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EXTRACTION AND ISOLATION OF BETALAINS FROM BOUGAINVILLEA GLABRA FLOWERS

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CHAPTER 1

INTRODUCTION

1.1 IMPORTANCE OF THE RESEARCH

- Sustainable and Eco-Friendly Alternatives

Research on the extraction of betalains from plants such as bougainvillea offers an eco-friendly alternative to synthetic dyes. Synthetic colorants are often associated with health risks and environmental pollution due to their chemical compositions and manufacturing processes. Betalains, being natural and biodegradable, present a sustainable solution that can help reduce the ecological footprint of various industries, particularly the food and cosmetic sectors.

- Health Benefits

Betalains are not just pigments; they possess significant health benefits. Their strong antioxidant properties help neutralize free radicals, reducing oxidative stress in the body. Additionally, their anti-inflammatory and potential anticancer activities make them valuable for developing functional foods and nutraceuticals. By harnessing these health-promoting properties, this research can contribute to public health and well-being.

- Industrial Applications

The demand for natural colorants is rising, driven by consumer preferences for clean-label products and regulatory pressures to limit synthetic additives. Betalains extracted from bougainvillea can provide a new source of natural colorants for the food and cosmetic industries, diversifying the supply and reducing dependence on traditional sources like beetroot. This can lead to the development of innovative products that meet consumer demand for natural and safe ingredients.

1.2 AIM AND SCOPE

Aim

This research aims to develop and optimize an efficient method for extracting betalains from bougainvillea flowers, characterize the extracted pigments, and evaluate their potential applications as natural colorants and antioxidants in various industries, including food and cosmetics.

Scope

- Extraction Method Development

Investigate various extraction methods to determine the most effective technique for isolating betalains from bougainvillea flowers. Optimize key parameters such as solvent type, pH, temperature, and extraction time to maximize yield and purity.

- Chemical Characterization

Identify and quantify the main betacyanins and betaxanthins present in the bougainvillea extracts.

- Stability Studies

Assess the stability of the extracted betalains under different environmental conditions, including various pH levels, temperatures, and light exposures. Determine the conditions that best preserve the integrity and color of the betalains.

- Comparative Analysis

Compare the efficiency, yield, and properties of betalains extracted from bougainvillea with those from other common natural sources like beets and amaranth. Highlight the advantages and potential limitations of using bougainvillea as an alternative source of natural pigments.

CHAPTER 2

2.1 MATERIALS AND METHODS

2.1.1 Collection and Authentication of Plant Materials:

The modified leaves of the paper plant Bougainvillea were collected from Ajjarkadu, Udupi, in the month March 2024. The taxon's identity was confirmed as Bougainvillea glabra by Mrs. Rajani Mathew, a lecturer in the Department of Botany at Dr. G. Shankar Women's Government First Grade College and P.G. Centre, Ajjarkadu, Udupi. Additionally, beetroot vegetables were collected from local farmers.

2.1.2 Extraction:

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids, semisolids or powders intended only for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts and powdered extracts. Such preparations popularly have been called Galenical, named after Galen, the second-century Greek physician.

The purposes of standardized extraction procedures for crude drugs are to attain the therapeutically desired portion and to eliminate the inert material by treatment with a selective solvent known as menstruum. The extract thus obtained may be ready for use as a medicinal agent in the form of tinctures and fluid extracts, it may be further processed to be incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual chemical entities such as ajmalicine, hyoscine and vincristine, which are modern drugs. Thus, standardization of extraction procedures contributes significantly to the final quality of the herbal drug.

2.1.3 Methods of Extraction of Medicinal Plants

- 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 or decantation after standing.

- Infusion

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

- 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.

- 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 water-soluble, heat-stable constituents. This process is typically used in the preparation of Ayurvedic extracts called “quath” or “kawath”. The starting ratio of crude drug to water is fixed, e.g. 1:4 or 1:16; 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.

- Percolation

This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstruum and allowed to stand for approximately 4 h in a well-closed container, after which the mass is packed and the top of the percolator is closed. Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 hr. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

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 of the Soxhlet apparatus. The extracting solvent in flask is heated, and its vapors condense in condenser. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber rises to the top of siphon tube, the liquid contents of chamber siphon into flask . 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.

Aqueous Alcoholic Extraction by Fermentation

Some medicinal preparations of Ayurveda (like asava and arista) adopt the technique of fermentation for extracting the active principles. The extraction procedure involves soaking the crude drug, in the form of either a powder or a decoction (kasaya), for a specified period of time, during which it undergoes

fermentation and generates alcohol in situ; this facilitates the extraction of the active constituents contained in the plant material. The alcohol thus generated also serves as a preservative. If the fermentation is to be carried out in an earthen vessel, it should not be new: water should first be boiled in the vessel. In large-scale manufacture, wooden vats, porcelain jars or metal vessels are used in place of earthen vessels. Some examples of such preparations are karpurasava, kanakasava, and dasmularista. In Ayurveda, this method is not yet standardized, but with the extraordinarily high degree of advancement in fermentation technology, it should not be difficult to standardize this technique of extraction for the production of herbal drug extracts.

Fig. 2.1 Simple Distillation Apparatus

2.2 EXTRACTION OF BETALAINS

Fig. 2.2 Bougainvillea Glabra flower

2.2.2 Apparatus and Equipment used:

- Round bottom flask, separating funnel, conical flask, beaker, Buchner funnel and filtration flask, glass plate, distillation adapters, Water condenser.
- Mixture, Weighing machine, Laboratory stirrer, Rotary evaporator, Reactor, Vacuum tray drier, UV chamber, Water bath.

2.3 PROCEDURE

1. 50 g of dry bougainvillea flower powder was taken in 200 ml of 10% aqueous alcohol, and the pH of the solvent was maintained at 3 to 5. The mixture was added to a 500 ml round bottom flask and refluxed for 3 to 4 hours at 60°C.
2. The mixture was then filtered and concentrated to a volume of 20 ml.
3. The insoluble part was taken in 100 ml of 10% aqueous alcohol (pH 3-5) and refluxed for 3 hours at 60°C.
4. The process was repeated two more times.
5. All four washes were combined and filtered through the Whatman filter paper. The filtrate was collected and concentrated to a volume of 15 ml.
6. 20 ml of hexane was added to the concentrated extract and mixed well.
7. The hexane layer was separated, collected in a round bottom flask, and concentrated to a thick paste to remove the hexane vapor.
8. 5.5 g of the paste was obtained, to which an equal amount of ethyl alcohol was added. The mixture was then evaporated to induce crystallization for 1 hour.
9. The ethyl alcohol layer was filtered, the solid layer was separated and kept for drying.
10. The sample was weighed.
11. Thin Layer Chromatography (TLC) was performed.

