A REVIEW: DETECTION OF BIOMARKERS IN POLYHERBAL FORMULATIONS BY DIFFERENT ANALYTICAL TECHNIQUES

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***Abstract*- Polyherbal Formulation is the use of more than one herb in a medicinal preparation. It contains biomarkers such as oleanolic acid, ursolic acid, mangiferin, gallic acid, quercetin, curcumin, piperine, 6-gingerol, berberine etc which enhances the therapeutic action and reduce the concentrations of single herbs, thereby reducing adverse events. The aim of this study was to examine biomarkers by different analytical techniques. This review focuses on the recent developments in analytical techniques for estimation of biomarkers in polyherbal formulations. This review will examine methods such as High-Performance Thin Layer Chromatography (HPTLC), Ultraviolet Visible Spectroscopy (UV), High Performance Liquid Chromatography (HPLC), Spectro fluorimetry, Ultra-Performance Liquid Chromatography (UPLC).**

***Keywords*- Analytical Techniques, Biomarkers, Polyherbal Formulation, HPTLC, HPLC, UV, UPLC, Spectro fluorimetry**

I. INTRODUCTION

Definition

- Polyherbal formulation (PHF) is the use of more than one herb in a medicinal preparation.
- The concept is found in Ayurvedic and other traditional medicinal systems where multiple herbs in a particular ratio may be used in the treatment of illness.[1]
- Polyherbal formulations enhance the therapeutic action and reduce the concentrations of single herbs, thereby reducing adverse events.[2]

Advantages

- Herbal medicines have long history of use and better patients' tolerance as well as acceptance.
- Medicinal plant has a renewable source.
- The cultivation and processing of medicinal herbs and herbal products is environmentally friendly.
- They do not provoke allergic reaction and do not have negative side effect. They are easily incorporated with skin and hair.
- With small quantity they are very effective as compared synthetic cosmetic.
- Extract of plant decreases the bulk property of cosmetics and gives appropriate pharmacological effects.
- Easily available and found in large variety and quantity.
- Easy to manufactures and chief in cost.[3]

General polyherbal formulation available in market

- Tablet
- Capsule
- Churna
- Syrup
- Vati etc.

INTRODUCTION TO BIOMARKER

Definition

- Biomarkers are therapeutically active compounds present in medicinal herbs.
- A biomarker is a measurable indicator of the severity or presence of some disease state. More generally a biomarker is anything that can be used as an indicator of a particular disease state or some other physiological state of an organism.
- It can be a substance that is introduced into an organism as a means to examine organ function or other aspects of health.
- Biomarkers help in early diagnosis, disease prevention, drug target identification, drug response etc.
- It may be used to see how well the body responds to a treatment for a disease or condition.[4]

Types of biomarkers

1. **Diagnostic biomarkers-** Used to confirm the presence of a disease or medical condition.
2. **Monitoring biomarkers-** Used to assess presence, status or extent of a disease or medical condition, used to evaluate the response to the intervention.
3. **Pharmacodynamic/Response biomarkers-** Used to evaluate the response to medical condition or clinical intervention.

4. **Predictive biomarkers-** Used to identify the probability of develop a clinical event (positive or negative) after the exposure to a medical product or environmental agent.
5. **Prognostic biomarkers-** Used to identify like hood of a clinical event, disease recurrence or progression in patients diagnosed with a disease or having a medical condition.
6. **Susceptibility/Risk biomarkers-** Used to measure the risk on an individual to develop a disease or medical condition in patients without the disease or medical condition.
7. **Safety biomarkers-** Used to predict toxic adverse events induced by drug, medical intervention or environmental agent exposure. [5]

Advantages of biomarker

1. Objective assessment.
2. Precision of measurement.
3. Reliable; validity can be established.
4. Less biased than questionnaires.
5. Disease mechanisms often studied.
6. Homogeneity of risk or disease.
7. Confirms absorption into human body.
8. Measures integrated exposure from all routes and all sources.
9. Very low-level exposures detectable.
10. Help to evaluate public health interventions.

