4.1 Introduction

4.1.1 Standardization of the powdered materials as per the WHO guidelines

4.1.1.1 Various Ash Values

a) **Ash values**-The types of ash determined are Total ash, Acid insoluble and water soluble. Ash value is used to determine the quality and purity of the drug and to establish its identity. Ash contains inorganic radicals lie phosphates, carbonates, and silicates of sodium, potassium, magnesium, calcium, etc. These are present in definite amount in a particular crude drug, hence quantitative determination in terms of various ash values helps in their standardization. Ash value is used to determine foreign inorganic matter present as impurity.

b) **Total Ash Value**-The method of total ash is designed to determine the amount of material that remains after ignition. Ash is classified as physiological ash which is derived from the plant tissue itself and non-physiological ash which is the residue after ignition of extraneous matter (e.g. sand and soil). It is carried out at low temperatures possibly because alkali chlorides, which are volatile at low temperatures, may be lost. The total ash consists of carbonates, phosphates, silicates, and silica.

c) **Acid insoluble ash**- Sometimes, inorganic variables like calcium oxalate, silica, and carbonate content of the crude drug affects Total ash value. Such variables are removed by treating with acid (as they are soluble in hydrochloric acid) and acid insoluble ash value is determined. Acid insoluble Ash, Water soluble ash and sulphated ash are also evaluated. [2]

4.1.2 Extraction

The commonly employed technique for separation of active substance from crude drug is called as ‘Extraction’ which involves the use of different solvents. The plant material used for extraction should be properly authenticated or identified. The choice of the plant material for extraction depends upon its nature and the components required being isolated. The dried powdered plant material is commonly used for extraction. The solvent used for extraction is called menstrum and the residue is known as marc.

4.1.2.1 Methods of extraction

Variation in extraction methods usually depends upon:

1. Length of the extraction period,
2. Solvent used,
3. pH of the solvent,
4. Temperature,
5. Particle size of the plant tissues
6. The solvent-to-sample ratio.

The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction. Earlier studies reported that solvent to sample ratio of 10:1 (v/w) solvent to dry weight ratio has been used as ideal.[3]

4.1.2.2 Conventional Extraction Techniques

a) **Maceration:** Maceration is a simple extraction method that involves soaking the plant prepared raw material in a coarse or powder form in a solvent of interest at room conditions for at least three days with intermittent agitation. After the extraction is completed, the mixture is strained either through sieves or a net with tiny holes. Subsequently, the marc is pressed, and the liquid extract is cleaned using either filtration or decantation after standing. Maceration is preferably carried out in a stoppered container to minimize solvent loss through evaporation.[4]

b) **Decoction:** This extraction technique is useful for phytochemicals that do not decompose or modify with increasing temperature. During decoction, plant material is boiled in water for 15 to 60 min. The duration of boiling will depend on the nature of plant tissues and the phytochemicals being extracted. Ordinarily, delicate plant parts such as leaves, roots, flowers, and tender stems are boiled for 15 min. For instance, phenols and flavonoids have been extracted using decoction and infusion from fruits, rhizomes and leaves at 100 °C. Instead, hard plant parts such as branches and tree barks can be subjected to boiling for an hour. After boiling, the mixture is cooled and then strained, adding cold water to obtain the required amount of solution. After the decoction process is completed, the

mixture is filtered to obtain the liquid extract. The extract produced using the decoction technique is likely to have many undesirable products. It may also be noted that it is not the ideal method for thermolabile compounds. It has been reported that the bark extract of *S. Cumini* using decoction as an extractive technique demonstrated significant antiglycation and antioxidant potential [5]

c) Infusion: Infusion is described as a dilute solution of easily soluble constituents of the plant material. It is an extraction technique in which the plant material is immersed in boiling solvent, particularly water, and left to stand in a stoppered container for about 15 min, after which time the extract (tea) is poured off and separated from the marc using a filter. Tea may be considered as the best example of an infusion. For example, Caffeine has been extracted from dried crushed leaves of tea brands alokozay, lipton, tapal, and tetley at brewing times ranging from 2 to 30 min within the temperature range of 30 to 90 °C. Further, phenolic compounds were extracted from fruits of Tilia cordata at an optimal temperature of 95 °C.

***d)* Digestion:** Digestion is an extractive method similar to maceration and uses slight warming in the extraction process. To avoid the temperature altering the bioactive phytochemicals of given plant material. Therefore, there is increased efficiency in using the extraction solvent due to warming. Mostly temperatures are kept in the range of 35 to 40 °C but may be increased to a maximum of 50 °C for tougher plant materials such as barks and materials containing dismally soluble phytochemicals. During extraction, desired plant parts are introduced in a container with the appropriate solvent pre-heated to the indicated temperatures. The optimum temperature is maintained for a period that may range from half an hour to 24 h with shaking the container at regular intervals.

e) Percolation: 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 menstrum 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 menstrum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstrum 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 menstrum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting. Percolation is the most popular procedure for preparing fluid extracts such as tinctures. Percolation means "to pass a liquid through a solid material drop by drop." During percolation, the solvent, commonly ethyl alcohol, is slowly passed through the plant material, gradually packing itself with phytochemicals, and is gradually propelled down by another fresh solvent added from the top. Before introducing plant material into the percolator, it must be carefully shredded, not making the particles too small. If particles are too fine, it will complicate separating the fine particles from the extraction solvent. Consequently, the extract would be cloudy with residue settling at the bottom of the percolator.

f) **Plant tissue homogenization:** Plant tissue homogenization in solvent has been widely used by researchers. Plant parts, whether dried or fresh, are finely ground using a blender. The resulting fine particles are then immersed in a specified quantity of solvent and either shaken vigorously for 5 to 10 minutes or left to stand for 24 hours. After this period, the extract is filtered. The filtrate can be dried under reduced pressure and then redissolved in the solvent to determine its concentration. In some cases, researchers further clarify the extract by centrifuging the filtrate.

g) **Serial Exhaustive Extraction-** This widely used extraction technique involves the sequential use of solvents with increasing polarity, starting from a non-polar solvent like hexane and progressing to a more polar solvent such as methanol. This approach ensures the extraction of a broad range of compounds with varying polarities. Some researchers prefer using Soxhlet extraction on dried plant material with organic solvents. [3]

4.1.3 Qualitative and Quantitative analysis

4.1.3.1 Qualitative analysis

Qualitative analysis of plant extracts involves identifying the different phytochemicals present in the extracts. This type of analysis is crucial for understanding the potential therapeutic properties, pharmacological activities, and safety profiles of the plant extracts. Here are some key aspects of qualitative analysis in this context:

a) **Purpose**:
   - **Identification of Compounds**: Determine the presence of specific bioactive compounds such as alkaloids, flavonoids, glycosides, tannins, saponins, terpenoids, and phenolic compounds.
   - **Phytochemical Screening**: Understand the chemical composition and potential health benefits of the plant extracts.
   - **Standardization**: Ensure consistency and quality of plant-based products.

b) **Methods**:
   - **Chemical Tests**: Use specific reagents that react with particular classes of compounds to produce visible changes (e.g., color change, precipitation).
     - **Alkaloids**: Detected using tests like Dragendorff's, Mayer's, or Wagner's reagent.
     - **Flavonoids**: Identified using tests such as the Shinoda test or with sodium hydroxide.
     - **Tannins**: Detected using Ferric chloride test.
     - **Saponins**: Froth test is used to identify saponins.
     - **Glycosides**: Detected using tests like the Keller-Kiliani test for cardiac glycosides.
   - **Chromatography**:
     - **Thin Layer Chromatography (TLC)**: Used for separating and identifying components based on their movement on a stationary phase.
     - **High-Performance Liquid Chromatography (HPLC)**: Used for more precise identification and quantification of compounds.
     - **Gas Chromatography-Mass Spectrometry (GC-MS)**: Used for identifying volatile compounds and complex mixtures.
   - **Spectroscopy**:
     - **UV-Visible Spectroscopy**: Used to identify and quantify compounds based on their absorbance of UV or visible light.
     - **Fourier Transform Infrared Spectroscopy (FTIR)**: Used to identify functional groups in compounds.
     - **Nuclear Magnetic Resonance (NMR) Spectroscopy**: Used for detailed structural analysis of organic compounds.

c) **Process**:
   - **Sample Preparation**: Extract plant material using appropriate solvents (e.g., water, ethanol, methanol) through methods like maceration, Soxhlet extraction, or ultrasound-assisted extraction.
   - **Preliminary Screening**: Conduct initial tests to detect the presence of broad classes of phytochemicals.
   - **Detailed Analysis**: Use advanced chromatographic and spectroscopic techniques for precise identification of specific compounds.
   - **Documentation and Interpretation**: Record observations and interpret results to draw conclusions about the chemical composition of the extracts.

d) **Applications**:
   - **Pharmacognosy**: Study of medicinal drugs derived from plants and their constituents.
   - **Herbal Medicine**: Development and standardization of herbal formulations.
   - **Quality Control**: Ensure the purity, potency, and safety of plant-based products.
   - **Research and Development**: Investigate new plant sources for potential therapeutic agents.

4.1.3.2 Quantitative analysis

Quantitative analysis of herbal extracts involves measuring the concentrations of specific bioactive compounds within the extracts. This type of analysis is crucial for standardizing herbal products, ensuring their quality, efficacy, and safety, and for understanding the dosage required for therapeutic effects. Here are some key aspects of quantitative analysis in this context:

1. **Purpose**:
   - **Determination of Concentration**: Measure the amount of specific compounds such as alkaloids, flavonoids, phenolics, glycosides, and other phytochemicals.
   - **Standardization**: Ensure consistent potency and quality of herbal products.
   - **Quality Control**: Verify that products meet specified standards and are free from contaminants.
   - **Dosage Formulation**: Determine appropriate dosages for therapeutic use.

2. **Methods**:
    - **Spectrophotometric Methods**:
        - **UV-Visible Spectrophotometry**: Quantify compounds that absorb UV or visible light.
        - **Fluorometry**: Measure fluorescence emitted by certain compounds.
    - **Chromatographic Techniques**:
        - **High-Performance Liquid Chromatography (HPLC)**: Separate and quantify components based on their interactions with a stationary phase and a mobile phase.
        - **Gas Chromatography (GC)**: Used for volatile compounds.
        - **High performance Thin Layer Chromatography (HPTLC)**: Quantify separated compounds by measuring their density on a TLC plate.
    - **Mass Spectrometry (MS)**:
        - **GC-MS**: Used for the precise quantification of volatile and semi-volatile compounds.
        - **LC-MS**: Combine HPLC with MS for highly sensitive and specific quantification.
    - **Nuclear Magnetic Resonance (NMR) Spectroscopy**: Quantitative NMR (qNMR) can be used for determining the concentration of compounds in a mixture.
    - **Titrimetric Methods**: Use chemical titrations to quantify specific groups of compounds (e.g., alkaloids, saponins).
    - **Enzyme-Linked Immunosorbent Assay (ELISA)**: Quantify specific proteins or other bioactive compounds using antibodies.
3. **Process**:
    - **Sample Preparation**: Extract plant material using appropriate solvents (e.g., water, ethanol, methanol) through methods like maceration, Soxhlet extraction, or ultrasound-assisted extraction.
    - **Calibration Curve Preparation**: Prepare standard solutions of known concentrations for the target compounds to create a calibration curve.

- **Analysis**: Measure the absorbance, peak area, or signal intensity of the sample and compare it to the calibration curve to determine the concentration of the target compounds.
- **Validation**: Validate the methods for accuracy, precision, sensitivity, specificity, and reproducibility.

4. **Applications**:
   - **Pharmacognosy**: Study the concentrations of active compounds in medicinal plants.
   - **Herbal Medicine**: Standardize herbal extracts and formulations to ensure consistent therapeutic effects.
   - **Quality Control**: Verify the content and purity of commercial herbal products.
   - **Research and Development**: Investigate the bioavailability and pharmacokinetics of active compounds.
5. **Challenges**:
   - **Complexity of Extracts**: Herbal extracts contain numerous compounds, making it challenging to isolate and quantify individual components.
   - **Interference**: Presence of other substances in the extract may interfere with the measurement of target compounds.
   - **Standardization**: Variability in plant material due to growing conditions, harvest times, and extraction methods can affect the consistency of the extracts.