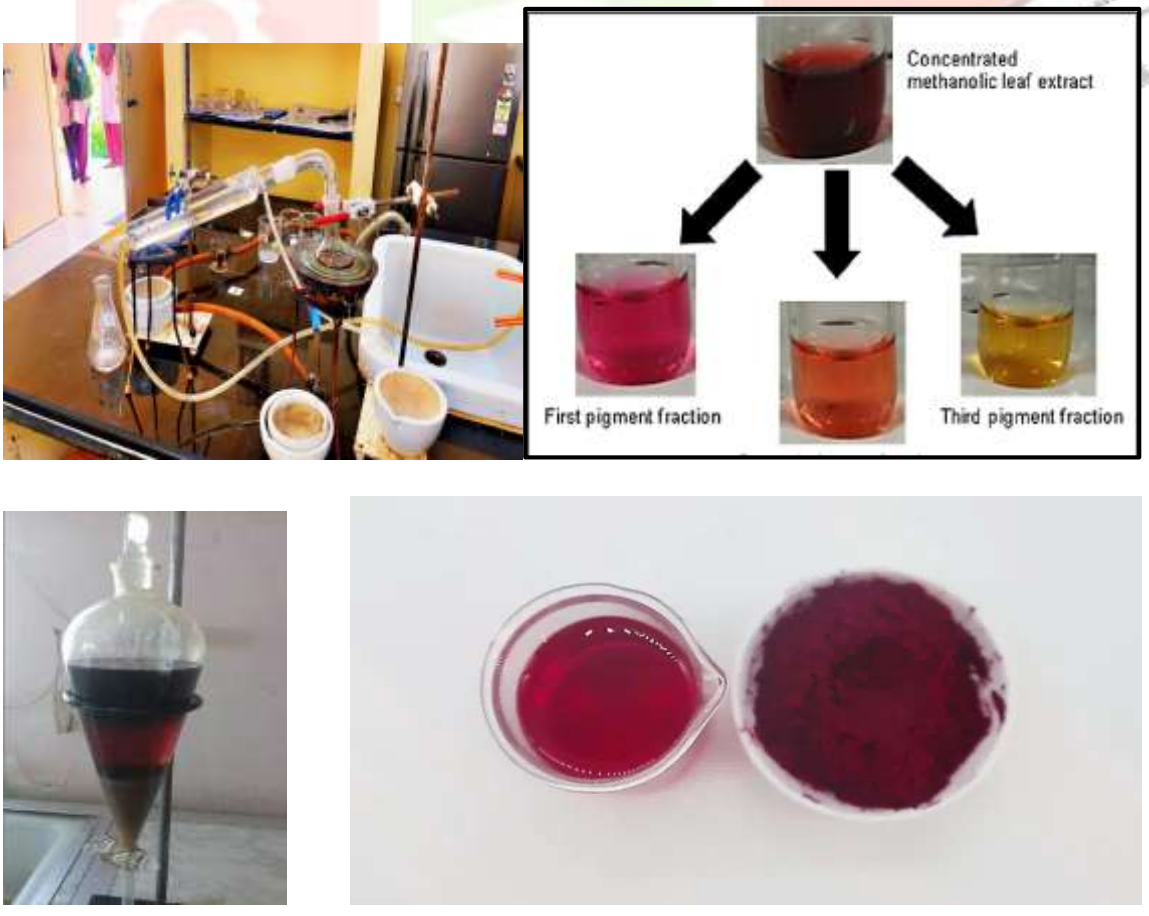
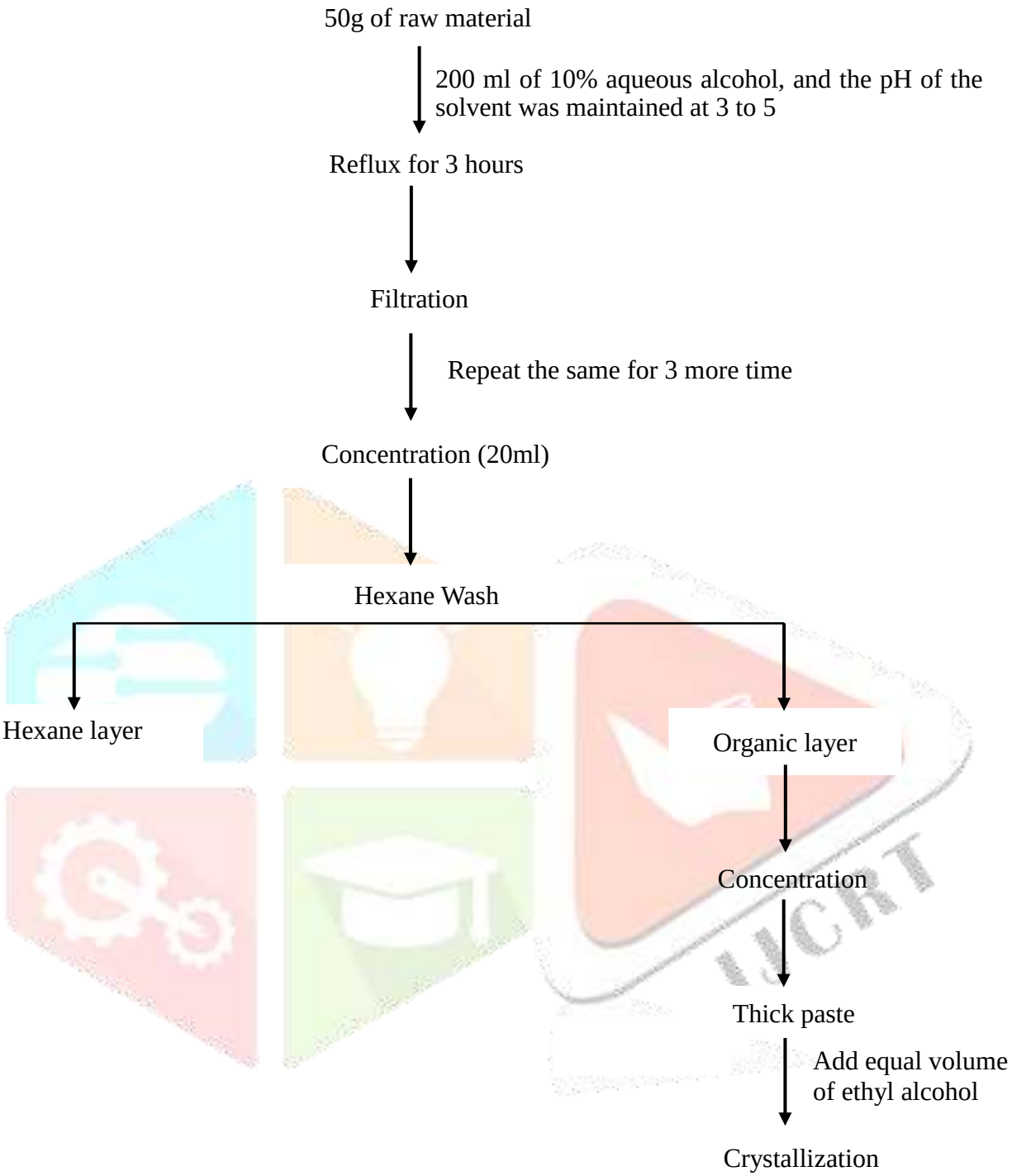