Disadvantages of biomarker

1. Timing is critical.
2. Expensive (costs for analyses).
3. Storage (longevity of samples).
4. Laboratory errors.
5. Normal range difficult to establish.
6. Ethical responsibility.[6]

II. METHODS

METHODS USED FOR THE ANALYSIS OF BIOMARKER

- High Performance Thin Layer Chromatography (HPTLC)
- Ultraviolet Visible Spectroscopy (UV)
- High Performance Liquid Chromatography (HPLC)
- Spectro fluorimetry
- Ultra-Performance Liquid Chromatography (UPLC)

High Performance Thin Layer Chromatography (HPTLC)

- HPTLC is the improved method of TLC which utilizes the conventional technique of TLC in more optimized way.
- It is also known as planar chromatography or Flat- bed chromatography.

Principle

- Chromatography is a physical process of separation in which the components to be separated are distributed between two immiscible i.e., the principle of separation is adsorption.
- The mobile phase solvent flows through because of capillary action. The components move according to their affinities towards the adsorbent.
- The component with more affinity towards the stationary phase travels slower. The component with lesser affinity towards the stationary phase travels faster.
- Thus, the components are separated on a chromatograph.[7]

Instrumentation

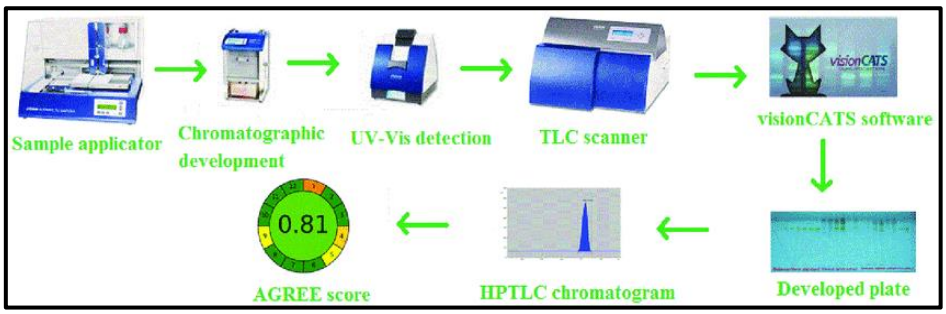

Fig. Schematic diagram of HPTLC Instrumentation [8]

Advantages

- More than one analyst works on the system simultaneously.
- HPTLC can be sharable, as it is not devoted to any sample.

- The pre-coated plates of HPTLC are available at low prices.
- There is less maintenance cost as compared to other equipment.
- HPTLC has a wide range of stationary phases.
- HPTLC has no risk of contamination, since the use of the freshly prepared mobile phase and stationary phase.
- Mobile phases are not required for filtration and degassing such as HPLC.
- It is highly sensitive, reproducible, and precise as compared with a thin layer chromatography.

Disadvantages

- Short separation bed is a major disadvantage of HPTLC
- A limited number of samples per plate can be tested.
- Sometimes silica gel is present during detection.

Applications

- Pharmaceutical industry- Quality control, identity purity test etc.
- Food Analysis- Quality control, additives, pesticides, stability testing etc.
- Clinical Applications- Metabolism studies, drug screening, stability testing etc.
- Industrial Applications- Process development and optimization etc.
- Forensic- Poisoning investigations.
- Biomedical Analysis- Separation of gangliosides.
- Environment Analysis-Pesticides in drinking water etc.
- Cosmetics- Hydrocortisone & cinchocaine in lanolin ointment etc.
- Natural products, plant ingredients- Glycosides in herbal drugs, Piperine in piper longum etc.
- Finger print Analysis-Finger prints for identification of liquorice, ginseng etc. [9]

Ultraviolet Visible (UV) Spectroscopy

Principle

- When ultraviolet radiations are absorbed, this results in the excitation of the electrons from the ground state towards a higher energy state.
- The more easily excited the electrons, the longer the wavelength of light they can absorb.
- The absorption of ultraviolet light by a chemical compound will produce a distinct spectrum that aids in the identification of the compound.[10]