Quantitative analysis of herbal extracts is essential for the development of safe and effective herbal medicines, ensuring product consistency, and advancing scientific research in phytotherapy. Qualitative and quantitative analysis of components contained is not only an imperative issue in the quality control of herbal medicines, but also a prerequisite for disclosing their overall benefits and risks. Great challenges lie in the global qualitative and quantitative analysis of herbal medicines, mainly because of the diversity in chemical structures and physiochemical properties, the wide range of contents, and also the lack of authentic standards, of its complex components contained. To address such challenges, numerous efforts have been made and almost all kinds of "state of the art" analytical tools such as mass spectrometers (MS), nuclear magnetic resonance spectrometer (NMR), and evaporative light scattering detectors (ELSD) have been used in the qualitative and quantitative analysis of herbal components in the

past decades. [7] Qualitative analysis of plant extracts is essential for validating their traditional uses, discovering new drugs, and ensuring the quality and efficacy of herbal products.

4.1.3.3 Introduction of High-performance thin layer chromatography (HPTLC)

HPTLC can handle more complex, crude herbal extracts without the need for extensive sample purification, whereas HPLC often requires purified or more concentrated extracts due to potential matrix effects.

The chromatographic techniques showed continuous improvement beginning from use of starch as a binder, silicic acid as sorbent, and use of silica as stationary phase in TLC. The TLC exhibited wide applicability in the separation science and gained quick response throughout the world. After the establishment of silica coated TLC as an efficient tool for quantitative techniques, effect of small sized silica coatings on Rf values and plate height were observed. The plates with small sized silica (termed as nano-plates) were found advantageous and were called as HPTLC plates. This led to the introduction of high-performance thin layer chromatography (HPTLC).

It is a type of planar (flatbed) chromatography and is sophisticated version of TLC with the enhancements like increased resolution of components, use of higher quality plates and small sized stationary phase. The modern HPTLC technique involves automated sample application and densitometric scanning. It is highly sensitive and is suitable for both the qualitative and quantitative analysis. It is better over other chromatographic techniques as it provides fingerprints for visualization and ability to store data in the form electronic library. [8]

A) Principle

The principle of HPTLC is same as TLC (i.e. separation by adsorption). In this technique, mobile phase is driven by capillary action and carries various components. Depending on their affinity towards adsorbent, the components exhibit adsorption on the stationary phase. The components with larger efficiency for stationary phase interact with the surface and move slowly in the mobile phase whereas components with lesser affinity for stationary phase move fast. This variation in the movement of components results in the separation of components on the chromatographic plate. [9]

B) Key features of HPTLC

Some of the important features of HPTLC are given below.

1. It produces complex information about the entire sample in the form of visible chromatograms at a glance.
2. Simultaneously, sample and standard can be analysed for better precision and accuracy.
3. Various samples can be analysed and compared simultaneously with the help of images.
4. It gives data in the form of visible chromatograms as well as peak data.
5. The data can be evaluated either by the image-based software Videoscan or by scanning densitometry with TLC Scanner, measuring the absorption and/or fluorescence of the substances on the plate.
6. The technique is cost-effective and has low cost for maintenance.
7. The sample preparation is simple and different types of samples can be analyzed by the technique.
8. Prior treatment of solvents i.e. filtration and degassing is not required.
9. The use of harmful solvents is less as compared to other chromatographic techniques.
10. The chances of contamination are less because it utilizes fresh stationary and mobile phases for each analysis. [10]

C) Working of HPTLC

The various steps of HPTLC are discussed below.

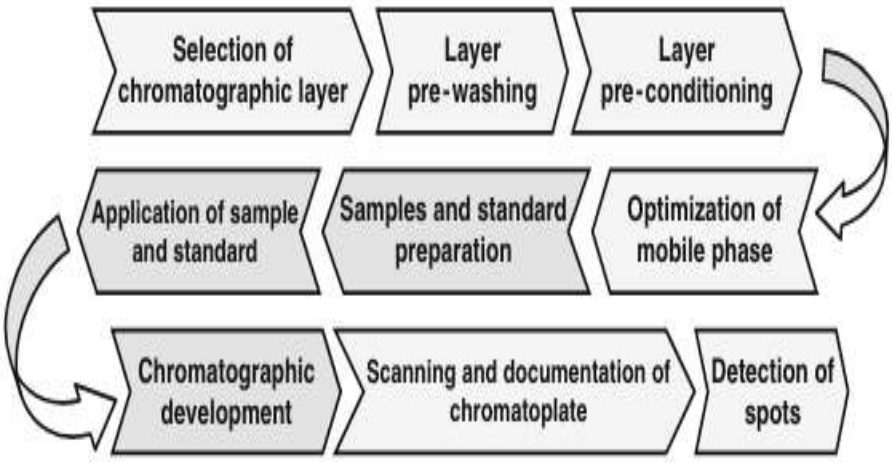

Figure 4.1 Steps of HPTLC working

D) Selection of HPTLC chromatographic layer

In HPTLC, plates coated with small sized particles and narrow size distribution is used. Thus, the surface area of plates is smooth. The size of the plates is comparatively small as compared to TLC i.e. (10-20 cm) and the development distance is 6 cm. The selection of HPTLC stationary phase is based upon the type of analyte. [11]

E) Application of sample

The sample is applied with the help of some applicators such as 1) capillary tubes 2) micro-bulb pipettes, 3) micro-syringes, and 4) automated sample applicators. Using these applicators, sample can be applied in the form of spot or band. The concentration range is 0.1-1µg /µL because above this range, separation becomes poor.

In case of applying a sample as a spot, a fixed volume pipette having capillary action is used. It is filled with the sample and touched on the surface of plate, thereby delivering the sample to the stationary layer. If variable volumes are needed, then, a syringe with micrometer control can be used. Proper care must be taken during the application of sample i.e. the spots should be precise in position and layer should not undergo any damage during application. In sample applicators, Camag Nanomat is a mechanized spotting device with fixed volume glass capillaries. These are lowered onto the layer with reproducible contact pressure, which controls the position of the spot. Similarly, Desaga PS 01 Sample Applicator uses microlitre syringes for the application of sample. Another method i.e. sample application as narrow bands provides the highest resolution in the separation. In this case, sample is filled in a syringe and vacated with the help of a motor. Both the syringe and plate move linearly to produce a band. Another applicator i.e. Linomat (Figure 4.3) allows sample application in narrow bands of variable length.

Figure 4.2 CAMAG instruments and tools (Basic kit)

Figure 4.3 Sample application (Semi-automatic)

Figure 4.4 Sample application (Automatic)

Figure 4.5 Plate development -Automatic developing chamber ADC2

Figure 4.6 Derivatizer

Derivatization- Apart from this, automatic samplers are computer controlled and can apply samples automatically. The samples are picked from the vials and transferred to plate with the help of steel capillary in the form of spots or bands. These devices offer choice to select the sample volume, dispensing speed and application pattern.

F) Chromatograph development

The chromatogram development is the most important step in the HPTLC procedure. The HPTLC chamber is pre-conditioned with solvent to get uniform vapours of the solvent in the chamber. The chromatograms can be developed in four ways 1) Vertical method, 2) Vario-method, 3) horizontal method and 4) automatic multiple development. The HPTLC plates are generally developed in twin-trough chambers, or horizontal-development chambers. The saturated twin-trough chambers fitted with filter paper offers the best reproducibility and avoids solvent vapor preloading and humidity.

In the cortical method, the lower edge of the plate is immersed in the developing chamber having solvent at the bottom. The solvent ascends in the plate and layer interacts with the vapours in the tank. This method does not provide reproducibility as the development of chromatograms varies with the dimensions of the plate. In the horizontal development, chromatogram is developed by applying the sample parallel to both opposing edges of the plate. The chromatogram is developed from both the sides towards the centre of the plate. Therefore, the number of samples can be doubled. In this method, the volume of solvent required for the development is very less, therefore, it is economical. In automatic development, the development of chromatogram can be controlled by an instrument. In offers the advantage to select various parameters like preconditioning, tank of sandwich configuration, solvent migration distance, etc prior to the development of chromatogram. In this case, software is used to decide the composition of developing solvent and developing distance. In addition, the volumes are measured with syringes and migration distance can be measured by sensors.

G) Detection of spots (Scanning) and documentation

The developed plates can be detected by using UV cabinet or chamber (Figure 4.7) which provides a non- destructive analysis. Alternatively, the spots are analyzed at 254 nm or 366 nm if the compounds are fluorescent. Moreover, fluorescent stationary phases may be used to if the compounds exhibit quenching properties. These days, design of UV cabinets is improved,

which allows fixing of digital camera for recording images of the plate. Further, the components may be quantified on the same plates.

Figure 4.7 Detection- TLC Scanner 4

H) Densitometer measurements

In densitometry, separation tracks are evaluated with the help of a light beam in the form of a slit with adjustable dimensions. The reflected light is measured by the photosensor (Figure 4.8) and the difference between optical response of blank and the sample zone is correlated with various sample zones. Nowadays, a planar chromatogram is evaluated by video technology.

Figure 4.8 Documentation (TLC Visualizer 2)

4.1.3.4 Validation process [12]

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Any developed method may be influenced by variables like different elapsed assay times, different days, reagent lots, instruments, equipment, environmental conditions like temperature, etc. Therefore, it is expected that after the method has been developed and before it is communicated or transferred from one lab to the other, it is properly validated and the result of validity tests are reported.

Method validation is required when:

- A new method is being developed.
- Revision of established method.
- When established methods are used in different laboratories, etc.
- When quality control indicates method changes.

Validation should not be seen separately from the development of a method. It starts from a clearly defined analytical goal, method selection, optimization, and development, which is called pre validation considerations before arriving at the elaboration of a validation protocol and is the starting point of the actual validation. After performing all the experiments described in the validation protocol, obtained data are evaluated and compared with the acceptance criteria. If all criteria are met, the method can be regarded as valid. In a less-formal approach, some validation data may be incorporated from experiments, which were conducted previously as part of the method development. The above approach is widely accepted for validation of qualitative HPTLC methods for identification during routine use. It is possible that the validation method in different situations may require some changes in the standard validation protocol. Such changes may include restrictions with respect to relative humidity, waiting times, precision, etc. The validation protocol is a key instrument for structuring, regulating and documenting the validation processes, depending on the quality management system. The following elements must be included:

a) Specificity

Specificity is the ability to assess unequivocally the analyte in presence of components which may be expected to be present. Typically, these might include impurities, degradation product, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). This definition has the following implications,

Identification: To ensure the identity of analyte

Purity Tests: To ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency): To provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

b) Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

c) Range

The range of an analytical procedure is the interval between upper and lower concentration (amounts) of analyte for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

d) Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed as trueness. Accuracy of an analysis is determined by systematic error involved. It is defined as closeness of agreement between the actual value and mean analytical value obtained by applying the test method a number of times. The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. This parameter is very important for formulated pharmaceutical dosage forms as it provides information about the recovery of the analyte from sample preparation and effect of matrix. It the recovery rate is found to be 100%, it implies that the proposed analytical method is free from constant and proportional systematic error. A blank matrix and known impurities must be available to test the accuracy of the method.

e) Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous

sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Precision provides an indication of random error. Its results should be expressed as relative standard deviation (RSD) or coefficient of variation (COV). Precession is observed in terms of replication: precision under same conditions, same analyst, same apparatus, short interval of time and identical reagents using the same sample; measurement of peak area: RSD should not be greater than 1%, based on seven times measurement of same spot; peak position: RSD should not be greater than 2% based on seven times repositioning the instrument after each measurement; sample application: equal volume applied as seven spots and RSD should not be greater than 3% and under different conditions, different analyte, different laboratory, and different days and reagents from different sources using the same sample. RSD should not be greater than 10% within laboratory reproducibility.

e.1) Repeatability

Repeatability expresses the precision under same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

e.2) Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

e.3) Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology.

f) Limit of Detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantities as an exact value. Lowest amount of analyte that can be detected is not greater than 10% of the individual impurity limit. If this is not possible, then amount of analyte to be applied has to be increased. Limit of detection (LOD) is determined on the basis of signal to noise ratio. Mean of 15 noise peak areas and their absolute SD values are determined. LOD is the amount of applied sample producing a peak area which is equal to the sum of mean blank area and three times standard deviation

g) Limit of Quantitation

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for determination of impurities and/or degradation products.

h) Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. This is one of the most important parameters for validation of HPTLC method. Experiments are usually recommended to evaluate ruggedness of a HPTLC method like sample preparation: composition, quantity of solvent, pH, shaking time, temperature and number of extractions; sample application: volume applied, spot shape and size, band and spot stability; separation: at least on three different plates; chromatographic conditions: chamber saturation, eluent composition, eluent volume, temperature, humidity and development distance; spot visualization: post chromatographic derivatization, spraying, dipping, reaction temperature and time; quantitative evaluation: drying of plates, detection and wavelength. Once the analytical method is developed, it should be performed independently by three analysts well conversant with practical aspects of the technique, analysing the same sample under same experimental conditions to check reproducibility of the method.