Fig. 2.3 Extraction of betalain

2.3.1 PROCESS FLOW CHART



2.4 THIN LAYER CHROMATOGRAPHY (TLC)

Thin Layer Chromatography (TLC) is a chromatographic technique used to separate non-volatile mixtures. TLC is performed on a sheet of glass, plastic, or aluminum foil coated with a thin layer of adsorbent material, usually silica gel, alumina, or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied to the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Different analytes ascend the TLC plate at different rates, achieving separation. The mobile phase has different properties from the stationary phase; for example, with silica

gel, a very polar substance, non-polar mobile phases such as heptanes are used. The mobile phase may be a mixture, allowing chemists to fine-tune the bulk properties of the mobile phase.

After the experiment, the spots are visualized. This is often done simply by projecting ultraviolet light onto the sheet; the sheets are treated with a phosphor, and dark spots appear on the sheet where compounds absorb the light impinging on a certain area. Chemical processes can also be used to visualize spots; for example, anisaldehyde forms colored adducts with many compounds, and sulfuric acid will char most organic compounds, leaving a dark spot on the sheet. To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase (the mobile phase must not be allowed to reach the end of the stationary phase). This ratio is called the Retention Factor or Rf.

2.4.1 Preparation of mobile phase:

Chloroform: acetone: formic acid 7.5:2: 0.1

Detection

Iodine Chamber and Spraying reagent: Aanisaldehyde-acetic acid reagent (AA)

Procedure:

A freshly prepared mobile phase is poured into a clean and dry 250ml beaker, which is covered with foil. A small amount of isolated betalains dissolved in a minimal amount of methanol is taken in a small test tube. In another test tube, the sample dissolved in a minimal amount of methanol is prepared. A suitable TLC plate is selected and marked with two spots, one for the standard and another for the sample. Two drops of the standard and sample solutions are applied to the spots using a capillary tube. The TLC plate is then placed in the beaker with the mobile phase, allowing the mobile phase to ascend the plate through capillary action. After the mobile phase has ascended sufficiently, the TLC plate is removed from the beaker and visualized under an Iodine chamber and Spraying reagent.

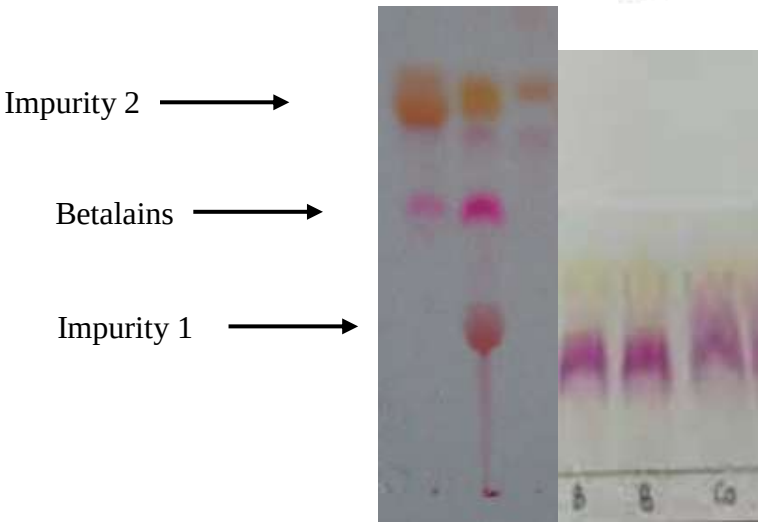


Fig. 2.4 Thin layer chromatography of bougainvillea extract

2.4.2 Result:

- Betacyanin (red-violet pigments) and betaxanthins (yellow-orange pigments) have distinct polarities and interactions with the silica gel and solvent.
- In a typical solvent system (e.g., water, ethanol, and acetic acid), betacyanins often have Rf values ranging from 0.2 to 0.5, while betaxanthins have Rf values from 0.6 to 0.8.
- BetalainsR_f values ranging from 0.5 to 0.7, the colour of the spot will be pink.
- TLC A (mobile phase: Chloroform: acetone: formic acid 7.5 :2: 0.1)
- Bougainvillea extract shows impurities ranging from 0.3% to 0.4% and 0.75%.
- The percentage of betalain purity is around 5-10%.
- To enhance the percentage of betalains in the extract, we need to isolate the betalains using chromatography techniques.

Sample	**Rf value**
Standard betalain Spot	0.56
Test sample	0.62
Impurity 1	0.36
Impurity 2	0.89

CHAPTER 3

3.1 ISOLATION OF BETALAINS FROM BOUGAINVILLEA EXTRACT

By isolating betalains from Bougainvillea extract using column chromatography,enhancing their concentration and various industries can harness the potential of these compounds for diverse applications, ranging from pharmaceuticals and cosmetics to food and nutritional supplements. This method supports the development of natural, safe, and effective products while promoting sustainability and innovation.

Separation of pure betalains can be done by Column chromatography method

3.2 COLUMN CHROMATOGRAPHY

Chromatographic separation carried out in long tube filled with stationary material is led column chromatography. In this method, the separation is carried out in the glass tubes where the mixture is poured at the top of column packed with stationary phase and phase is allowed to pass through the column. The components of mixture get separated because of different component of mixture having different affinity towards stationary and mobile phase as a result of which components of mature travel with different rates in the column The technique of column chromatography was invented by the American chemist D. T. Day

in 1900 In 1906, the Polish botanist, M. S. Tsweet used adsorption columns in the oration of plant pigments; hence column chromatography is also known as adsorption Chromatography Glass tubes having diameter of 40 to 50 mm are used as chromatographic columns in column chromatography. The glass tubes are filled with stationary phases having particle diameters 150 to 200 um. The column dimension is not critical. It ranges from millimeters to a few centimeters and lengths from centimeters to meters. For a given set of columns, greater efficiency will be obtained with long narrow column than with a short thick one but if the column is too long the flow rate will be very low.

