontal Development or descending chromatography

In this TLC developing method, the plate is positioned horizontally inside the chamber, and the solvent is applied using a wick or capillary slit. Development can be performed from one or both sides of the TLC plate.

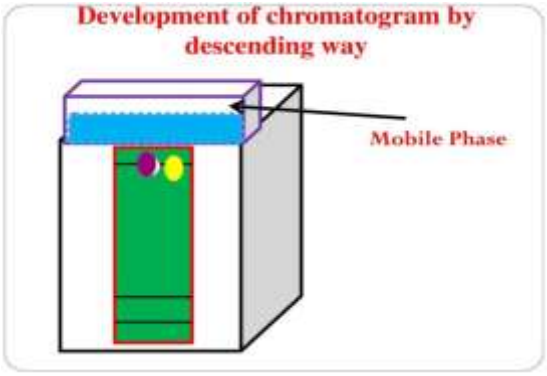


Fig. 17: Descending method.

3) Two-Dimensional Development

In two-dimensional TLC development, the sample is applied to a starting point in a corner of the TLC plate. The plate is placed in a normal chamber and developed once from bottom to top. After drying, the plate is turned 90° and placed in another chamber with a different solvent and developed again. The chromatogram track from the first development is used as the starting line for the second development. Two-dimensional TLC offers the advantage of running a standard with either development. However, the standard cannot be developed in two dimensions on the same chromatogram, since it would mix with the sample.

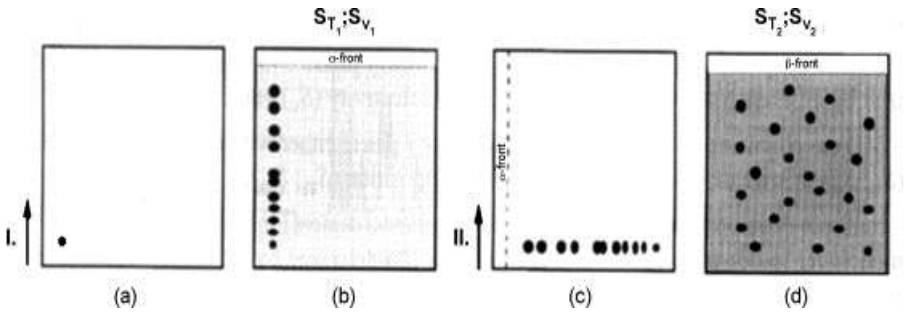


Fig. 18: Two dimensional method.

4) Multiple development

In this method, the TLC plate undergoes multiple developments with drying between each cycle. The solvent travels repeatedly through the layer, re-concentrating and deforming the spots, often producing elliptical shapes or narrow bands. This significantly improves resolution for substances with Rf values below 0.5. Multiple development can be performed over different separation distances, using the same solvent or different solvents of varying polarity.

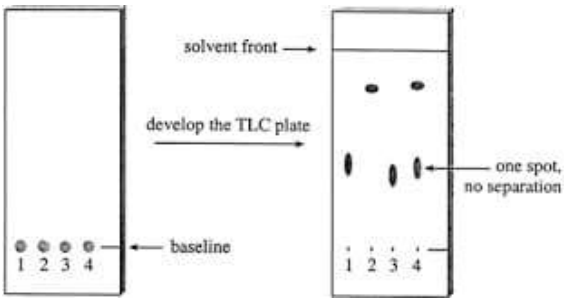


Fig. 19: Multiple development method.

9) Developing chamber

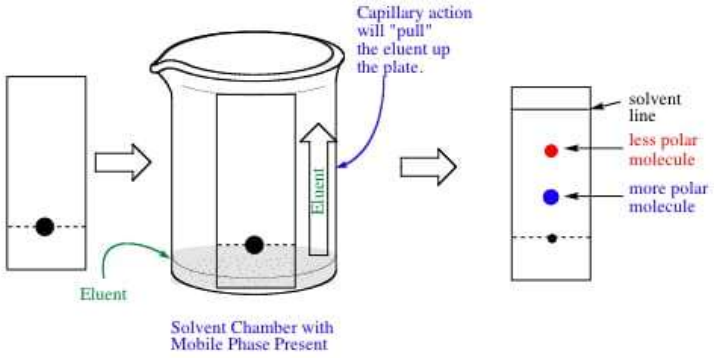


Fig. 20: Developing chamber.