Instrumentation

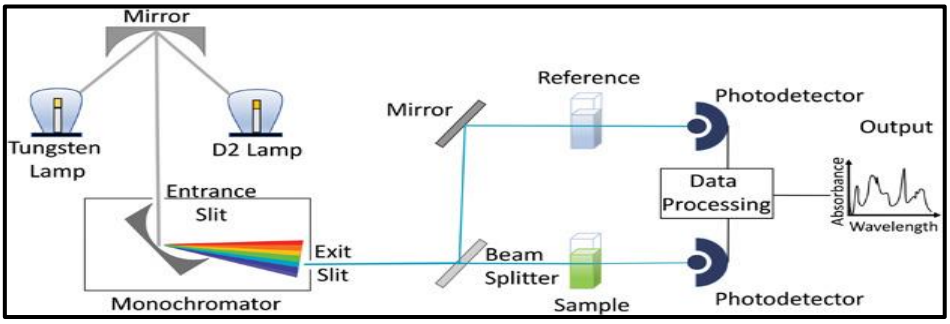

Fig. Schematic diagram of UV Instrumentation [11]

Advantages

- The UV-VIS spectrometer is easy to handling and use.
- Provide robust operation.
- UV-VIS spectroscopy is simple to operate.
- Cost effective instrument.
- Cover the entire of ultraviolet and visible.
- It can be utilized in the qualitative and quantitative analysis.
- The Derivative graph can be obtained by UV-VIS spectrophotometer.
- It can be used in the degradation study of drug.
- Only possible for the analytes which have a chromophore.

Disadvantages

- Only those molecules are analysed which have chromophores
- The results of the absorption can be affected by pH, temperature, contaminants, and impurities.
- Only liquid samples are possible to analyse.
- It takes time to get ready to use it.
- Cuvette handling can affect the reading of the sample.

Applications

- It is one of the best methods for the determination of impurities in organic molecules.
- Additional peaks can be observed due to impurities in the sample and it can be compared with that of standard raw material.
- By also measuring the absorbance at a specific wavelength, the impurities can be detected.
- UV absorption spectroscopy can be used for the quantitative determination of compounds that absorb UV radiation.
- UV absorption spectroscopy can characterize those types of compounds that absorb UV radiation thus used in the qualitative determination of compounds. Identification is done by comparing the absorption spectrum with the spectra of known compounds.
- This technique is used to detect the presence or absence of a functional group in the compound. The absence of a band at a particular wavelength is regarded as evidence for the absence of particular group.
- Kinetics of reaction can also be studied using UV spectroscopy. The UV radiation is passed through the reaction cell and the absorbance changes can be observed.
- Many drugs are either in the form of raw material or in the form of the formulation. They can be assayed by making a suitable solution of the drug in a solvent and measuring the absorbance at a specific wavelength.
- Molecular weights of compounds can be measured spectrophotometrically by preparing the suitable derivatives of these compounds.[12]

High Performance Liquid Chromatography (HPLC)

Principle

- The sample is injected into the mobile phase flow from the pump to the separation column using a syringe.
- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.
- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.
- The chromatogram allows the identification and quantification of the different substances.[13]

Instrumentation

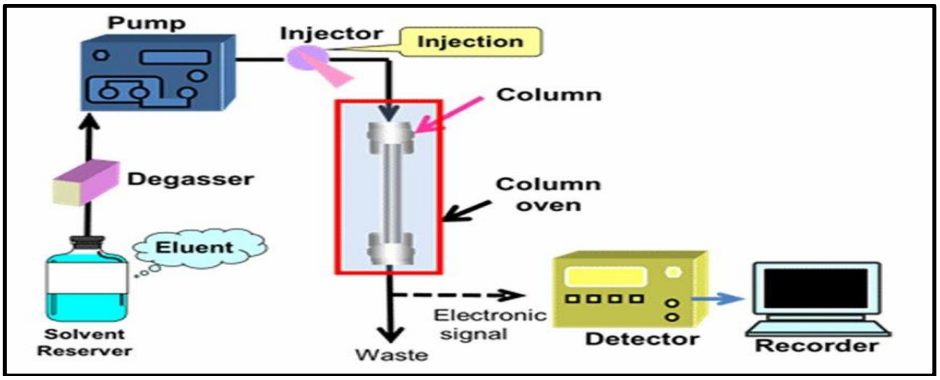

Fig. Schematic diagram of HPLC Instrumentation [14]

Advantages

- HPLC offers a rapid, automated and highly precise method to recognize certain chemical components in a sample.
- HPLC offers a fast and precise quantitative analysis.
- A gradient solvent system can be applied in certain methods.
- It is highly reproducible.
- HPLC can be upgraded to mass spectroscopy (MS).
- The HPLC is very rapid, efficient, and delivers high resolution as compared to other chromatographic techniques.
- Manages all areas of analysis to increase productivity.