PRINCIPLE

Components of the mixture are having different affinity Le different distribution coefficient or partition coefficient towards stationary and mobile phase, on the basis of which separation of the mixture can be achieved. This is the basic principle of column chromatography In column chromatography, the column is packed with stationary phase and the mixture to be separated is dissolved in a suitable solvent and allowed to pass through the adsorbent column. The rate of adsorption varies with the type of adsorbent (stationary phase) filled in the column and the type of mixture to be separated. The component from the mixture, which has greater adsorbing power, is adsorbed at the upper part of the column, while the component having less adsorbing power than the first component is adsorbed at the lower portion of the column. Thus, column chromatography works on the principle of selective adsorption. The adsorbed sample components form a distinct band in the column, travel down along the mobile phase, and get eluted. The portion of the column which is occupied by a particular substance is called its zone. Different zones appear in the adsorbent column, which is termed a chromatogram, and the chromatographic operations are called the development of a chromatogram. Those are: a) Two common procedures may be adopted to estimate various constituents of the sample mixture. After development, the column of adsorbent may be pushed out of the tube, various zones are cut with a knife at boundaries, and the substances present in zones are extracted with suitable solvents. This process of recovery of constituents from the chromatogram is known as elution. b) After development, the column may be washed with more solvent, now termed the eluent, and each component is collected separately as it reaches the end of the column. The process of separation of a mixture of two substances, A and B, has been represented in the

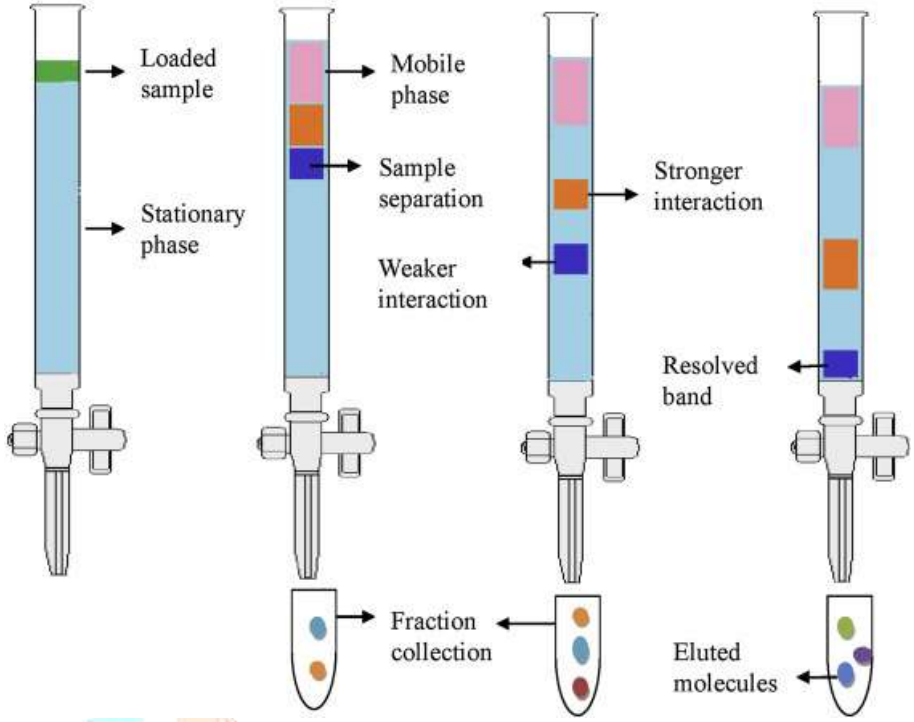


Fig. 3.1 Diagram of Column chromatography

Types of column chromatography:

Based onthe type of stationary phase used in the separation, column chromatography is classified into three types: 1) Adsorption column chromatography, 2) Partition column chromatography, and 3) Gel column chromatography.

1. Adsorption Column Chromatography: In adsorption column chromatography, the separation is achieved by allowing the mixture to pass through the stationary and mobile phase, in which the stationary phase is solid and the mobile phase is liquid solvents. In this method, the mixtures are made to adsorb on the surface of the adsorbent, and the mobile phase is allowed to flow through it.

2. Partition Column chromatography: In this technique, the mixture is to be separated and distributed between the liquid stationary phase and the liquid mobile phase. Partitioning of the mixture between two liquid phases takes place in this method. Depending upon the affinity of compounds towards the liquid phase, compounds get separated in the column. The liquid is coated on a solid support to render it stationary. Experimental detailed both types of column chromatography are almost the same, but they differ only in the use of stationary material for the separation.

3. Gel Chromatography: Gel chromatography, also called Gel Filtration, in analytical chemistry, technique for separating chemical substances by exploiting the differences in the rates at which they pass through a bed of a porous, semisolid substance. The method is especially useful for separating enzymes, proteins, peptides, and amino acids from each other and from substances of low molecular weight. The separation of the components of a mixture by gel chromatography is based on the differences in the molecular sizes of the components. Small molecules tend to diffuse into the interior of the porous particles so that their flow is

restricted, while large molecules are unable to enter the pores and tend to flow unhindered. Thus, the components of the highest molecular weight leave the bed first, followed by successively smaller molecules. The bed materials most extensively used are polyacrylamide and a polymer prepared from dextran and epichlorohydrin.

3.3 PROCEDURE: -

Column purification of betalains

- The glass Column was packed with a slurry of silica gel (mesh size, 60-120) with water.
- A sample (4 gm) of bougainvillea extract was first dissolved in Methanol and carefully applied by pipette at the top of the prepared column. Immediately after the application of the sample, run with water up to 1 to 10 fractions
- Run with a gradient of water: Methanol (98:2, 95:5, 90:10, 80:20, 70:30) finally Methanol and 50 fractions (F1-F12) were collected. 70:30 shows pure betalains on TLC
- Thereafter, (from 70: 30) collected fractions of solvent was removed by evaporation at room temperature.
- After evaporation of solvent from the fractions F30 to F 40, pinkish brown powder was isolated. The sample of fractions was concentrated at 60^0 C. The concentrated fractions were kept in a freezer at 4 to 6^0C. The identity of the sample was confirmed by spectroscopic analysis.