1. Non-polar solvents will force non-polar compounds to the top of the plate, because the compounds dissolve well and do not interact with the polar stationary phase.
2. Allow the solvent to travel up the plate until ~1 cm from the top.
3. Take the plate out and mark the solvent front immediately. Do not allow the solvent to run over the edge of the plate. Next, let the solvent evaporate completely.
4. A TLC plate can be developed in a beaker or closed jar. Place a small amount of solvent (mobile phase) in the container.
5. A small spot of solution containing the sample is applied to a plate, about 1cm from the base.
6. The plate is then dipped in to a suitable solvent, such as hexane or ethyl acetate, and placed in a sealed container.
7. The solvent moves up the plate by capillary action through the vertical, horizontal, two dimensional and multiple development meets the sample mixture, which is dissolved and is carried up the plate by the solvent.
8. Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase, and because of differences in solubility in the solvent.
9. By changing the solvent, or perhaps using a mixture, the separation of components (measured by the RF value) can be adjusted.
10. The lower edge of the plate is then dipped in a solvent. The solvent (eluent) travels up the matrix by capillarity, moving the components of the samples at various rates because of their different degrees of interaction with the matrix (stationary phase) and solubility.

10) Visualization:

When the solvent front has moved to within about 1 cm of the top end of the adsorbent (after 15 to 45 minutes), the plate should be removed from the developing chamber, the position of the solvent front marked, and the solvent allowed to evaporate. If the components of the sample are coloured, they can be observed directly. They can sometimes visualized by shining ultraviolet light on the plate or by allowing the plate to stand for a few minutes in a closed container in which the atmosphere is saturated with iodine vapor.

11) Analysis:

The components, visible as separated spots, are identified by comparing the distance they have travelled with those of the known reference materials. Measure the distance of the start line to the solvent front. Then measure the distance of centre of the start line. Divide the distance the solvent moved by the distance the individual spot moved. The resulting ratio is called Rf- value.

RF VALUE:

RF is the “retardation factor”, which is the ratio of the distance travelled by a compound in a mobile phase compared with the distance travelled by the front of the mobile phase itself. It is always greater than or equal to zero, and less than equal to 1.

APPLICATIONS:

1) Analysis of drug residues and antibiotic in food and environment samples.
2) Identification and qualification of natural product like volatile oil, fixed oil, waves, alkaloids, glycosides, steroids etc.
3) Quality control and purity testing of pharmaceutical formulation.
4) TLC can be used in forensic studies where body fluids, such as urine and blood can be tested for the presence of drugs.
5) Separation of inorganic ions such as cations, anions, purely covalent species.

Advantages of TLC

1) Thin- layer chromatography (TLC) has many advantages over other chromatographic methods, the most important including the simplicity of the equipment, low costs, small requirement as regard the training of personnel and, in many instances, a fairly short time needed for obtaining the results.
2) The separation is done in a very short time as the components elute rapidly. All components of UV light are achievable to visualize. The non-volatile

compounds can be separated by this method. The components of complex mixtures easily separate and recover.

1.6 Extraction methods[43]

1.6.1 Maceration:

In this process, the whole or coarsely powdered crude drug is placed in a stoppered Container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration decantation after standing.