Disadvantages

- HPLC can be an expensive method, it required a large number of expensive organics, needs a power supply, and regular maintenance is required.
- It can be complicated to troubleshoot problems or develop new methods.
- The lack of a universal detector for HPLC, however, the UV-Vis detector only detects chromophore compounds.
- The separation in HPLC has less efficiency than GC.
- It is more difficult for the beginner.
- HPLC pump process reliability relies on of cleanliness of the sample, mobile phase, and proper operation of the system.

Applications

- Analysis of drugs.
- Analysis of synthetic polymers.

- Analysis of pollutants in environmental analytics.
- Determination of drugs in biological matrices.
- Isolation of valuable products.
- Product purity and quality control of industrial products and fine chemicals.
- Separation and purification of biopolymers such as enzymes or nucleic acids.
- Water purification.
- Pre-concentration of trace components.
- Ligand-exchange chromatography.
- Ion-exchange chromatography of proteins.
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides.[15]

Spectro fluorimetry

Principle-

- Fluorescence is the emission of light by an atom, or molecule, following the absorption of light, or other radiation, by the molecule. The emitted light arises due to the transition of the excited electrons from the first singlet level to ground level.[16]

Instrumentation

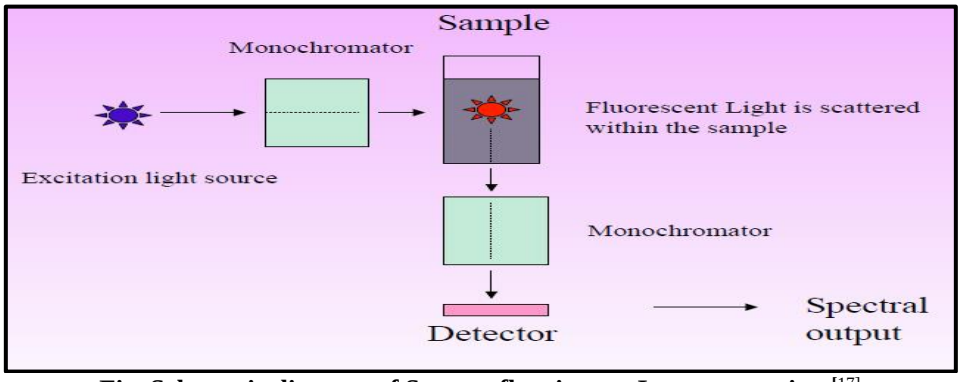

Fig. Schematic diagram of Spectro fluorimetry Instrumentation [17]

Advantages

- **Sensitivity**: It is more sensitive as concentration is low as µg/ml or ng/ml.
- **Precision:** Upto 1 % can be achieved.
- **Specificity:** More specific than absorption method where absorption maxima may be same for two compounds.
- **Range of application:** Even non fluorescent compounds can also be converted to fluorescent compounds by chemical compounds.

Disadvantages

- Not useful for identification.
- Not all compounds fluorescence.
- Contamination can quench the fluorescence and hence give false/no results.

Applications

- Widely used method of quantitative analysis in the chemical and biological sciences.
- It is a very accurate and sensitive technique.
- To detect environmental pollutants such as polycyclic aromatic hydrocarbons.
- Generally used to carry out qualitative as well as quantitative analysis for great aromatic compounds present in cigarette smoking, air pollutant concentrates & automobile exhausts.

➢ **Analytical chemistry-**

- To detect compounds from HPLC flow.
- TLC plates can be visualized if the compounds or a coloring reagent is fluorescent.
- Plant pigments, steroids, proteins, naphthol etc. can be determined at low concentrations.