Identification of isolated crystals

The isolated constituent of Betalains(pinkish brown) was identified through thin-layer chromatography.

Preparation of mobile phase:

Chloroform: acetone: formic acid 7.5:2: 0.1

Detection:

Iodine Chamber and Spraying reagent: Aanisaldehyde-acetic acid reagent (AA)

Procedure:

A freshly prepared mobile phase is poured into a clean and dry 250ml beaker, which is covered with foil. A small amount of isolated betalains dissolved in a minimal amount of methanol is taken in a small test tube. In another test tube, the sample dissolved in a minimal amount of methanol is prepared. A suitable TLC plate is selected and marked with two spots, one for the standard and another for the sample. Two drops of the standard and sample solutions are applied to the spots using a capillary tube. The TLC plate is then placed in the beaker with the mobile phase, allowing the mobile phase to ascend the plate through capillary action. After the mobile phase has ascended sufficiently, the TLC plate is removed from the beaker and visualized under an Iodine chamber and Spraying reagent.

Sample	Rf value
Standard betalain Spot	0.56
Test sample	0.59

Fig. 3.2 Purification and fractions of betalain

3.4 CHARACTERIZATION

The isolated betalains were characterized by spectrophotometer and HPLC method

3.5 HPLC [HIGH PRESSUER LIQUID CHROMATOGRAPHY]

The schematic of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile

phase. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or based on mass spectrometry. Most HPLC instruments also have a column oven that allows for adjusting the temperature at which the separation is performed.

High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

HPLC has been used for manufacturing (e.g. during the production process of pharmaceutical and biological products), legal (e.g. detecting performance enhancement drugs in urine), research (e.g. separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and medical (e.g. detecting vitamin D levels in blood serum) purposes. Chromatography can be described as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (e.g. silica, polymers, etc.), 2–50 micrometres in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the absorbent particles. The pressurized liquid is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination.

HPLC is distinguished from traditional ("low pressure") liquid chromatography because operational pressures are significantly higher (50–350 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the column. Due to the small sample amount separated in analytical HPLC, typical column dimensions are 2.1–4.6 mm diameter, and 30–250 mm length. Also, HPLC columns are made with smaller sorbent particles (2–50 micrometre in average particle size). This gives HPLC superior resolving power (the ability to distinguish between compounds) when separating mixtures, which makes it a popular chromatographic technique.

Principle:

HPLC is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. All chromatographic separations, including HPLC operate under the same basic principle; separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase & the stationary phase used in the separation.

Types: there are following variants of HPLC, depending upon the phase system (stationary) in the process:

1. Normal phase HPLC: This separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase & non-polar mobile phase. Therefore, the stationary phase is usually silica & typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether and mixtures of these. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

2. Reverse phase HPLC: The stationary phase is non-polar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions hence the more non-polar the material is, the longer it will be retained.

3. Size-exclusion HPLC: The column is filled with material having precisely controlled pore sizes, & the particles are separated according to its molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later.

4. Ion-exchange HPLC: The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionisable samples. The stronger the charge on the sample, the stronger it will be attached to the ionic surface & thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH & ionic strength are used to control elution time. HPLC instrumentation includes a pump, column, injector, detector, and integrator or acquisition and display system. The heart of the system is the column where separation occurs.

Solvent reservoir:

Mobile phase contents are present in a glass reservoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending upon the composition of the sample.

Pump:

A pump aspirates the mobile phase from the solvent reservoir and forces it through the system's column and detector. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressure of up to 42000 Kpa (about 6000 psi) can be generated.

Sample injector:

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100ml of volume with high pressure (upto 4000 psi).

Column:

Columns are usually made of polished stainless steel, are between 50 & 300mm long and have an internal diameter of between 2 & 5mm. They are commonly filled with a stationary phase with particle size of 3-10 micrometre. Columns with internal diameters of less than 2mm are often referred to as microbore columns. Ideally the temperature of the mobile phase and the column should be kept constant during an analysis.

Detector: Detector is located at the end of the column, detects the analyte as they elute from the chromatographic column. Commonly used detectors are UV-Spectroscopy, Fluorescence, Mass spectrometric & electrochemical detectors.

Data Collection Devices: Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

Fig. 4.1 Instrument of High-Performance Liquid chromatography

3.6 ANALYSIS OF BETALAINS BY SPECTROPHOTOMETER

Introduction

Betalains are water-soluble pigments found in the plant order Caryophyllales, providing vibrant red and yellow colours. These pigments are categorized into two main groups: betacyanin's (red-violet) and betaxanthins (yellow-orange). Betalains are valued for their antioxidant properties and potential health benefits, making them important for food, cosmetic, and pharmaceutical industries. Accurate quantification and analysis of betalains are essential for understanding their properties and applications. Spectrophotometry is a common technique used to analyse betalains due to its accuracy and simplicity.

3.6.1 Spectrophotometer

A spectrophotometer is an instrument that measures the amount of light that can pass through a solution. It is apparent that less light is allowed to pass through a highly turbid or colored solution than through a clear solution. Spectrophotometer is the device that can quantify the amount of light transmitted through solutions. Inside a spectrophotometer, light is focused through a lens system to an entrance slit. The light rays are refocused by a second lens onto an exit slit. Between the second lens and the exit slit is a monochromatic grating which separates the white light into its component wavelengths in much the same fashion as a prism. By proper rotation of the monochromatic grating, specific light wavelengths may be passed on through the exit slit to a photocell. This cell is connected directly to a galvanometer which

translates the electrical output of the activated photocell into a specific transmittance value. In between the exit slit and the photocell is a chamber where samples may be placed. A clear specimen will yield 100% transmittance, while a turbid sample will deflect a considerable portion of the light rays and will have a lower percent transmittance. The greater the density, the lower the percent transmittance. The wavelength selection is important and depends on the colour of the suspension medium. However, it should not be changed during the experiment. It is customary to use 420 nm wavelength if the blank is nearly colourless, and 550 nm if it is yellowish. When light is transmitted through a solution, some of it may be absorbed. If the absorption occurs in the ultraviolet or infrared regions of the electromagnetic spectrum, the solution will appear colourless. But if the absorption occurs in the visible region of the spectrum, the solution will appear coloured. To give a simple example, a solution has a blue colour when viewed by a transmitted white light (light containing all visible wavelengths and perhaps some UV and IR) because it absorbs a greater proportion of the nonblue wavelengths and transmits most of the blue wavelengths to one's eyes. Thus, white light entering this solution will emerge diminished in intensity and consist of a preponderance of blue wavelengths. Quantitative photometric measurement of the absorption process is based on two observations, Lambert's Law and Beer's Law.