4.2 Materials and methods

4.2.1 Collection and authentication of Plant materials

The leaves of *Calotropis procera* and leaves of *Adhatoda vasica* were obtained from medicinal garden of The Maharaja Sayajirao University of Baroda and flower petals of *Rosa indica* were obtained from market at Vadodara. All the plant materials were identified by Botany Department, The Maharaja Sayajirao University of Baroda. The voucher specimens of the herbs have been deposited in the Pharmacy department, The Maharaja Sayajirao University of Baroda.

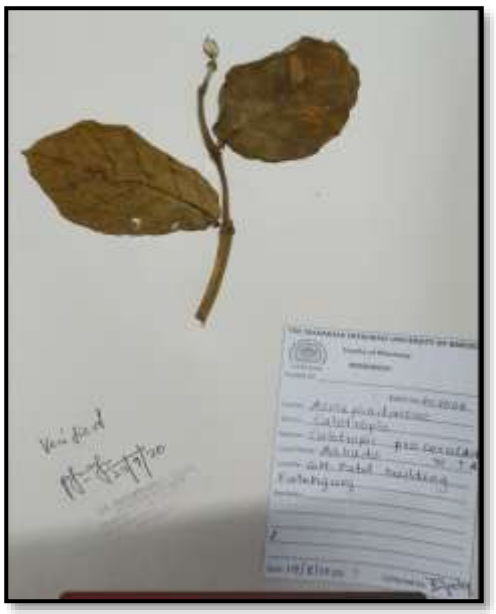

Figure 4.9A

Figure 4.9B

Figure 4.9C

Figure 4.9A Authentication of leaves of *Calotropis procera*

Figure 4.9B Authentication of flowers of *Rosa indica*

Figure 4.9C Authentication of leaves of *Adhatoda vasica*

4.2.2 Preparation of powdered material

The selected plant materials were collected, cleaned to remove any adhering material and then dried in shade. The large, dried plant parts were then subjected to size reduction to coarse powder and used for further studies.

4.2.3 Standardization of the powdered materials as per the WHO guidelines

a) Determination of ash

After ignition, the remaining ash from medicinal plant materials was measured in terms of total ash, acid-insoluble ash, and water-soluble ash.

1. **Total Ash**- Approximately 2 grams of the ground plant material was accurately weighed into a previously ignited and tared crucible. The sample was spread into an even layer and ignited by gradually raising the temperature to 500-600°C until it turned white, indicating the absence of carbon. The ash was then cooled to room temperature and weighed. The total ash content was expressed as a percentage of the air-dried material.
2. **Acid-Insoluble Ash**- To the total ash, about 25 ml of 70% hydrochloric acid (HCl) was added, and the mixture was covered with a watch glass. After boiling for 5 minutes, the insoluble residue was collected on ashless filter paper, transferred to the crucible, and ignited to a constant weight. The residue was then weighed, and the acid-insoluble ash content was calculated as a percentage of the air-dried material.
3. **Water-Soluble Ash**- The total ash was mixed with 25 ml of water and boiled for 5 minutes. The insoluble residue was collected on ashless filter paper, and the crucible was ignited to a constant weight. The weight of the residue was subtracted from the weight of the total ash, and the water-soluble ash content was calculated as a percentage of the air-dried material.

4.2.4 Preparation of Extracts: As aqueous and methanolic extract contains almost all phytoconstituents for the study and they are also safe. Therefore, prepared both methanolic and aqueous extracts. Aqueous extracts and methanolic extracts of three selected plants were obtained using Soxhlet apparatus but individual bioactive compounds /Phytochemicals/bioactive constituents were not extracted and aqueous and methanolic extract were used for further studies.

For the preparation of aqueous extracts, Soxhlet apparatus was used because the advantage of Soxhlet apparatus to extract a wide variety of compounds from the plant material, including fats, oils, waxes, and other hydrophobic molecules. The dried powder of plant was extracted with two solvents 1) Methanol and 2) Water

4.2.4.1 Preparation of Methanolic Extract

About 20 gm of dried powder of plant part was subjected to Soxhlet apparatus for 36 hours. The temperature of Soxhlet apparatus was set 40℃ for the methanolic extraction. The solvents were removed by heating at low temperature on water bath and got semi solid mass.

4.2.4.2 Preparation of Aqueous Extract

About 20 gm of dried powder of plant part was subjected to Soxhlet apparatus for 36 hours. The temperature of Soxhlet apparatus was set 100℃ for the aqueous extraction. The solvents were removed by heating at low temperature on water bath and got semi solid mass.

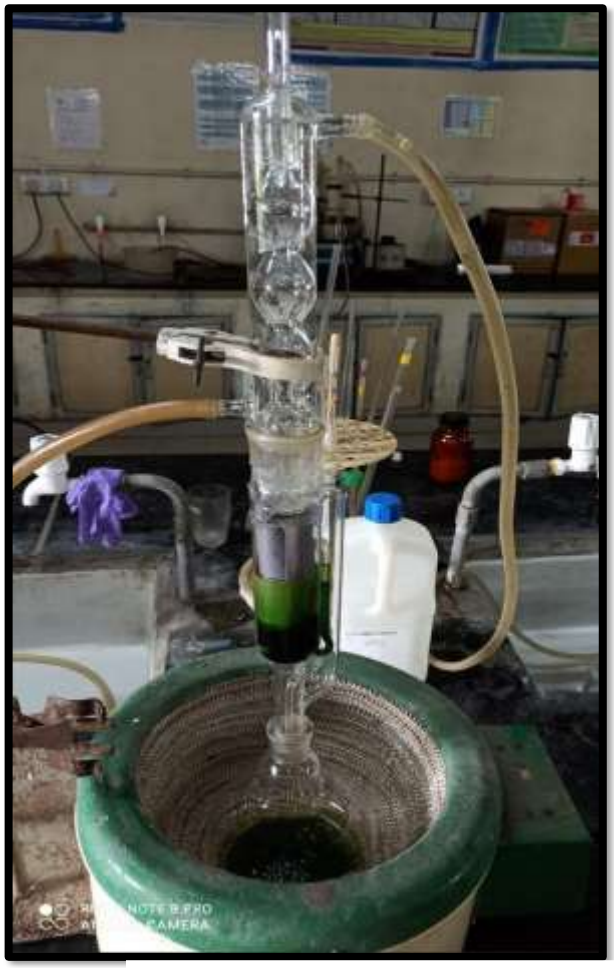

Figure 4.10 – Soxhlet apparatus

Figure 4. 11 Aqueous and Methanolic extract of *Rosa indica*

4.2.5 Qualitative Analysis of extracts [13]

Phytochemical examinations were carried out for the extracts as per the standard methods.

- **Detection of alkaloids:** Extracts were dissolved individually in dilute Hydrochloric acid and filtered.
  - ❖ **Mayer's Test:** Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a syellow-coloured precipitate indicates the presence of alkaloids.
  - ❖ **Wagner's Test:** Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.
  - ❖ **Dragendroff's Test:** Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.
  - ❖ **Hager's Test:** Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.
- **Detection of carbohydrates:** Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.
  - ❖ **Molisch's Test:** Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.
  - ❖ **Benedict 's test:** Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.
  - ❖ **Fehling's Test:** Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.
- **Detection of glycosides:** Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.
  - ❖ **Modified Borntrager's Test:** Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with

ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

- ❖ **Legal's Test:** Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

- **Detection of saponins**
  - ❖ **Froth Test:** Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.
  - ❖ **Foam Test:** 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.
- **Detection of phytosterols**
  - ❖ **Salkowski's Test:** Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.
  - ❖ **Libermann Burchard's test:** Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.
- **Detection of phenols**
  - ❖ **Ferric Chloride Test:** Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.
- **Detection of tannins**
  - ❖ **Gelatin Test:** To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.
- **Detection of flavonoids**
  - ❖ **Alkaline Reagent Test:** Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

- ❖ **Lead acetate Test:** Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

- **Detection of proteins and amino acids**
  - ❖ **Xanthoproteic Test:** The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.
  - ❖ **Ninhydrin Test:** To the extract, 0.25% w/v Ninhydrin reagent was added and boiled for few minutes. Blue colour indicates the presence of amino acid.

4.2.6 Quantitative Analysis by High Performance Thin Layer Chromatography

Aqueous extracts of three plants were standardised based on marker compound. From MTT assay, (Details were mentioned in Chapter 6 includes in-vitro studies of extract) it was concluded that methanolic extracts having lethality. Therefore, standardized only aqueous extracts of *Calotropis procera, Rosa indica, Adhatoda vasica.*

4.2.6.1 Method-1 Method development and validation for Gallic acid in *Calotropis procera* extract by HPTLC.

- **Chemicals**- Methanol, Standard Gallic acid. Gallic acid reference standard was purchased from Sigma–Aldrich GmbH, Germany. All other solvents and chemicals were of the highest analytical grade.
- **Apparatus**

  CAMAG Linomat 5 - Applicator, 100 mL syringe (Hamilton, Bonaduz, Switzerland), glass twin trough chamber (20 cm × 10 cm × 4 cm) (CAMAG), TLC Scanner 3 linked to Win Cats software (CAMAG), 0.2 mm thickness pre-coated with silica gel 60 F_{254} (Merck) were used in this study. The experiment was carried out under the conditions with temperature of (25 ± 2) ^{0}C and relative humidity of 40%.

- **Preparation of Standard solution of Gallic acid**

  Weigh accurately 2 mg of Standard Gallic acid in 2 ml volumetric flask. To it add 1 ml of Methanol and sonicate till the standard gets dissolved completely. Then, make up the volume up to 2 ml with methanol. Use the standard solution thus obtained for HPTLC fingerprinting.

- **Preparation of Solution of Extract**

Weigh accurately 1 g of extract of Calotropis procera in 250 ml reflux flask. To it add 10 ml of Methanol and reflux on water bath for 60 minutes. On completion of time, remove the reflux flask from the water bath and allow to cool. Filter with the help of Whatman filter paper no. 1 in a 10 ml volumetric flask. Then, make up the volume up to 10 ml with Methanol. Use the test solution thus obtained for HPTLC fingerprinting.