1.6.2 Infusion:

Fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drug

1.6.3 Digestion:

This is a form of maceration in which gentle heat is used during the process of extraction. It is used when moderately elevated temperature is not objectionable. The solvent efficiency of the menstruum is thereby increased

1.6.4 Decoction:

In this process, the crude drug is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting watersoluble, heats table constituents. This process is typically used in preparation of Ayurvedic extracts called “quath” or “kawath”. The starting ratio of crude drug to water is fixed,the volume is then brought down to one-fourth its original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further

1.6.5 Percolation:

The apparatus used in this process is called percolator. It is a narrow-cone-shaped glass vessel with opening at both ends. A dried, grinded, and finely powdered plant material is moistened with the solvent of extraction in a clean container. More quantity of solvent is added, and the mixture is kept for a period of 4h. Subsequently, the content is then transferred into percolator with the lower end closed and allow to stand for a period of 24h. The solvent of extraction is then poured from the top until the drug material is completely saturated. The lower part of the percolator is then opened, and the liquid allowed to drip slowly. Some quantity of solvent was added continuously, and the extraction taken place by gravitational force, pushing the solvent through the drug material downward. The addition of solvent stopped when the volume of solvent added reached 75% of the intended quantity of the entire preparations. The extract is separated by filtration followed by decantation. The marc is then expressed and final amount of solvent added to get required volume

1.6.6 Hot Continuous Extraction (Soxhlet)

In this method, the finely ground crude drug is placed in a porous bag or “thimble” made of strong filter paper, which is placed in chamber E of the Soxhlet apparatus. The extracting solvent in flask A is heated, and its vapors condense in condenser D. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber E rises to the top of siphon tube C, the liquid contents of chamber E siphon into flask A. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This effects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale.

1.7 Phytochemical analysis[50]

1.7.1 Test for alkaloids

a. Mayer’s test

To a few ml of plant sample extract, two drops of Mayer’s reagent are added along the sides of test tube. Appearance of white creamy precipitate indicates the presence of alkaloids.

b. Wagner’s test

A few drops of Wagner’s reagent are added to few ml of plant extract along the sides of test tube. A reddish-Brown precipitate confirms the test as positive.

1.7.2 Test for Amino acids

The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through Wattman No. 1 filter paper and the filtrate is subjected to test for Amino acids.

a. Ninhydrin test

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) are added to 2 ml of aqueous filtrate. Appearance of purple color indicates the presence of amino acids.

1.7.3 Test for carbohydrate

a. Molisch’ s test

To 2 ml of plant sample extract, two drops of alcoholic solution of α- naphthol are added. The mixture is shaken well and few drops of concentrated sulphuric acid is added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates.

c. Benedict’s test

To 0.5 ml of filtrate, 0.5 ml of Benedict’s reagent is added. The mixture is heated on a boiling water bath for 2 minutes. A characteristic-colored precipitate indicates the presence of sugar.

1.7.4 Test for Fixed Oils and Fats

a. Spot test

A small quantity of extract is pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oils.

b. Saponification test

A few drops of 0.5 N alcoholic potassium hydroxide solution is added to a small quantity of extract along with a drop of phenolphthalein. The mixture is heated on a water bath for 2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

1.7.5 Test for Glycosides

For 50 mg of extract is hydrolyzed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate is subjected to the following tests

a. Borntrager's test

To 2 ml of filtered hydrolysate, 3 ml of chloroform is added and shaken, chloroform layer is separated and 10% ammonia solution is added to it. Pink color indicates presence of glycosides. b. Legal's test 50 mg of extract is dissolved in pyridine, sodium nitroprusside solution is added and made alkaline using 10% NaOH. Presence of glycoside is indicated by pink color.

1.7.6 Test for Phenolic Compounds and Tannins

a. Ferric Chloride test

The extract (50 mg) is dissolved in 5 ml of distilled water. To these few drops of neutral 5% ferric chloride solution are added. A dark green color indicates the presence of phenolic compound.

b. Gelatin test

The extract (50 mg) is dissolved in 5 ml of distilled water and 2 ml of 1% solution of Gelatin containing 10% NaCl is added to it. White precipitate indicates the presence of phenolic compounds.

c. Lead acetate test

The extract (50 mg) is dissolved in of distilled water and to this 3 ml of 10% lead acetate solution is added. A bulky white precipitate indicates the presence of phenolic compounds

d. Alkaline reagent test

An aqueous solution of the extract is treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.

e. Magnesium and Hydrochloric acid reduction

The extract (50 mg) is dissolved in 5 ml of alcohol and few fragments of magnesium ribbon and concentrated hydrochloric acid (drop wise) are added. If any pink to crimson color develops, presence of flavanol glycosides is inferred.