Lambert's Law: Lambert was working on the transmission of monochromatic light (light composed of only one wavelength) by homogeneous solid substances. He observed that the thickness of the solid material played a major role in the amount of light being transmitted through it; i.e., each successive unit layer of the medium absorbed an equal fraction of the light passing through it. The thickness of the medium is called the "light path length".

Beer's Law: When Beer applied Lambert's Law to solutions, he found that both the concentration as well as the length of the light path affected light transmission through the solution. He demonstrated that the intensity obtained when light passed through a solution of concentration C and length L was equal to that obtained when that light passed through a solution of the same substance at concentration 2C and length L/2. Beer's Law is thus a generalized Lambert's Law.

And simply states that light absorbance is proportional to the number of molecules of absorbing substance through which the light passes. In Beer's Law, O.D. takes into account the concentration of the solution, the length of the light path, as well as the wavelength of the incident light. O.D. can be read directly off a spectrophotometer. At a fixed wavelength and fixed light path length, O.D. is proportional to the concentration of the light-absorbing molecules; it is this fact that allows us to use the spectrophotometer so advantageously in experiments. Since O.D. is a ratio of two numbers having the same dimensions, it is obviously a dimensionless quantity.

The fixed wavelength at which absorption measurements are made upon a given molecule is not arbitrary. It is customary to make such measurements at the wavelength (λ) of maximum absorption, the so-called λmax of the molecule. The value of λmax for a given molecule is determined experimentally by taking an absorption spectrum; i.e., the O.D. of a solution of the molecule (at a fixed concentration) is measured at successive closely spaced wavelengths, and these O.D. values are plotted as a function of wavelength. The

λmax is immediately evident from such a graph. There are two advantages in using λmax to make experimental absorption measurements of a molecule:

(1) λmax is the wavelength at which there is the greatest change in O.D. per unit change in concentration (the experimental variable we are inevitably interested in if we are using O.D.) and (2) since the slope of the absorption spectrum is zero at λmax only small errors in measurement of O.D. will be introduced if the wavelength dial of the spectrophotometer is slightly inaccurate or set slightly inaccurately.

(2) Absorbance and Transmittance Transmittance is simply the percentage of light impinging on a solution that passes through the solution and emerges to be detected by the instrument. It is zero for a completely opaque solution and 100% when all the light is transmitted. Transmittance and absorbance measure the same quantity, but the scales are reversed, and they are divided differently. Quite simply:

$$O.D= A=\log_{10} 1 / T$$

A spectrophotometer contains the following:

• A light source (visible, UV, or both)

• A monochromator (to obtain a single fixed wavelength)

• A sample compartment (which holds a sample tube or cuvette of fixed path length)

• A photomultiplier (to magnify emergent light because its intensity is too diminished to be accurately read)

• And a phototube (to measure this light and display a reading on the meter panel; i.e., adjusts for the magnification relative to the original light source and gives accurate O.D. readings). Operation of the Spectrophotometer:

1. Turn on the instrument by rotating the Power Switch/Zero Control knob clockwise. This should be done 10 min before the measurements are to be made to give the machine enough time to warm up.

2. Select the proper wavelength. Turn the Wavelength Control knob, which is on top of the instrument, so that it registers the wavelength at which you want to measure your sample. Adjust the meter needle to "0" (left margin) by turning the Zero Control knob on the left.

3.Adjust the meter needle to "0" (left margin) by turning the Zero Control knob on the left.

4. Insert a cuvette containing 5 ml of control blank into the Sample Compartment. Adjust the meter needle to 100% transmittance by turning the Transmittance/ Absorbance Control knob (on the right). This knob regulates the amount of light passing through the exit slit. Now that the meter is adjusted for zero and 100 % transmittance, turbidity measurements can be made.

5. Remove the blank and insert the sample to be read and note the O.D. (bottom scale) or percent transmittance (top scale). Continue to read any other samples you may have. If you have a large number of samples to measure, recheck and adjust the transmittance using your blank after every ten readings. When you have finished your measurements, turn off the spectrophotometer, remove your blank or sample, and clean the instrument

Fig. 4.2 Spectrophotometer

RESULT AND DISCUSSION

HPLC METHOD

Assay: Betacyanins

Mobile phase A: 0.1 ^{0}/0 v/v Trifluro acetic acid Mobile phase B: Acetonitrile (HPLC grade)

(Gradient program)	
Time (Min)	Solvent -B
0.01	50
10.00	50
15.00	95
25.00	95
28.00	50
35.00	50
35.01	stop
Column	Phenomenex Luna C 18 (250 X 4.6mm)I particle size-511
Flow rate	1.0 ml/min
Wavelength	530 nm for betalains
Injection volume	10 Ill
Column Temperature	350C
Run time	35 minutes

Details of working standards:

Name of working standard	% Purity
betalains	94.47%w/w

- Preparation of Mobile phase-A (On 1 0/0 v/v TFA): Take 500 ml purified water into a 1000 ml volumetric flask, add 1 ml of Trifluro acetic acid and make the volume up to the mark with purified water, shake vigorously and sonicate for 5 minutes.
- Preparation of Mobile phase-B (Acetonitrile): Take 100 % Acetonitrile.
- Diluent preparation (1 000/0 Methanol): Take Methanol (HPLC grade) and use as a diluent-

- Standard stock solution of betalains (1.0 mg/ml): Weigh about 100 mg equivalent of standard betalains and transfer in a 100 ml volumetric flask. Add 70 ml methanol and sonicate for 10 minutes, make the volume up to the mark with methanol and mix well.
- Working mixed standard: Take 10m' of betalains standard stock solution to this 100ml volumetric flask and make the volume up to the mark with methanol and mix well. Discard the first 2mL of the filtrate and use subsequent filtrate for the analysis.
- Sample Preparation (04 mg/ml):
  Weigh accurately about 400mg of powdered test substance (passed through 60#) in to 100 ml volumetric flask. Add 70 ml of Methanol and dissolve by sonication for 10 minutes. Make the volume up to the mark with methanol. Pipette out 1ml of this solution into a 10ml volumetric flask and make the volume up to the mark with methanol. Filter the solution through 0.451-1. Syringe nylon filter. Discard the first 2mL of the filtrate and use subsequent filtrate for the analysis.
- Chromatographic procedure: Stabilize the instrument with the mobile phase till the baseline is satisfactory. Inject the standard solution two times and record the chromatogram. The % RSD between the results should be less than 2 0/0. Inject the sample solution and record the Chromatogram. The analysis should comply for the following system suitability parameters. Theoretical plate: More than 2000; and tailing factor: Less than 2