Table 4.1 Chromatographic conditions for quantification of Gallic acid

Chromatographic Conditions:	
Application Mode	CAMAG Linomat 5 - Applicator
Filtering System	Whatman filter paper No. 1
Stationary Phase	MERCK - TLC / HPTLC Silica gel 60 F254 on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from plate base
Standard Application Volume	10.0 µL
Sample Application Volume	10.0 µL (Extract)
Distance Between Tracks	13.3 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Time	30 minutes
Mobile Phase (MP)	Toluene: Ethyl acetate: Formic acid (10: 7: 1 v/v)
Visualization	@ 254 nm
Quantification	@ 278 nm
Drying Mode, Temp. & Time	TLC Plate Heater Preheated at 100 ± 5^0C for 3 minutes

❖ **Validation of the developed method for Gallic acid**

Developed Method is validated according to the ICH Guidelines and data complying with the standards were obtained. All validation parameters were check for the method. Validation parameter includes Accuracy, Precision, Specificity, Linearity, LOD, LOQ, and Robustness.

a) Linearity and range

The linearity of Rutin was evaluated by analyzing a series of different concentrations of Rutin. In present study, five different concentrations of Rutin were selected within the linearity range, and each was repeated three times. A linear relationship was found between the peak area and the concentration of the Gallic acid in the range 20-100 µg/ml. The characteristics such as linearity range and regression equation (slope, intercept and correlation coefficient) were determined for the method.

b) Detection and Quantitation Limits

The LOD and LOQ was performed through linear regression.

c) Precision

The precision is determined by replicate analysis. For intraday precision, Gallic acid is analyzed at three-time intervals in a single day for three different concentrations. For interday precision, each Gallic acid is analysed at three consecutive days for five different concentrations. The variation in the data is represented as %RSD.

d) Accuracy

The accuracy of the method was determined by the Standard Addition Method. Known amount of Markers were added to the sample at three different levels (80, 100 and 120 %) and the mixture was analysed. Percent Recovery was calculated as the mean of three determinations at each standard addition level.

e) Robustness

In this parameter the method is explored under different conditions like changes in the saturation time and wavelength for quantification.

f) Specificity

As per the ICH guideline for specificity, the method is specific when the results are unaffected by presence of other constituents in the extract.

4.2.6.2 Method-2 Method development and validation for Rutin in *Rosa indica* extract by HPTLC

- **Preparation of Standard Solution:** Weigh accurately 2 mg of Standard Rutin in 1 mL volumetric flask. To it add 1 mL of Methanol and sonicate till the standard gets dissolved completely. Then, make up the volume up to 1 mL with Methanol. Use the standard solution thus obtained for HPTLC fingerprinting.

- **Preparation of Test Solution (Extract):** Weigh accurately 1 g of sample in a 250 mL reflux flask. To it add 10 mL of Methanol and reflux on water bath for 60 minutes. On completion of time, remove the reflux flask from the water bath and allow to cool. Filter with the help of Whatman filter paper no. 1 in a 10 mL volumetric flask. Then, make up the volume up to 10 mL with Methanol. Use the Test Solution thus obtained for HPTLC fingerprinting.

Table 4.2 Chromatographic conditions for quantification of Rutin

Chromatographic Conditions:	
Application Mode	CAMAG Linomat 5 - Applicator
Filtering System	Whatman filter paper No. 1
Stationary Phase	MERCK - TLC / HPTLC Silica gel 60 F254 on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from plate base
Standard Application Volume	10.0 µL
Sample Application Volume	10.0 µL
Distance Between Tracks	13.3 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Time	30 minutes
Mobile Phase (MP)	Toluene: Ethyl acetate: Formic acid: Methanol
Visualization	@ 254 nm
Quantification	@ 257 nm
Drying Mode, Temp. & Time	TLC Plate Heater Preheated at 100 ± 5^0C for 3 minutes

❖ **Validation of the developed method for Rutin -**Developed Method is validated according to the ICH Guidelines and data complying with the standards were obtained. All validation parameters were check for the method. Validation parameter includes Accuracy, Precision, Specificity, Selectivity, Linearity, LOD, LOQ, and Robustness.

a) Linearity and range

The linearity of Rutin was evaluated by analyzing a series of different concentrations of Rutin. In present study, five different concentrations of Rutin were selected within the linearity range, and each was repeated three times. A linear relationship was found between the peak area and the concentration of the Rutin in the range 20-100 µg/ml. The characteristics such as linearity range and regression equation (slope, intercept and correlation coefficient) were determined for the method.

b) Detection and Quantitation Limits

The LOD and LOQ was performed through linear regression.

c) Precision

The precision is determined by replicate analysis. For intraday precision, Rutin is analyzed at three-time intervals in a single day for three different concentrations. For interday precision, Rutin is analysed at three consecutive days for three different concentrations. The variation in the data is represented as %RSD.

d) Accuracy

The accuracy of the method was determined by the Standard Addition Method. Known amount of Markers were added to the sample at three different levels (80, 100 and 120 %) and the mixture was analysed. Percent Recovery was calculated as the mean of three determinations at each standard addition level.

e) Robustness

In this parameter the method is explored under different conditions like changes in the saturation time and wavelength for quantification.

f) Specificity

As per the ICH guideline for specificity, the method is specific when the results are unaffected by presence of other constituents in the extract.

4.2.6.3 Method-3 Method development and validation for in *Adhatoda vasica* extract by HPTLC

- **Preparation of Standard Solution:** Weigh accurately 1 mg of Standard Vasicine in 1 mL volumetric flask. To it add 0.5 mL of Methanol and sonicate till the standard gets dissolved completely. Then, make up the volume up to 1 mL with Methanol. Use the standard solution thus obtained for HPTLC fingerprinting.

- **Preparation of Test Solution (Extract):** Weigh accurately 1 g of sample in a 250 mL reflux flask. To it add 10 mL of Methanol and reflux on water bath for 60 minutes. On completion of time, remove the reflux flask from the water bath and allow to cool. Filter with the help of Whatman filter paper no. 1 in a 10 mL volumetric flask. Then, make up the volume up to 10 mL with Methanol. Use the Test Solution thus obtained for HPTLC fingerprinting.

Table 4.3 Chromatographic conditions for quantification of Vasicine

Chromatographic Conditions:	
Application Mode	CAMAG Linomat 5 - Applicator
Filtering System	Whatman filter paper No. 1
Stationary Phase	MERCK - TLC / HPTLC Silica gel 60 F254 on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from plate base
Standard Application Volume	10.0 µL
Sample Application Volume	10.0 µL
Distance Between Tracks	13.3 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Time	30 minutes
Mobile Phase (MP)	Dioxane: Toluene: Methanol: Ammonia (5: 2 : 2 : 1 v/v)
Visualization	@ 254 nm
Quantification	@ 254 nm
Drying Mode, Temp. & Time	TLC Plate Heater Preheated at 100 ± 5^0C for 3 minutes

❖ **Validation of the developed method for Vasicine**

Developed Method is validated according to the ICH Guidelines and data complying with the standards were obtained. All validation parameters were check for the method. Validation parameter includes Accuracy, Precision, Specificity, Linearity, LOD, LOQ, and Robustness.

a) Linearity and range

The linearity of Vasicine was evaluated by analyzing a series of different concentrations of Rutin. In present study, five different concentrations of Rutin were selected within the linearity range, and each was repeated three times. A linear relationship was found between the peak area and the concentration of the Rutin in the range 100-500 µg/ml. The characteristics such as linearity range and regression equation (slope, intercept and correlation coefficient) were determined for the method.

b) Detection and Quantitation Limits

The LOD and LOQ was performed through linear regression.

c) Precision

The precision is determined by replicate analysis. For intraday precision, Vasicine is analysed at three-time intervals in a single day for three different concentrations. For interday precision, Vasicine is analysed at three consecutive days for three different concentrations. The variation in the data is represented as %RSD.

d) Accuracy

The accuracy of the method was determined by the Standard Addition Method. Known amount of Markers were added to the sample at three different levels (80, 100 and 120 %) and the mixture was analysed. Percent Recovery was calculated as the mean of three determinations at each standard addition level.

e) Robustness

In this parameter the method is explored under different conditions like changes in the saturation time and wavelength for quantification.

f) Specificity

As per the ICH guideline for specificity, the method is specific when the results are unaffected by presence of other constituents in the extract.

4.3 Results and Discussion

4.3.1 Results of Standardization of plant powder

The physico-chemical parameters like Total ash value,Acid insoluble ash and Water-soluble ash, were observed as shown in Table. 4.4

Table 4.4 Standardization of the powdered materials

Name of Extract	**Total ash (%)**	**Water-soluble ash (%)**	**Acid insoluble ash (%)**
***Rosa indica* extract**	8.34	5.44	2.21
***Calotropis procera* extract**	19.51	3.92	2.52
***Adhatoda vasica* extract**	12.53	4.41	1.52

The inorganic content of the plant materials was determined by the ash value. The acid insoluble ash is the total soil or siliceous matter present in the plants. Thus, ash value of the plant material determines the raw material quality of the plant materials. The results showed that the ash values are within the limits.

Results of % Yield of Extracts

Name of Extract	**Extraction yield (%)**
Aqueous extract of *Rosa indica*	5.3 %
Methanolic extract of *Rosa indica*	4.7%
Aqueous extract of *Adhatoda vasica*	20.2%
Methanolic extract of *Adhatoda vasica*	17.4%
Aqueous extract of *Calotropis procera*	16 %
Methanolic extract of *Calotropis procera*	14.6 %

Each value is average of three determinations

4.3.2 Results of Qualitative Phytochemical Screening of extracts

Preliminary phytochemical screening was performed to find out the phytoconstituent present in the extracts.

Table 4.5 Phytochemical screening results of extracts

	AVM	**AVA**	**RIM**	**RIA**	**CPM**	**CPA**
Alkaloids	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**
Saponin	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**

Carbohydrates	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
Phenolic glycoside	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
Tannins	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
Proteins	**-ve**	**-ve**	**-ve**	**-ve**	**+ve**	**+ve**
Flavonoids	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
Volatile Oil	**-ve**	**-ve**	**+ve**	**+ve**	**+ve**	**-ve**
Fixed Oil	**-ve**	**-ve**	**+ve**	**+ve**	**+ve**	**-ve**

- AVM- *Adhatoda vasica* Methanolic extract
- AVA- *Adhatoda vasica* Aqueous extract
- RIM- *Rosa indica* Methanolic extract
- RIA- *Rosa indica* Aqueous extract
- CPM- *Calotropis procera* Methanolic extract
- CPA- *Calotropis procera* Aqueous extract

The Physico-chemical parameters evaluation showed presence of saponins, alkaloids, flavonoids glycosides and other chemical classes in both aqueous and alcoholic extracts of all three plants. (Table 4.2)