1.7.7 Test for proteins

The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through Whatman No. 1 filter paper and the filtrate is subjected to test for proteins.

a. Million's test

To 2 ml of filtrate few drops of Million's reagent are added. A white precipitate indicates the presence of proteins.

b. Biuret test

2 ml of filtrate is treated with 1 drop of 2% copper sulphate solution. To this 1 ml of ethanol (95%) is added, followed by excess of potassium hydroxide pellets. Pink color methanolic layer indicates the presence of protein

1.7.8 Test for Saponins

The extract (50 mg) is diluted with distilled water and made up to 20 ml. The suspension is shaken in a graduated cylinder for 15 minutes. A two cm layer of foam indicates the presence of saponins.

1.7.9 Test for gum and Mucilage's

The extract (100 mg) is dissolved in 10 ml of distilled water and to this 2 ml of absolute alcohol is added with constant stirring. White or cloudy precipitate indicates the presence of Gums and Mucilage

1.8 Plant description

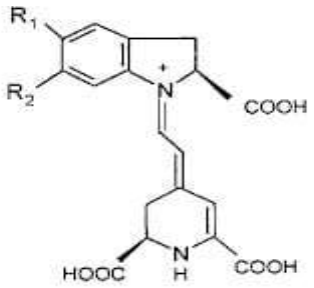
1.8.1 Beta vulgaris

Biological source: Beta species is obtained from the tap roots of the Beta vulgaris.

Geographical source: The coasts of Western Europe and the Mediterranean Sea to Near and Middle East. India, Western Asia, Europe.

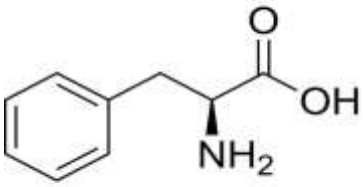
Scientific classification:
Family: Chenopodiaceae
Ayurvedic name: Raktagrnjana
English name: beet root
Hindi name: Chukandar
Parts used: Roots
Genus: Beta
Species: B. vulgaris
Botanical name: Beta vulgaris

Chemical constituents:



Betacyanins

Beta vulgaris roots consist of saccharose and the same element and vitamins found the leaves like vitamin A and C and riboflavin as well as folate, zinc and magnesium. The color is mainly derived from water soluble nitrogenous pigments called betalanins: Betacyanins (red) or beta xanthin's (yellow) including betanin, betanidin, betalmic acid and vulgaxanthin. Amino acids like leucine, tryptophan, valine, alanine, phenylalanine, tyrosine, glutamine, kaempferol glycosides.



Phenylalanine

Pharmacological activity:
- Antioxidant Properties:
- Antihypertensive effect
- Anti-cancer
- Anti-microbial
- Anti depressive
- Anti diabetic

Medicinal uses
Beet root is used for anti-tumor, carminative, laxative hemostatic properties, genital tumor and also used in numerous cancer types: leukemia, breast cancer, prostate, rectum, spleen, stomach, and uterus cancers.
Beet root is also used as anti-oxidant, anti-hypertensive, anti-diabetic, anti-ulcer.

2. MATERIALS AND METHODS

2.1 Plant material collection

The plants were collected from local market and areas of Jagan's college of pharmacy campus. The collected leaves of plants were made thoroughly free from any foreign organic matter, dried under shade, powdered and stored in a well closed container.