➢ **Biochemistry-**

- Used generally as a non-destructive way of tracking or analysis of biological molecules (proteins).
- Fingerprints can be visualized with fluorescent compounds such as ninhydrin.

➢ **Medicine-**

- Blood and other substances are sometimes detected by fluorescent reagents, particularly where their location was not previously known.

➢ **Pharmacy-**

- Possible direct or indirect analysis drugs such as: vitamins.
- To measure the number of impurities, present in the sample.[18]

Ultra-Performance Liquid Chromatography

- It improves in chromatographic resolution, speed and sensitivity analysis.
- It uses fine particles and saves time and reduces solvent consumption.
- UPLC comes from High Performance Liquid Chromatography (HPLC).

Principle

- The UPLC is based on the principal of use of stationary phase consisting of particles less than 2.5 µm (while HPLC columns are typically filled with particles of 3 to 5 µm).
- Mobile phases at high linear velocities decreases the length of column, reduces solvent consumption and saves time.[19]

Instrumentation

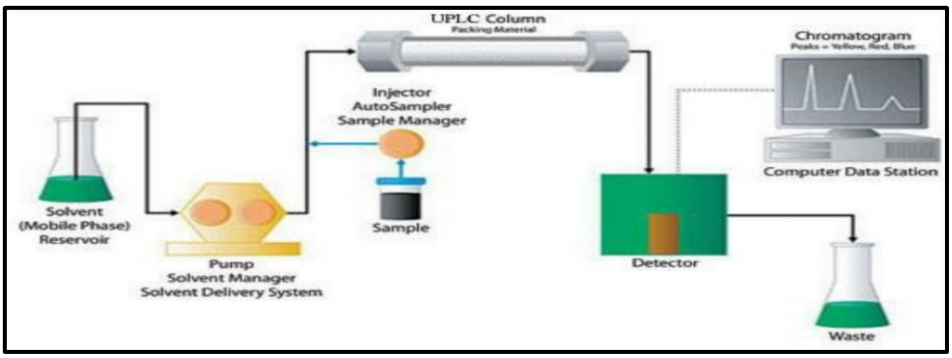

Fig. Schematic diagram of UPLC Instrumentation [20]

Advantages

- Decreases run time and increases sensitivity.
- Reducing analysis time so that more product can be produced with existing resources.
- Provides the selectivity, sensitivity, and dynamic range of LC analysis
- Maintains resolution performance.
- Fast resolving power quickly quantifies related and unrelated compounds.
- Operation cost is reduced.
- Less solvent consumption.

Disadvantages

- Due to increased pressure requires more maintenance and reduces the life of the columns of this type.
- In addition, the phases of less than 2 µm are generally non- regenerable and thus have limited use.

Applications

- Analysis of natural products and traditional herbal medicine.
- Identification of metabolite.
- Study of metabonomic/metabolomics.
- Bio analysis/bioequivalence studies.
- Manufacturing/QA/QC.
- Impurity profiling.
- Forced Degradation Studies.
- Dissolution Testing.
- Toxicity Studies.[21]

III. LITERATURE REVIEW

1. Potawale R, Parker H, Karwande A (2022) has developed **HPTLC** method for determination of **gymnemagenin and gallic acid** in herbal formulation **(tablet)** with densitometric detection. Separation was attained on Merck aluminium HPTLC plates precoated with silica gel 60 F_{254}. The solvent system which is optimized contained toluene: ethyl acetate: methanol: acetic acid: formic acid (10.4: 4: 4: 0.4: 0.3, v/v/v/v). Developed plates were derivatized by 5% sulphuric acid reagent followed by heating at 110°C for 4 min in a preheated oven followed by scanning at 456 nm in reflectance-absorbance mode. The R_f (Retention factor) was found to be 0.58±0.02, for gymnemagenin and 0.41±0.02, for gallic acid. Results were found to be linear over a range of 200-1000 ng band-1 and 80-240 ng band-1 for gymnemagenin and gallic acid respectively with correlation coefficient 0.999 for both. LOD was found to be 33.35 ng band-1 and 6.05 ng band-1 repectively, while LOQ was found to be 101.08 ng band-1 and 18.35 ng band-1 respectively. The percent relative standard deviation (% RSD) for precision was found to be less than 2%. Percentage recovery study was found to be 99.55-100.72% for gymnemagenin and 99.16-101.65% for gallic acid.[22]