4.3.3 Results of Quantitative analysis by HPTLC method

4.3.3.1 Quantification of Gallic acid in *Calotropis procera* extract

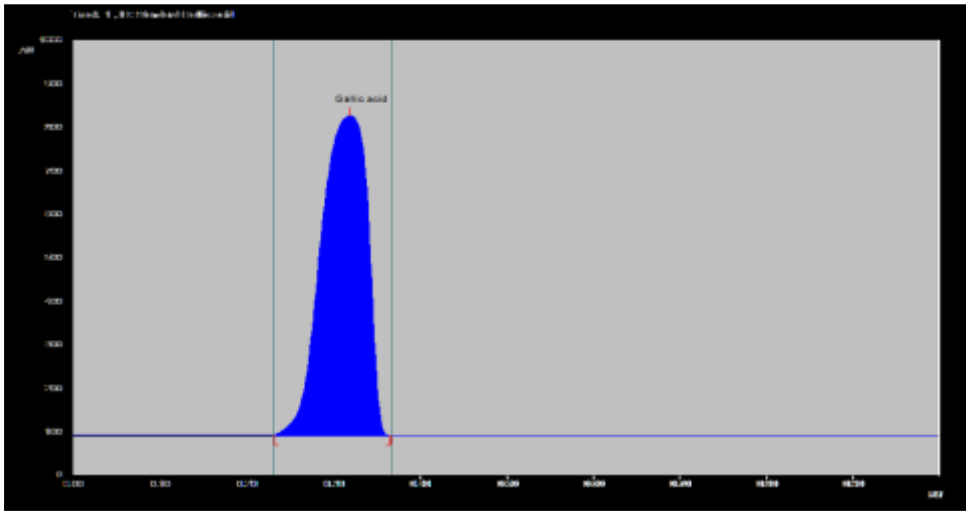

Figure 4.12 2D Chromatogram of Standard Gallic acid

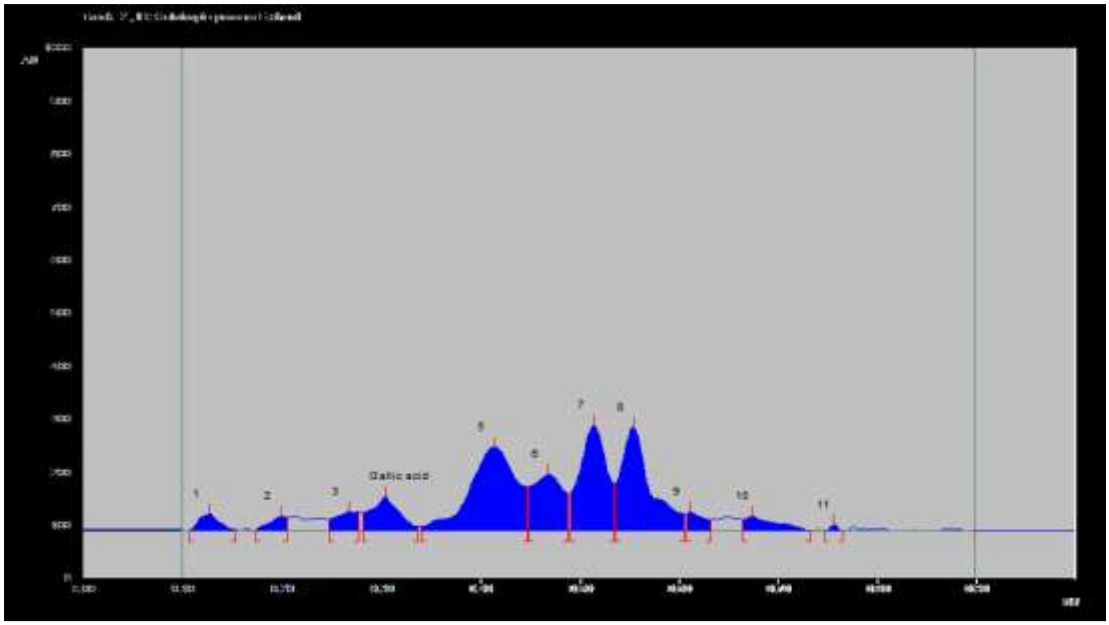

Figure 4.13 2D Chromatogram of *Calotropis procera* Extract

A spot with an R_f value of 0.31 was obtained for standard Gallic acid in the HPTLC chromatogram (Figure 4.12) and Gallic acid in the aqueous extract of *Calotropis procera*. (Fig. 4.13). This indicates that the Gallic acid was well separated from other components in the extract, which did not interfere with its detection. The %w/w of Gallic acid was determined to be 0.077 %, calculated using the peak area.

The method developed for the analysis of Gallic acid proved to be highly effective and reliable. The precision and accuracy of this HPTLC method make it suitable for routine analysis of Gallic acid in plant extracts. The clear separation of Gallic acid from other extract components demonstrates the method's specificity, ensuring that the quantification of Gallic acid is not affected by other substances present in the extract.

Overall, the HPTLC method's good performance highlights its potential for regular use in quality control and standardization of herbal formulations containing Gallic acid.

❖ **Validation of developed HPTLC Method for Gallic acid [19]**

a) Linearity and Range

The linearity of proposed method was evaluated by analyzing series of five different concentrations of marker. The standard solutions were analyzed in triplicate for the establishment of calibration curve. The calibration curve was plotted by using the value of peak area v/s concentration of compound. (Figure 4.14)

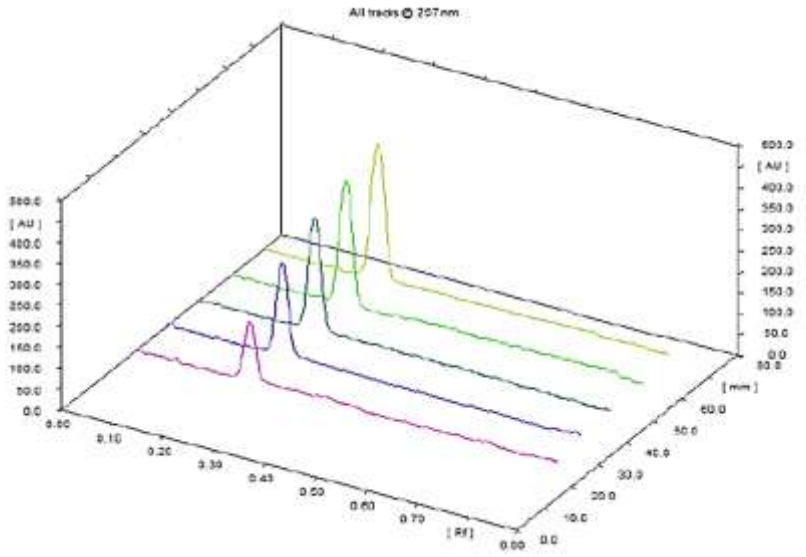

Figure 4.14 HPTLC 3D Graph for linearity of Gallic acid

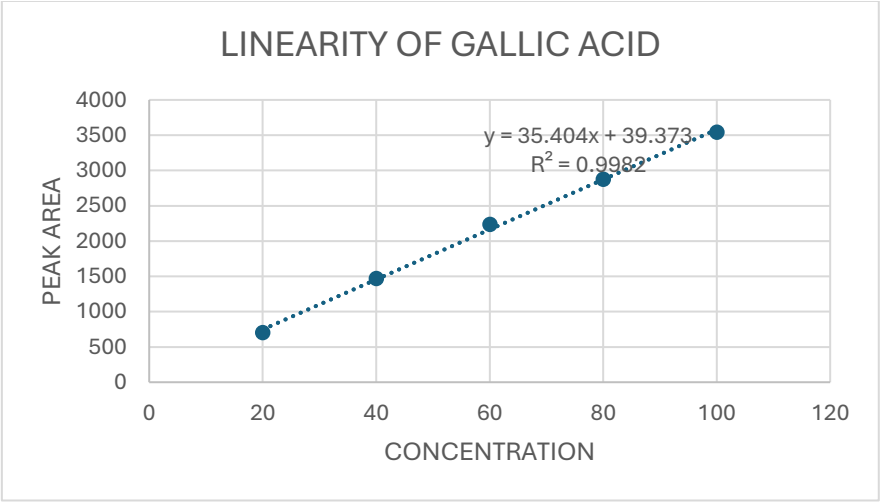

Figure 4.15 Calibration curve for Linearity of Gallic acid

Table 4.6 Linearity and Range for Gallic acid

Marker Gallic acid (20-100 µg/mL)		
Conc (µg/mL)	Avg peak area ± SD (n=6)	RSD
20	702.02±11.55	1.64
40	1467.858±25.53	1.73
60	2235.406±38.91	1.74
80	2872.46± 21.11	0.73
100	3539.896±24.93	0.70

The linearity and range for Gallic acid were found in the 20-100 µg/mL range with the R^2 of 0.9982, indicating that the method is linear in the above-specified range. Table 4.6 demonstrates the linearity Gallic acid. Figure 4.15. depict the calibration plot for Gallic acid.

b) LOD and LOQ

Table 4.7 LOD and LOQ of Gallic acid

Marker	**R_f Value**	**Regression equation**	**R^2**	**Linear range (µg/ml)**	**LOD (µg/ml)**	**LOQ(µg/ml)**
Gallic acid	0.31	y = 35.404x + 39.373	0.998	20-100	5.094	9.262

c) Precision data:

Intra-day and inter-day precision were performed using five concentrations in order to evaluate intermediate precision.

The intraday test was determined by injection of the same standard solutions thrice a day. And interday was determined by analysing same standards thrice each day for 3 days.

Precision was expressed as relative standard deviation (RSD). Generally, the values of RSD within 2% are acceptable. The results of precision test for each analyte are summarized below.

Table 4.8 Interday and Intraday precision data of Gallic acid

Marker	Conc. (µg/ml)	**Intraday**(n=3)			**Interday**(n=3)		
		Area	**SD**	**% RSD**	**Area**	**SD**	**% RSD**
Gallic acid	20	685.877	8.321	1.213	672.820	6.569	0.976
	40	1421.087	12.001	0.844	1475.420	18.160	1.231
	60	2250.773	18.262	0.811	2181.533	14.472	0.663
	80	2857.377	15.272	0.534	2823.377	19.129	0.678
	100	3527.047	23.823	0.675	3546.823	24.883	0.702

d) Accuracy

Table 4.9 Accuracy data of HPTLC method validation for Gallic acid

Level	**Amount of drug from sample (mg)**	**Amount of drug spiked (mg)**	**Total amount of drug (mg)**	**Amount of spiked drug recovered (mg)**	**Mean % recovery**	**SD**	**%RSD**
80 %	40	32	72	71.05	98.68	0.51	0.52
100%	40	40	80	78.42	99.20	0.58	0.59
120%	40	48	88	86.49	98.28	1.43	1.45

e) Robustness

Table 4.10 Robustness data for HPTLC method of Gallic acid

Factor	**Gallic acid (40 µg/ml)**		
	Mean Area	SD	%RSD
Saturation time			
30 min	1490	20	1.344
60 min	1450.66	28.746	1.981
Wavelength			
257nm	1529	24.758	1.619
280nm	1494.66	13.051	0.873