2.1.1 Glass Wares used

S. N.	Glass wares	Materials
1	Conical flask	Borosilicate glass
2	Beakers	Borosilicate glass
3	Funnel	Borosilicate glass
4	Tripod stand	Aluminum
5	Glass rod	Borosilicate glass
6	Glass slides	Borosilicate glass
7	Pipette	Borosilicate glass
8	Measuring cylinder	Polypropylene
9	Capillary tube	Borosilicate glass
10	Test tubes	Pyrex glass
11	Motor and pestle	Ceramic

2.1.2 Chemicals:

S. No	Chemicals	Company
1)	Ethanol	Simhapuri chemicals
2)	Chloroform	Simhapuri chemicals
3)	Acetic acid	Simhapuri chemicals

4)	Silica gel	Simhapuri chemicals
5)	Mayer's reagent	Simhapuri chemicals
6)	Molisch's reagent	Simhapuri chemicals
7)	NaOH	Simhapuri chemicals

2.2 Preparation of plant extractions:

2.2.1 Maceration method

5gm of the powdered rhizomes of curcuma longa and powdered leaves of coriander were extracted separately with 100 ml of water for 7 days and kept in air tight container until the extracts were obtained. These solvent extracts were subjected to phytochemical screening and thin layer chromatography technique.

2.3 Phytochemical screening:

The photochemical screening of the three plants showed the presence of different primary and secondary bioactive molecules like carbohydrates, proteins, fixed oils, alkaloids, flavonoids, tannins and saponins.

2.3.1 Test for alkaloids

a) **Meyer's test:** To 1ml of each of the sample solution few drops of Meyer's reagent (potassium mercuric chloride solution) was added. A creamish white precipitate was formed indicating the presence of alkaloids.

b) **Wagner's test:** To few ml of each of the sample solution, Wagner's reagent (Iodine in potassium iodide) was added, which resulted in the formation of reddish-brown precipitate indicating the presence of alkaloids.

2.3.2 Test for glycosides

Concentrated sulphuric acid test: Conc.H2SO4 was added to test sample which resulted in appearance of reddish color.

2.3.3 Test for tannins

a) **Gelatin test:** When gelatin and water were added to test samples formation of white precipitate was resulted.

b) **Alkaline reagent:** When sodium hydroxide solution was added to the sample solution results in the formation of yellow to red precipitate.

2.3.4 Test for saponins:

Foam test: 5ml of extract was shaken vigorously to obtain a stable persistent froth. The froth was then mixed with three drops of olive oil and observed for the formation of an emulsion, which indicated the presence of saponins.

2.3.5 Test for Fats and Oils

Stain test: Press the small quantity of each extract between two filter papers, the stain on filter papers indicates the presence of the oils.

2.3.6 Test for sterols

Libermann-Buchard test: When samples were treated with few drops of acetic anhydride, boiled and few drops of concentrated sulphuric acid from the sides of the test tube were added, shows a brown ring at the junction of two layers and the upper layer turns green which shows the presence of steroids.

2.3.7 Test for flavonoids

Alkaline reagent test: When sodium hydroxide solution was added to the test samples formation of intense yellow color, which turns to color less on addition of few drops of dilute acid indicates the presence of flavonoids.

2.3.8 Test for carbohydrates

1) **Molisch test:** When alpha naphthal and concentrated H2SO4 were added to test samples reddish violet ring at junction of two layers was resulted.

2) **Benedict's Test:** Add 1ml of Benedict's reagent to test tube C and heat the mixture to boiling in a water bath for 2 minutes. The formation of an orange red precipitate due to the formation of copper (I) oxide indicates the presence of reducing sugar.

2.4 Thin layer chromatography:

2.4.1 Stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is uniform thickness and is in fine particle size. Here artificially coated stationary plates were used.

2.4.2 Mobile phase: This comprises a solvent and solvent mixture. The mobile phase used should be particulate-free and of the highest purity for the proper development of TLC spots.

S. No.	Plants	Mobile phase	Ratio
1	Beta vulgaris	Chloroform: acetic acid: ethanol	7:2:3

2.4.3 Capillary spotters: Place a melting point capillary in the dark blue part of the Bunsen burner flame. Hold it there until it softens and starts to sag. Allow the capillary to cool down and then break it in the middle. Make sure to break off the closed end on one of them

2.4.4 Spotting the plate: The thin end of the spotter is placed in the dilute solution; the solution will rise up in the capillary (capillary forces). Touch the plate briefly at the start line.