2. Patel M, Chauhan S, Patel V, Soni H and Trivedi V (2021) has developed a simple, quick, and accurate **RP-HPLC** technique used for simultaneous assessment of **monoammonium glycyrrhizinate and sennoside-B** in a **polyherbal laxative tablet**

(Vasulax). The phytomarkers were effectively quantified by RP-HPLC system on C18 analytical column using gradient mobile phase consisting of phosphate buffer to acetonitrile. The detector wavelength was set at 254 nm. The Rt was found to be 21.49 for monoammonium glycyrrhizinate and 12.52 for sennoside-B. The linearity of calibration curves for monoammonium glycyrrhizinate and sennoside-B was established in range of 5– 25 µg/mL with correlation coefficient 0.9993 and 0.9996 respectively. LOD were found to be 0.17 µg/ml and 0.61 µg/ml and LOQ was found to be 0.51 µg/ml 1.85 µg/ml respectively. Precision studies were carried out using intra-day and inter-day intervals and RSD values were found to be less than 2.00%. The method was found to be accurate, which was evident from 98.96 to 101.39% and 99.17 to 100.67% recovery of monoammonium glycyrrhizinate and sennoside-B, respectively, when the formulation was spiked with the respective phytomarkers.[23]

3. **Malathi S, Brintha N, Lakshmi P (2021)** has developed a simple, reliable and rapid **HPTLC** method for quantitative estimation of **Gallic acid** in the Methanol extract of **Triphala Churna**. Pre coated silica gel on aluminum sheets were used as the stationary phase. Toluene: Ethyl acetate: Methanol: Formic acid (12:6:0.4:0.8) constituted the mobile phase. The percentage of Gallic acid was estimated through densitometry scanning using a TLC Scanner III (Camag, Switzerland) with win CATS software. The amount of Gallic acid present in the sample was found to be 1.1 mg/gm. The linearity range was found to be 200- 1000ng/spot. Correlation coefficient was found to be 0.98. LOD and LOQ were found to be 100ng/ml and 200-1000ng. The percent relative standard deviation (% RSD) for precision was found to be less than 2%.[24]

4. **Kaur P, Gupta R, Dey A, Malik T and Pandey D (2020)** has developed **HPTLC** method for validation and quantification of **oleanolic acid (OA), ursolic acid (UA), mangiferin (M), gallic acid (GA), quercetin (Q) and curcumin (C)** in commercial **Mahasudarshan Churna** formulation. Mobile phase- hexane: ethyl acetate: acetone (16.4: 3.6: 0.2, v/v) was used for the separation of OA and UA; ethyl acetate: glacial acetic acid: formic acid: water (20: 2.2: 2.2: 5.2 v/v) for the development of M; and toluene: ethyl acetate: formic acid (13.5: 9: 0.6 v/v) for the separation of GA, Q and C in crude sample extracts. Visualization and scanning were performed at λ = 530 nm for OA and UA, at λ = 254 nm for M and at λ = 366 nm for GA, Q and C. In addition, HPLC-PDA analysis was used to confirm the HPTLC results. Major bio-active compounds in MC formulations were oleanolic acid (1.54–1.78%), mangiferin (1.38–1.52%) and gallic acid (1.01–1.15%); followed by ursolic acid (0.79–0.98%), curcumin (0.45–0.67%) and quercetin (0.22– 0.34%). Calibration curve was found to be linear at 200–800 ng/band for mangiferin, 200–3000 ng/band for triterpenoids (OA and UA), 500–2000 ng/band for gallic acid, 300–1500 ng/band for quercetin and 100–1000 ng/band for curcumin with correlation coefficient (r^2) 0.987, 0.997, 0.991, 0.988, 0.992, 0.99 respectively. Limit of detection (LOD) (ng) was found to be 25, 27, 30, 40, 45, 30 respectively. Limit of quantitation (LOQ) (ng) [10 x SD/S] 75, 81, 90, 125, 140, 90 respectively. Rf value was found to be 0.53, 0.59, 0.38, 0.28, 0.48, 0.5 respectively. The percent relative standard deviation (% RSD) for precision was found to be less than 2%. Percentage recovery study was found to be within acceptable limit.[25]