f) Specificity

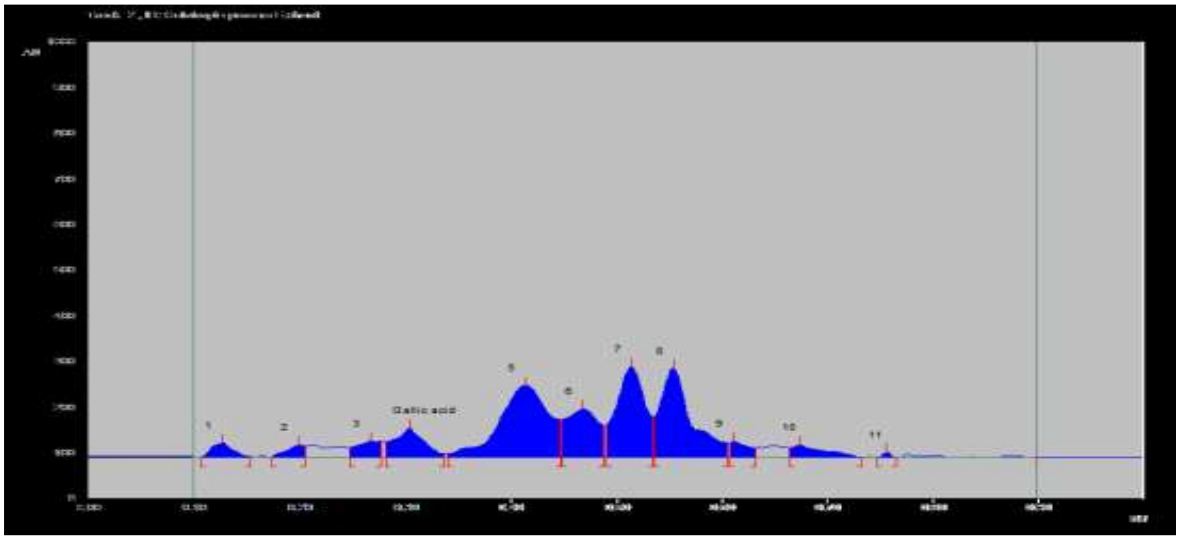

Figure 4.16 2D graph of Standard Gallic acid for Specificity

4.3.3.2 Quantification of Rutin in *Rosa indica* extract

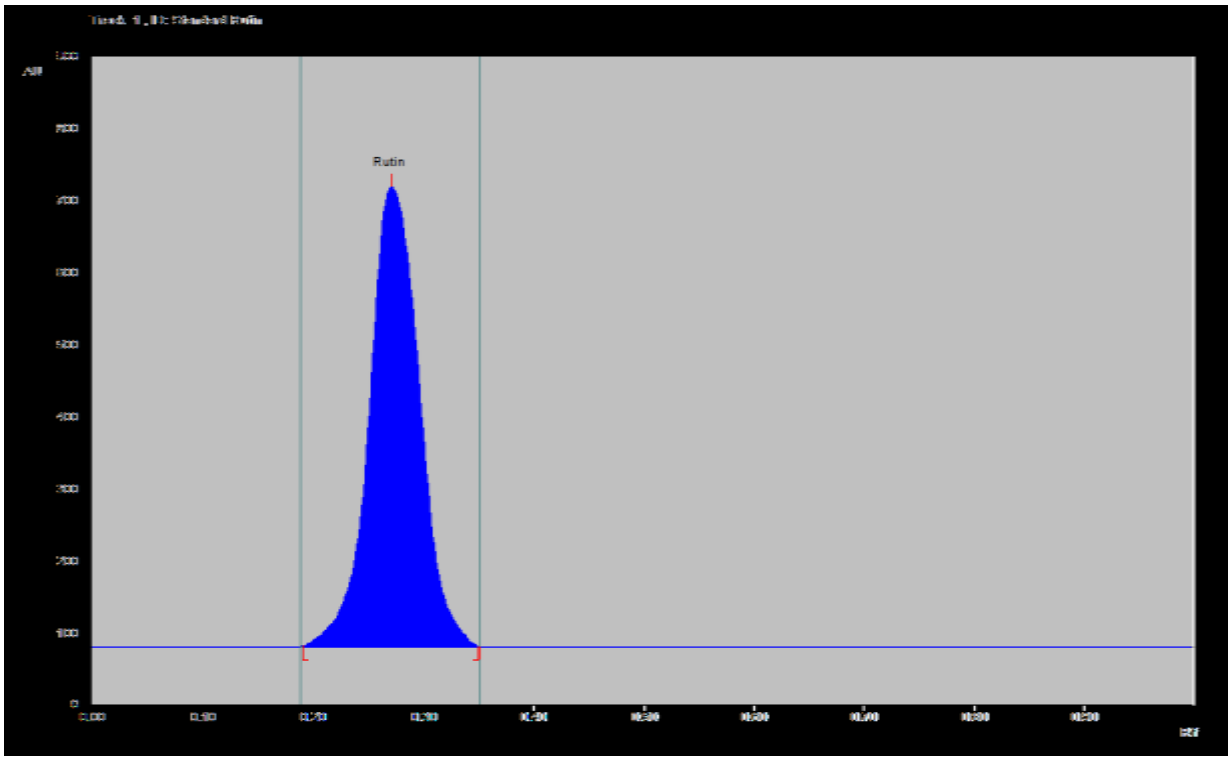

Figure 4.17 2D Chromatogram of Rutin Standard

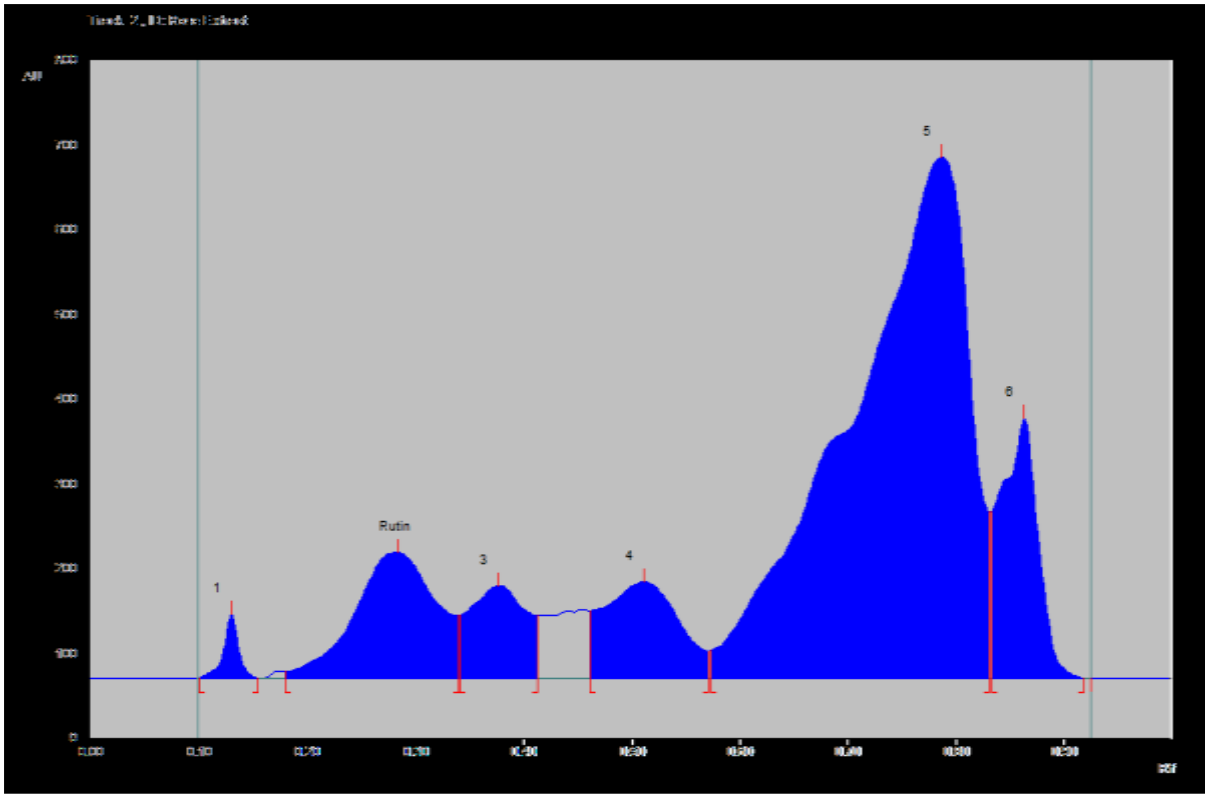

Figure 4.18 2D Chromatogram of *Rosa indica* extract

A spot with an R_f value of 0.25 was obtained for standard Rutin in the HPTLC chromatogram (Figure 4.17) and Rutin in the aqueous extract of *Rosa indica*. (Fig. 4.18). This indicates that the Rutin was well separated from other components in the extract, which did not interfere with its detection. The %w/w of vasicine was determined to be 0.319 %, calculated using the peak area.

❖ **Validation of developed HPTLC Method for Rutin:**

a) Linearity and Range

The linearity of proposed method was evaluated by analyzing series of five different concentrations of marker. The standard solutions were analyzed in triplicate for the establishment of calibration curve. The calibration curve was plotted by using the value of peak area v/s concentration of compound.

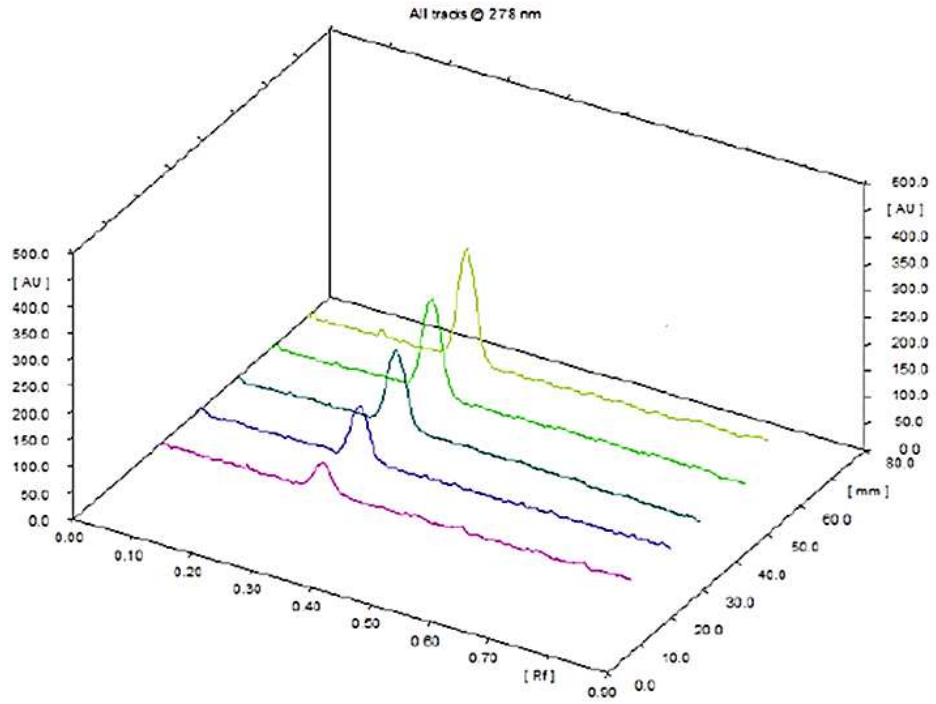

Figure 4.19 HPTLC 3D Graph for linearity of Rutin

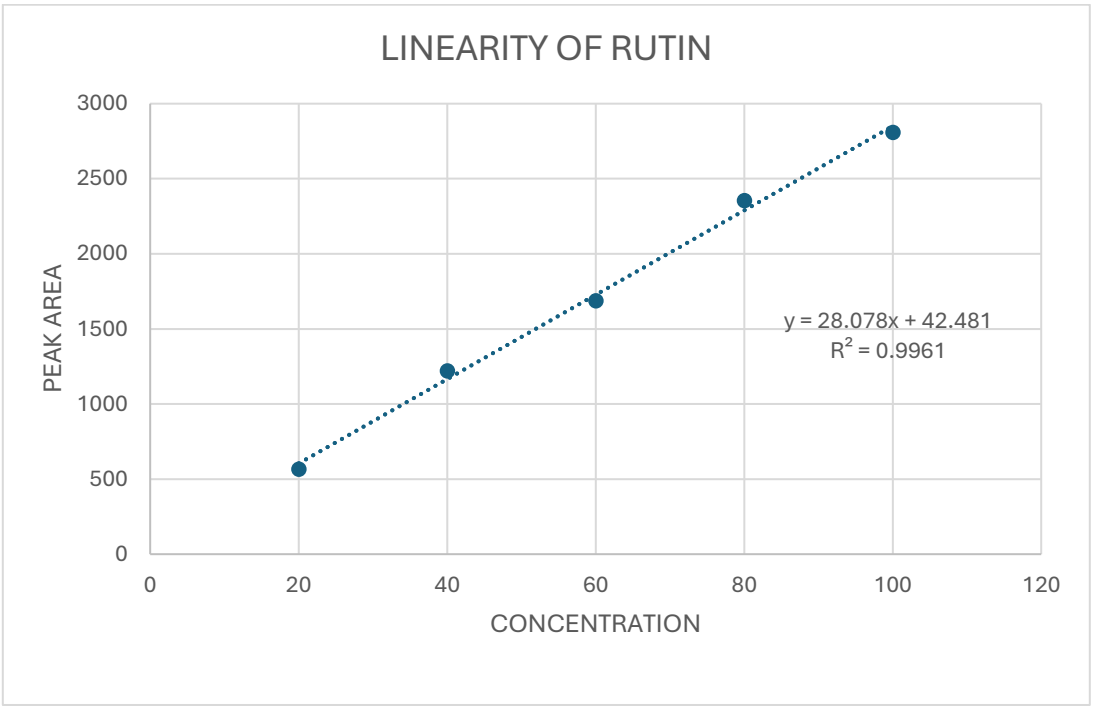

Figure 4.20 Calibration curve for the linearity of Rutin

Table 4.11 Linearity and Range for Rutin

Marker Rutin (20-100 µg/mL)		
Conc (µg/mL)	Avg peak area ± SD (n=6)	RSD
20	566.14 ±9.702	1.71
40	1220.764± 13.130	1.07
60	1687.504±29.952	1.77
80	2354.286± 33.987	1.44
100	2807.214±34.23082	1.21

The linearity and range for Rutin were found in the 20-100 µg/mL range with the R^2 of 0.9961, indicating that the method is linear in the above-specified range. Table 4.11 demonstrates the linearity Rutin. Figure 4.20. depict the calibration plot for Rutin.

b) LOD and LOQ

Table 4.12 LOD and LOQ for Rutin

Marker	**R_f Value**	**Regression equation**	**R^2**	**Linear range (µg/ml)**	**LOD (µg/ml)**	**LOQ(µg/ml)**
Rutin	0.25	y = 28.078x + 42.481	0.996	20-100	7.496	13.63

c) Precision data:

Intra-day and inter-day precision were performed using standard solutions at 5 concentrations in order to evaluate intermediate precision.