2.4.5 Developing chamber:

1) A TLC plate can be developed in a beaker or closed jar. Place a small quantity of solvent (mobile phase) in the container.
2) A small spot of solution containing the sample is applied to a plate, about 1cm from the base
3) The plate is then dipped into a suitable solvent such as hexane or methyl acetate, and placed in a sealed container.
4) The solvent moves up the plate by capillary action through the ascending order and meets the sample mixture, which is dissolved and is carried up the plate by solvent. **Advantages:** Ascending order requires a very small amount of sample and takes only about a half an hour or so for chromatographic separations
5) By changing the solvent, or perhaps using a mixture, the separation of components (measured by the Rf value) can be adjusted.
6) Non -polar solvents will force non polar compounds to the top of the plate because the compounds dissolve well and don't interact with the polar stationary phase. And don't interact with the polar stationary phase and don't interact with the stationary polar phase allow the solvent to travel up the plate until 1cm from the top.
7) Take the plate out and mark the solvent front immediately don't allow the solvent to run over the edge of the plate. Next, let the solvent evaporate.

2.4.6 Visualization under visible light:

The plate should be removed from the developing chamber, the position of the solvent front marked, and the solvent allowed to evaporate. If the components of the sample are coloured, they can be observed.

2.4.7 Analysis of TLC Plates:

- The components, visible as separated spots, are identified by comparing the distances they have travelled with those of the known reference materials.
- Measure the distance of the start line to the solvent front. Then measure the distance of the centre of the spot to the start line.
- Divide the distance the solvent moved by the distance the individual spot moved.
- The resulting ratio is called Rf-value.

2.4.8 R f Value:

Rf is the "Retardation Factor", which is the ratio of the distance travelled by a compound in a mobile phase compared with the distance travelled by the front of the mobile phase itself. It is always greater than or equal to zero, and less than or equal to one.

$$Rf = \frac{distance\ travelled\ by\ the\ compound}{distance\ travelled\ by\ the\ solvent\ front}$$

3. RESULT AND DISCUSSION

Table 1: Phytochemical analysis of plants.

Bio active groups	Beeta vulgaris
Alkaloids	Absent
Glycosides	Present
Tannins	Absent
Saponins	Present
Fats and Oils	Absent
Sterols	Present
Flavonoids	Absent
Carbohydrates	Present

Table 2: Rf value of beta vulgaris.

Sample Id	Plant species	No. of spots detected	Rf value
1	Beta vulgaris	3 spots	0.9230 0.9038 0.9423

- Bio active compounds of two plant extracts were identified by using phytochemical analysis test like Mayer's test, Molisch's test, Borntrager's test, And Ferric chloride test. Phytochemicals like alkaloids tannins, glycosides, carbohydrates, amino acids, flavonoids and saponins are identified in these plants. Most extracts that we handled are Beta vulgaris should give a single spot on two TLC plates ideally each compound in a mixture will produce distinct spot as a sample with two extracts will give two different spots and so on. Mixture of components is separated identically in TLC method.
- The Rf value is dependent on both the compound and the solvent used for development if we analyse two compounds and they give the Rf value with the same solvent system

4. SUMMARY AND CONCLUSION

The present study was designed to extract, identify the Phyto-chemically active constituents in Beta vulgaris by using TLC method.

On the basis of the results obtained, the following salient findings have emerged

Preliminary qualitative phytochemical analysis made for the root of Beta vulgaris revealed that the presence of glycosides, carbohydrates, sterols, and saponins so these species are expected to have many medicinal uses.

TLC offers several benefits over the other chromatographic separation. The biggest advantage offered to the chromatography is time saving when compared to other chromatographic techniques. The non-volatile compounds can be separated by this method, where in gas chromatography and other chromatographic techniques only volatile compounds can be separated. All components of UV Light are achieved to visualise microlite `quantity of sample can also be separated in TLC.

TLC proved to be successful method of identifying unknown compounds and determining the polarity of the substance. the manual error is accepted in TLC up to 1% but also this provides accurate results where as in other or HPTLC Methods the same error may give different results and also damage the equipment.

The Rf value helps to identify the different phytochemical constituents present in the chloroform extraction of Beta vulgaris.

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