Calculation:

The percentage of Betalains in w/w content can be calculated using the formula

$$\%w/w \text{ of Betalains} = \frac{A1}{A2} \times \frac{W2}{100} \times \frac{10}{100} \times \frac{100}{W1} \times \text{percentage purity of standard betalains}$$

Where,

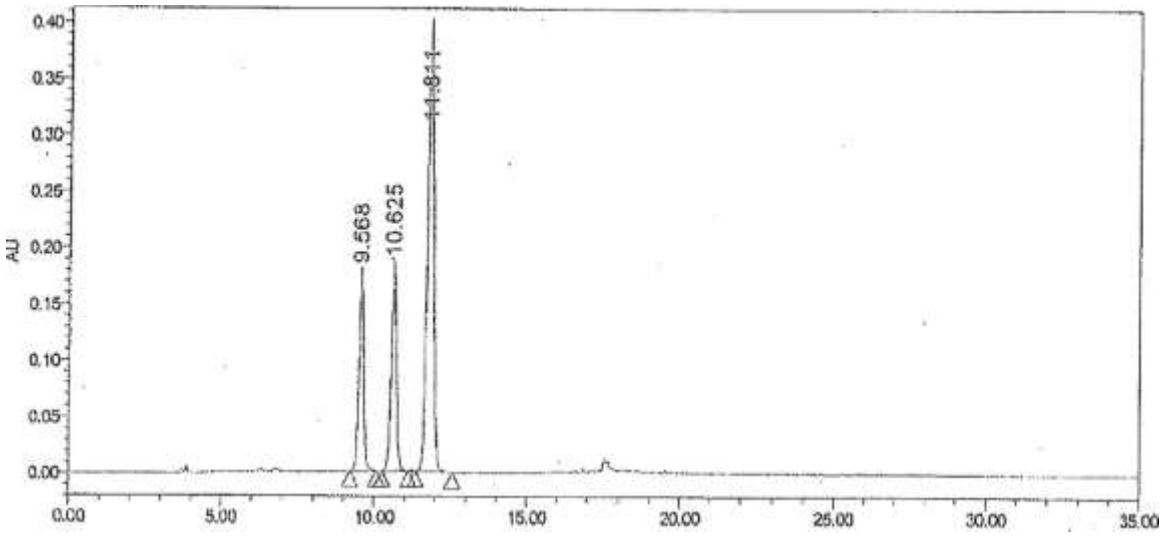
Al =AUC of peak in the sample solution corresponding to (sum of three major peaks)

A2 = AUC Standard betalains (Sum of three major peaks)

WI =Weight of sample (mg)

W2 =Weight of standard Betalains (mg)

SANDARD INFORMATION



SAMPLE INFORMATION

Sample Name: MIX ST D-1 (Betacyanin+ Betalains)

Sample Type: Unknown Acq. Method Set: BetalainslM

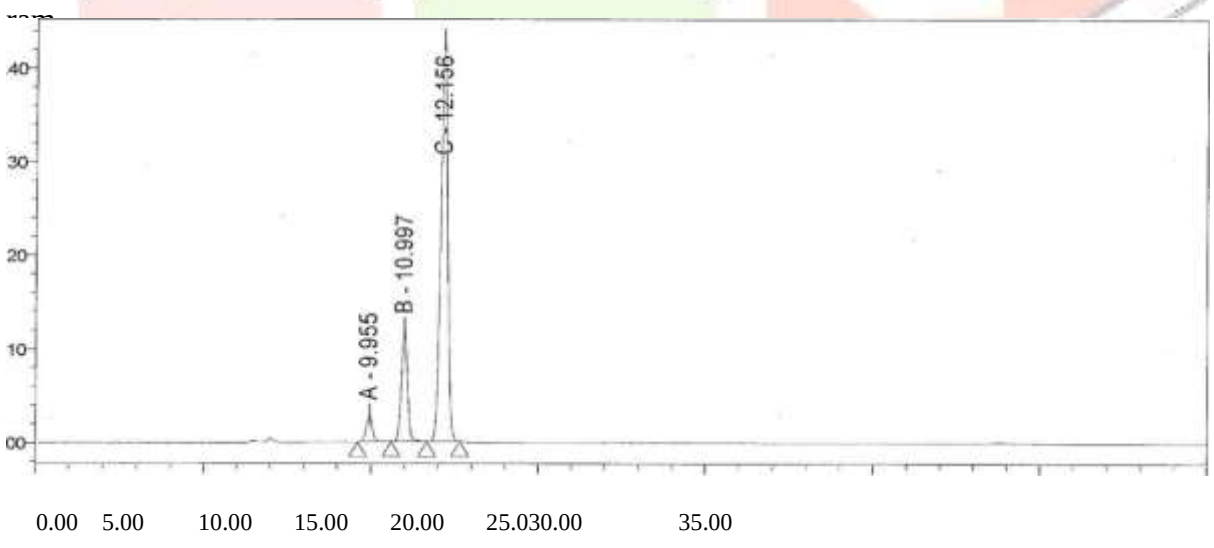
Vial: 26

Injection #: 1 Processing Method Betalains 420PM

Injection Volume: 1 0.00 ul Channel Name: 2998 Ch2 420nm@1 .2nm

Run Time: 35.0 Minutes Proc. Chnl. Descr..2998 Ch2 420nm@1.2nm

Auto Scaled Chromatog



Result

	Name	RT	AREA	USP Plate Count	USP Tailing	Resolution
1	A	9.95	345545	16835	1.12	3.1
2	B	10.9	1544875	17695	1.04	3.3
3	C	12.1	5944945	17972	1.01	3.4

4.3.2 Spectrophotometer method

The betalain pigments responsible for this red colour have been used in cosmetic and food industries. In this study, the spectrophotometric properties of aqueous and ethanol extracts will be determined.

Materials: Bougainvillea flower extract containing betalains

Chemicals: Methanol (analytical grade) Standard betalain solution

Apparatus: Spectrophotometer (UV-Visible) Cuvettes, Volumetric flasks, Pipettes, BeakersSample Preparation

Extraction of Betalains: Extract betalains from bougainvillea flowers as described in the extraction procedure.