The intraday test was determined by injection of the same standard solutions thrice a day. And interday was determined by analysing same standards thrice each day for 3 days.

Precision was expressed as relative standard deviation (RSD). Generally, the values of RSD within 2% are acceptable. The results of precision test for each analyte are summarized below.

Table 4.13 Interday and Intraday precision data of Rutin

Marker	Conc. (µg/ml)	Intraday (n=3)			Interday (n=3)		
		Area	SD	% RSD	Area	SD	% RSD
Rutin	20	586.267	7.921	1.351	575.300	10.512	1.827
	40	1233.233	8.861	0.718	1231.470	14.366	1.167
	60	1752.167	18.566	1.060	1723.627	28.595	1.659
	80	2362.867	37.454	1.585	2365.737	39.465	1.668
	100	2803.233	42.687	1.523	2825.983	44.783	1.585

d) Accuracy

Table 4.14 Accuracy data HPTLC method of Rutin

Level	Amount of drug from sample (mg)	Amount of drug spiked (mg)	Total amount of drug (mg)	Amount of spiked drug recovered (mg) ± SD	Mean % recovery	SD	%RSD
80 %	40	32	72	71.56	99.38	0.58	0.58
100%	40	40	80	79.07	98.84	0.54	0.54
120%	40	48	88	87.31	99.22	0.89	0.90

e) Robustness

Table 4.15 Robustness data for HPTLC method of Rutin

Factor	Rutin		
	Area	SD	%RSD
Saturation time			
30 min	1370.667	22.898	1.670
60 min	1434.667	25.006	1.743
Wavelength			
278 nm	1562.333	10.785	0.690
300 nm	1501.333	19.756	1.315

f) Specificity

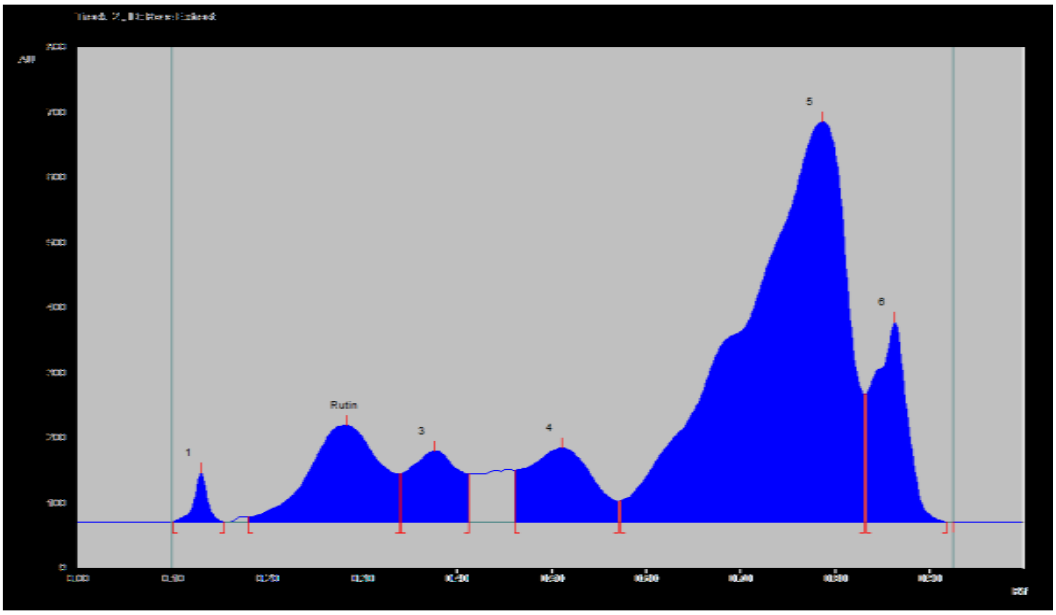

Figure 4.21 2D graph of Standard Rutin for Specificity

4.3.3.3 Quantification of Vasicine in *Adhatoda vasica* extract

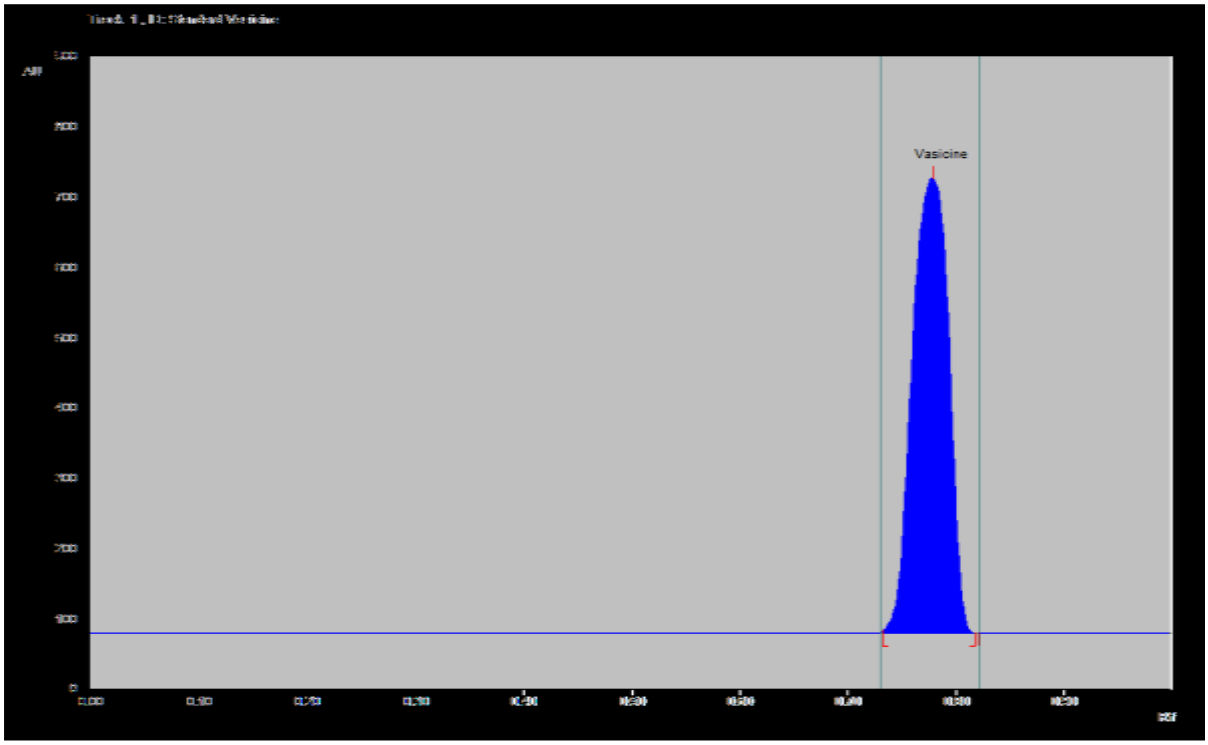

Figure 4.22 2D Chromatogram of Vasicine Standard

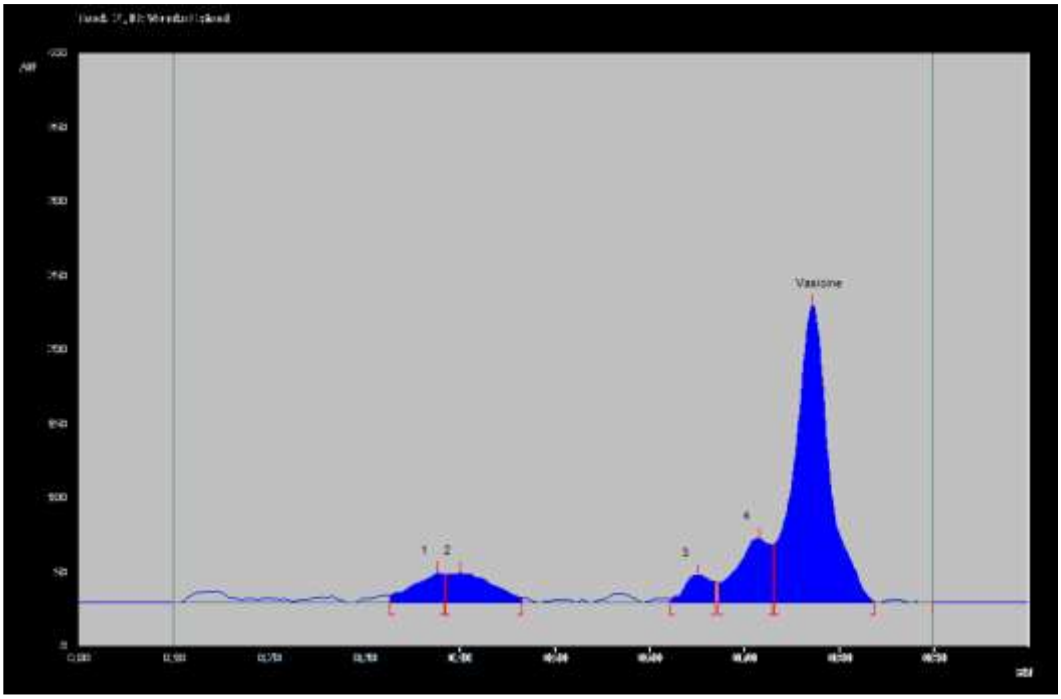

Figure 4.23 2D Chromatogram of Vasicine in *Adhatoda vasica* extract

❖ **Validation of developed HPTLC Method for Vasicine:**

a) Linearity and Range

The linearity of proposed method was evaluated by analyzing series of five different concentrations of marker. The standard solutions were analyzed in triplicate for the establishment of calibration curve. The calibration curve was plotted by using the value of peak area v/s concentration of compound.