Preparation of Standard Solution: Prepare a standard betalain solution by dissolving a known amount of pure betalain in methanol. Serial dilutions are made to obtain different concentrations for calibration.

Preparation of Sample Solution: Dissolve a known quantity of the extracted betalains in a minimal amount of methanol. If necessary, further dilute with methanol to fall within the spectrophotometer's range.

Spectrophotometric Analysis

- Wavelength Selection

Determine the absorption maxima (λmax) of betacyanin and betaxanthins by scanning the standard betalain solution over a range of wavelengths (400-700 nm).

Record the wavelengths with the highest absorbance, typically around 536 nm for betacyanin’s and 480 nm for betaxanthins.

- Calibration Curve

Prepare a series of standard betalain solutions with known concentrations. Measure the absorbance of each standard solution at the determined λ_{max} for betacyanins and betaxanthins.

Plot absorbance against concentration to create a calibration curve.

- Measurement

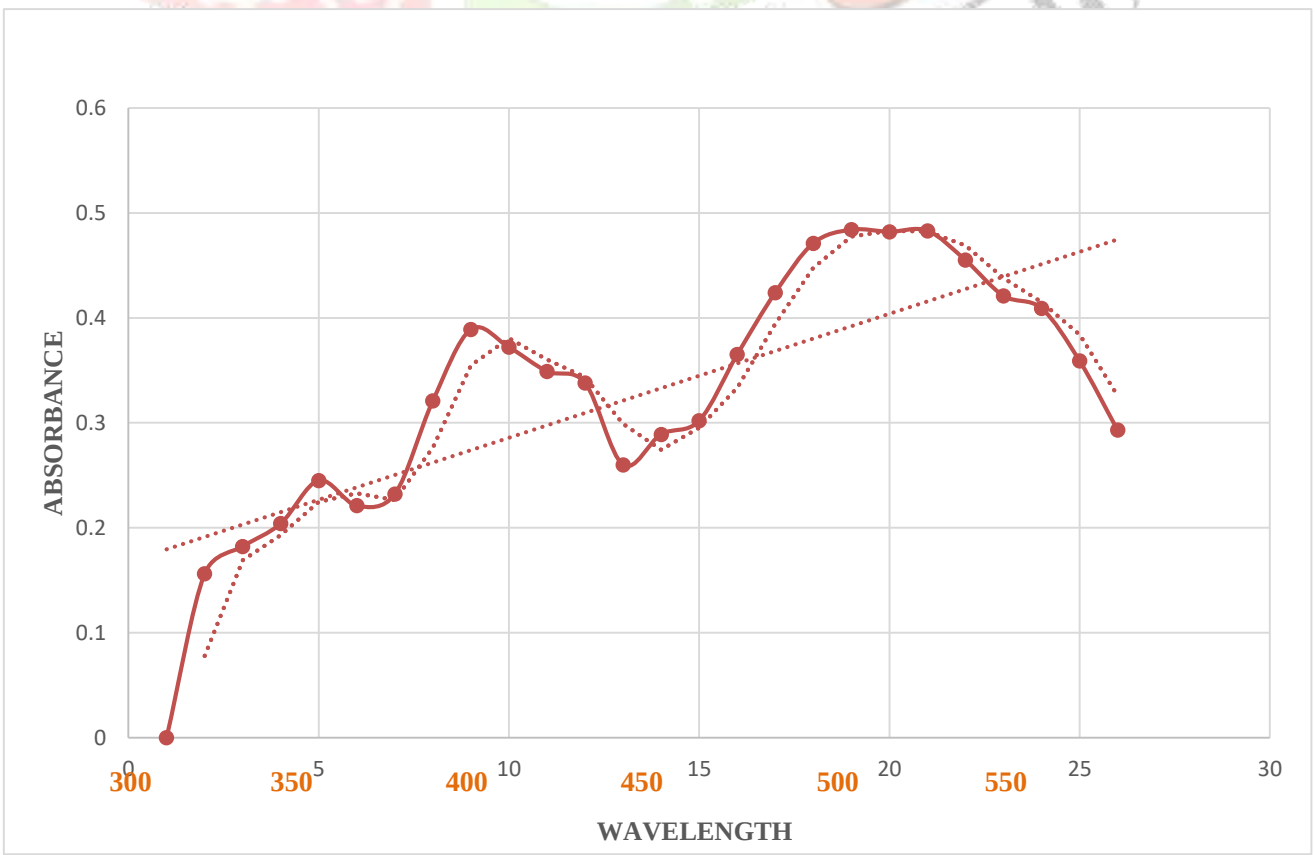
Measure the absorbance of the sample solution at the λ_{max} for betacyanins and betaxanthins.

Ensure that the spectrophotometer is zeroed with a blank solution (methanol) before taking measurements.

Observation

Wavelength (nm)	Absorbance
350	0.156
360	0.182
370	0.204
380	0.245
390	0.221
400	0.232
410	0.321
420	0.389
430	0.372
440	0.349
450	0.338
460	0.26
470	0.289
480	0.302
490	0.365
500	0.424
510	0.471
520	0.484
530	0.482
540	0.483
550	0.437
560	0.455
570	0.421
580	0.409
590	0.359
600	0.293

Spectra of betalain pigment



CONCLUSION:

TLC analysis confirmed that bougainvillea contains betalains and their derivatives, validating its potential as a valuable source of these natural pigments. The present work was taken up with the view to completely standardize the herb in accordance with the parameters of World Health Organization (WHO) Guidelines and standard laboratory procedures. Standardization and phytochemical screening were done to evaluate the qualitative and quantitative parameters of Bougainvillea glabra. The study of the whole plant of Bougainvillea glabra was thoroughly investigated for its organoleptic characteristics, physicochemical characteristics, and major active constituents to analyze its superiority.Quantitative data for betacyanin determined by a spectrophotometric method and a high-performance liquid chromatography (HPLC) method were compared. Pigment solutions at pH 4.0, 5.0, and 6.0 were heat treated under a nitrogen atmosphere and analyzed for pigment losses at appropriate intervals. Fresh, blanched and canned beets were measured for pigment content by the two methods. Quantitative determinations of pigment in all purified or undegraded samples compared well when analyzed by either method. Discrepancies between the results of the two procedures occurred and increased up to 15% with extended heat treatment of the pigment. The differences were attributed to the formation of degradation products or interfering substances. Of the two, the HPLC method is preferred when interfering substances are present.UV–visible absorption spectrophotometry of the beet extract confirmed that betanin has an absorption maximum varying from 430 nm to 520 nm.

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