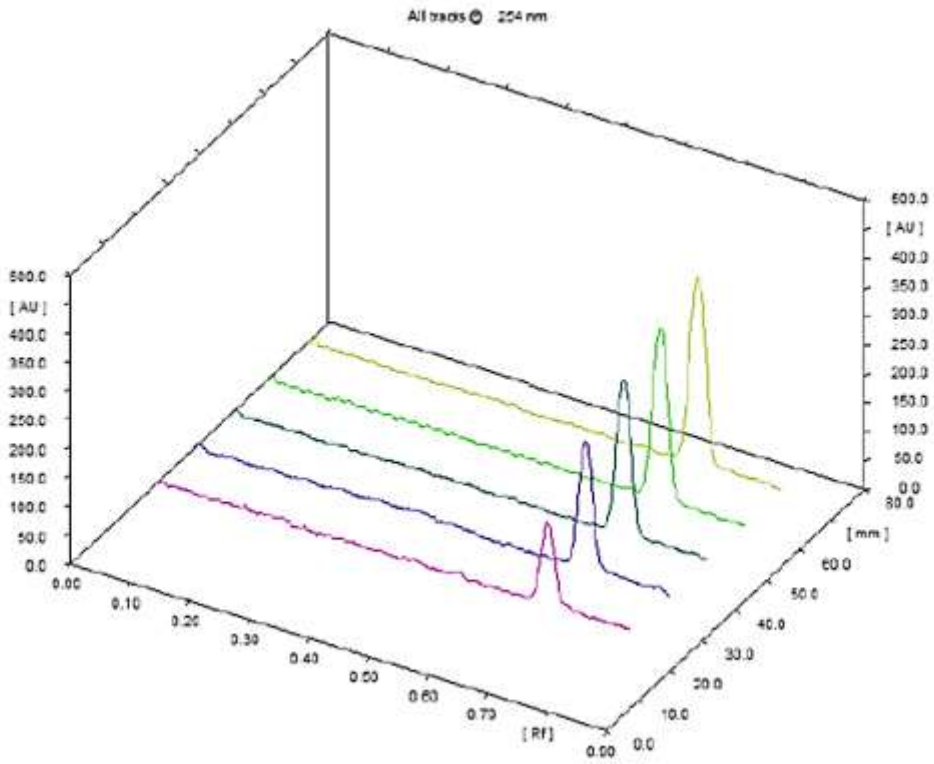

Figure 4.24 HPTLC 3D Graph for linearity of Vasicine

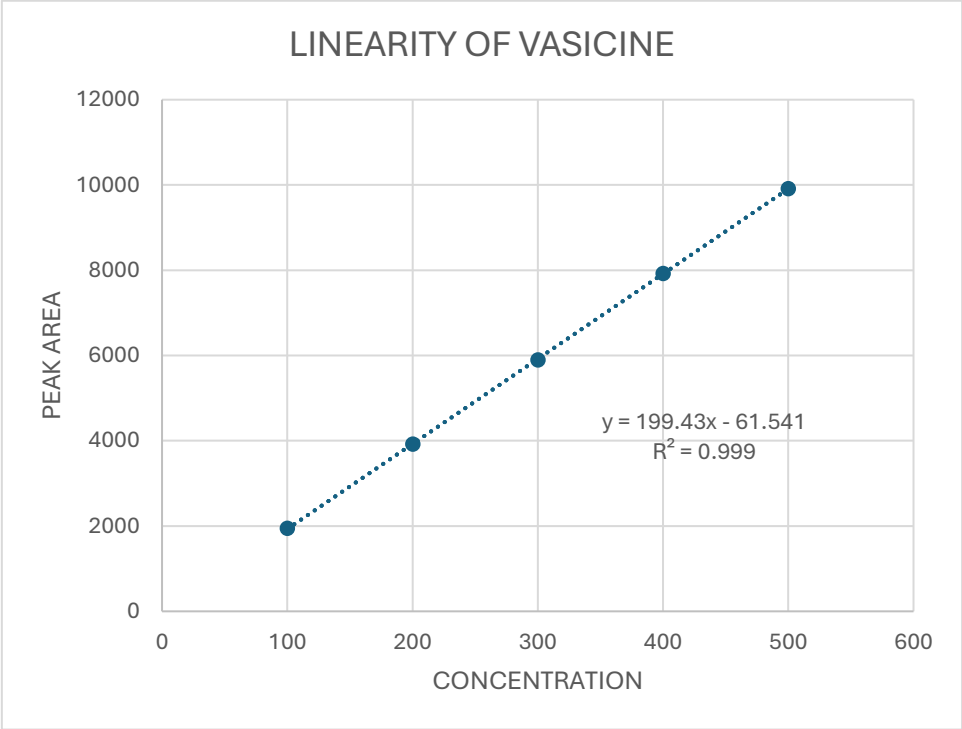

Figure 4.25 Calibration curve for the linearity of Vasicine

Table 4.16 Linearity and Range for Vasicine

Marker Vasicine (100-500 µg/mL)		
Conc (µg/mL)	Avg peak area ± SD (n=6)	RSD
100	1946.52±24.55	1.26
200	3919.98± 41.160	1.05
300	5893.936± 63.470	1.07
400	7924.24 ± 55.69	0.70
500	9915.78± 45.57	0.45

The linearity and range for Vasicine were found in the 100-500 µg/mL range with the R^2 of 0.999, indicating that the method is linear in the above-specified range. Table 4.16 demonstrates the linearity Rutin. Figure 4.25. depict the calibration plot for Vasicine.

b) LOD and LOQ

Table 4.17 LOD and LOQ for Vasicine

Marker	**R_f Value**	**Regression equation**	**R^2**	**Linear range (µg/ml)**	**LOD (µg/ml)**	**LOQ (µg/ml)**
Vasicine	0.78	y = 199.43x - 61.541	0.999	100-500	4.332	13.130

c) Precision

Table 4.18 Interday and Intraday precision data for Vasicine

Marker	**Conc. (µg/ml)**	**Intraday**(n=3)			**Interday**(n=3)		
		Area	**SD**	**% RSD**	**Area**	**SD**	**% RSD**
Vasicine	100	1961.600	19.010	0.969	1956.833	28.446	1.454
	200	3943.467	28.834	0.731	3959.403	22.318	0.564
	300	5920.033	36.742	0.621	5904.093	19.979	0.338
	400	7943.733	32.589	0.410	7860.337	26.752	0.340
	500	9908.600	71.033	0.717	9888.517	48.341	0.489

d) Accuracy

Table 4.19 Accuracy data for HPTLC method for Vasicine

Level	Amount of drug from sample (mg)	Amount of drug spiked (mg)	Total amount of drug (mg)	Amount of spiked drug recovered (mg)	Mean % recovery	SD	%RSD
80 %	200	160	360	356.68	99.08	0.89	0.90
100%	200	200	400	398.13	99.53	0.73	0.73
120%	200	240	440	435.42	98.96	0.25	0.25

e) Robustness

Table 4.20 Robustness data for HPTLC method of Vasicine

Factor	Vasicine		
	Mean Area	SD	%RSD
Saturation time			
30 min	3754.286	33.987	1.443
60 min	3835.406	38.917	1.740
Wavelength			
254 nm	3831.621	10.654	0.477
280 nm	3933.233	8.863	0.391

f) Specificity

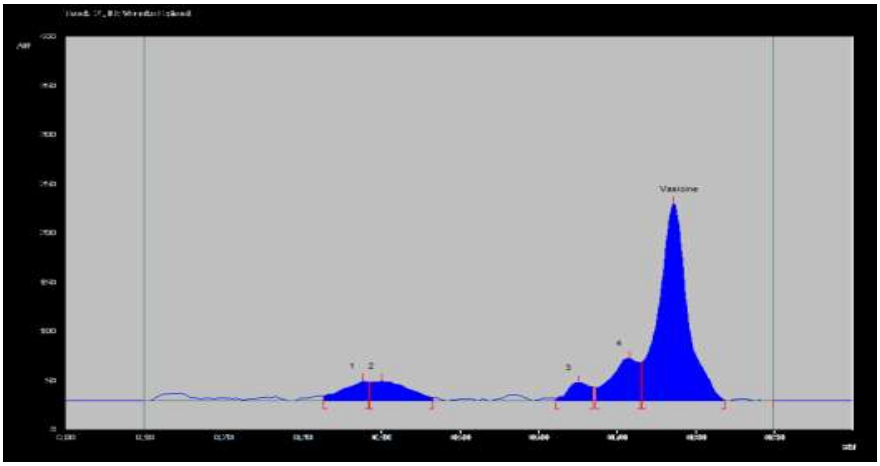

Figure 4.26 2D graph of Standard Vasicine for Specificity

Summary

Table 4.21 Summary of all validation parameters of three standards

Parameters	Gallic acid	Rutin	Vasicine
R_f value	0.31	0.25	0.78
Detection wavelength	278 nm	257 nm	254 nm
Range(µg/ml)	20-100	20-100	100-500
Linearity (R^2Correlation coefficient)	0.998	0.996	0.999
Regression equation	y= 35.404x +39.373	y = 28.078x + 42.481	y = 199.43x - 61.541
LOD (µg/ml)	5.094	7.496	4.332
LOQ(µg/ml)	9.262	13.630	13.130
Precision	0.663-1.231	1.167-1.827	0.338-1.45
Accuracy	< 2% RSD	< 2% RSD	< 2% RSD
Robustness	Robust	Robust	Robust
Specificity	Specific	Specific	Specific

4.4 Conclusion

The plant materials were studied as per the WHO guidelines for the determination of different ash values. The plant materials were extracted in methanol and distilled water by Soxhlet apparatus. These extracts were then subjected to preliminary phytochemical analysis using chemical tests which revealed the presence of alkaloids, saponins, carbohydrates, glycosides, proteins and amino acids, phytosterols, flavonoid and phenolics in aqueous and methanolic extracts of *Calotropis procera, Adhatoda vasaka* and *Rosa indica*.

The chemical marker Gallic acid, Rutin and Vasicine were used for the standardization of leaves of *Calotropis procera*, flowers of *Rosa indica* and leaves of *Adhatoda vasica*. The HPTLC methods (Method I, II and III) provide accurate and reproducible quantitative analysis for determination of Gallic acid, Rutin and Vasicine. A spot with an R_f value of 0.78 was obtained for standard vasicine in the HPTLC chromatogram and Vasicine in the aqueous extract of *Adhatoda vasica*. This indicates that the vasicine was well separated from other components in the extract, which did not interfere with its detection. The %w/w of vasicine was determined to be 0.716%, calculated using the peak area. A spot with an R_f value of 0.31 was obtained for standard Gallic acid in the HPTLC chromatogram and Gallic acid in the aqueous extract of *Calotropis procera*. This indicates that the Gallic acid was well separated from other components in the extract, which did not interfere with its detection. The %w/w of Gallic acid was determined to be 0.077 %, calculated using the peak area. A spot with an R_f value of 0.25 was obtained for standard Rutin in the HPTLC chromatogram and Rutin in the aqueous extract of *Rosa indica*. This indicates that the Rutin was well separated from other components in the extract, which did not interfere with its detection. The %w/w of vasicine was determined to be 0.319 %, calculated using the peak area.

4.5 References

1. Quality control of herbal drugs. 4th ed. Business Horizons, New Delhi, P. 184-219.
2. Ansari S.H. 2006. Essentials of Pharmacognosy. 1st ed. Birla Publications, New Delhi, p. 581- 596
3. Das K, Tiwari RKS, Shrivastava DK. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. Journal of Medicinal Plants Research 2010; 4(2): 104-111.
4. Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African Journal of Biotechnology 2008; 7 (12): 1797-1806.
5. Remington JP. Remington: The science and practice of pharmacy, 21st edition, Lippincott Williams & Wilkins, 773-774.
6. Handa SS, Khanuja SPS, Longo G, Rakesh DD. Extraction Technologies for Medicinal and Aromatic Plants. International centre for science and high technology, Trieste, 2008, 21-25.
7. Liang Wu, Haiping Hao and Guangji Wang, LC/MS Based Tools and Strategies on Qualitative and Quantitative Analysis of Herbal Components in Complex Matrixes, Current Drug Metabolism, 2012, 13, 1251-1265
8. D.A. Skoog; F. J. Holler, T.A. Nieman (1998). *Principles of Instrumental Analysis*, 5th edition. Orlando, FL: Harcourt Brace College Publishers.
9. R.W. Murray, Analytical Chemistry is what analytical chemists do, Editorial, Anal. Chem., 66 (1994) 682A.
10. J. Mendham, R.C. Denney, J.D. Barnes, M.J.K. Thomas, Vogel's Quantitative Chemical Analysis (6th Edition)
11. Harish Chandra Andola, Vijay Kant Purohit, Nature and Science, High Performance Thin Layer Chromatography (HPTLC): A Modern Analytical tool for Biological Analysis, 8(2010) 10.
12. MM. Srivastava (ed.), High-Performance Thin-Layer Chromatography (HPTLC),DOI 10.1007/978-3-642-14025-9_1, # Springer-Verlag Berlin Heidelberg 2011
13. Prashant Tiwari, Bimlesh Kumar, Mandeep Kaur, Gurpreet Kaur, Harleen Kaur, Phytochemical screening and Extraction: A Review, International Pharmaceutica Sciencia, 2011, V0l. 1, Issue 1, p 98-106.

14. Prabhu C, Subramanian G, Karthik A, Kini S, Rajan S, Udupa N**.** Determination of Telmisartan by HPTLC - A stability indicating assay. Journal of Planar Chromatography—Modern TLC. 2007;20(6):477–481.
15. Shethi PD. High Performance Thin Layer Chromatography-Quantitative analysis of Pharmaceutical Formulations. 1sted. New Delhi: CBS Publishers; 1996. pp 1-68.
16. ICH Harmonized Tripartite Guideline. Pharmaceutical Quality System Q10, International Conference on Harmonization, Geneva: Switzerland; 2005.
17. ICH Harmonized Tripartite Guideline. Quality Risk Management Q9, International Conference on Harmonization, Geneva: Switzerland; 2005.
18. ICH Harmonized Tripartite Guideline. Pharmaceutical Development Q8, International Conference on Harmonization, Geneva: Switzerland; 2005.
19. Shah, V. D.; Chauhan, B.; Satani, B., Development And Validation Of Hptlc Method For The Estimation Of Vasicine, Curcumin And Cucurbitacin B In An Ayurvedic Formulation: Panchtikta Ghrit, International Journal Of Pharmaceutical Research (09752366), 2018, Vol 10, Issue 2, P138.