5. **Pancham Y, Patil N, Girish B and Bhuvaneshwari S (2020)** has developed **UV-Visible spectrophotometric** method for simultaneous estimation of **Curcumin and Gallic acid** in its **bulk powder combination**. UV-Spectrophotometer of Shimadzu UV-1900 with Lab solutions software & Shimadzu UV-1800 with UV probe software was used for determination of Curcumin and Gallic acid. Curcumin and Gallic acid displayed maximum absorbance wavelength at 422nm and 264nm respectively. Curcumin and Gallic acid exhibited linear response between the concentration range of 1-6 µg/mL and 2-10 µg/mL respectively with regression equation of 0.998 and 0.993 for Curcumin and Gallic acid respectively. The %RSD values for the parameters carried out were found to be less than 2%. LOD was found to be 0.34 µg/mL and 1.08 µg/mL respectively, while LOQ was found to be 1.03 µg/mL and 3.26 µg/mL respectively.[26]

6. **Kurangi B, Jalalpure S (2020)** has developed a **RP-HPLC** method for quantitative analysis of **piperine** in Ayurvedic marketed formulation **(Trikatu churna, Ajmodadi churna and Chitrakadi gutika)**, black pepper and cubosome nanoformulation. HPLC system used was (LC-20AD prominence equipment, Shimadzu, Japan). The method was established by using Luna C18 HPLC column using a mobile phase consisting of acetonitrile: 0.01% ortho phosphoric acid (60:40, v/v; pH 3), delivered isocratically with flow rate of 1 mL/min and detected at 340 nm. The developed method was observed to be specific, linear (r^2> 0.999) over the selected range of concentration 0.5 to 20 µg/mL, precise (percentage relative standard deviation < 2%), with the detection and quantification limit as 0.015 and 0.044 µg/mL respectively. The relevancy of the developed method was analyzed on the piperine entrapped cubosome nanoformulation, which was formulated by fragmentation technique. The entrapment efficiency of piperine for prepared cubosome was observed to be 87.01 %. The method was implemented for the estimation of piperine in black pepper. The concentration of piperine in marketed formulation was found to be similar with the labeled concentration. The analyte peak was found to be complete resolved without any interference of additives and degrading products.[27]

7. **Qadir A, Ansari S, Ahmad S, Ahmad A and Taleuzzaman M (2020)** has developed a **TLC** method for the quantification of **piperine** in **Trikatu Churna** formulation. TLC was performed on TLC pre-coated plates of silica gel $60F_{254}$ with a uniform thickness of 200µm. Solvent system used are Toluene: Ethyl acetate: Formic acid (7: 2: 1). Scanning wavelength–350 nm. Prepared a calibration curve in the concentration range (100-800 ng/spot) with correlation coefficients r^2 (0.997) with R_f value 0.46 ± 0.03. The Limit of detection (LOD) and Limit of quantification (LOQ) value has 100 ng and 329 ng, respectively. The %RSD for intra and inter day precision was found to be less than 2%. The % recoveries were found out to be 99.18%. The maximum degradation found in the bench top (98%) follow with an acidic condition (86%), almost similar in the range of condition basic, oxidation and wet (75-77%), and a minimum in dry heat condition (49%). The freeze-thaw stability study, the accelerated stability study, the real-time stability study result of these conditions almost is the same range (92-99%).[28]

8. **Sahani S, Jain V (2019)** has developed e a novel, simple, rapid, precise and accurate **RP-HPLC** method for simultaneous quantitative estimation of **berberine, quercetin, and piperine** in Ayurvedic formulation **(capsule)**. The chromatographic separation was achieved using a stationary phase C18 shim-pack (150 mm x 4.6 mm, 5µ) column and mobile phase consisted of acetonitrile: 0.04 M potassium dihydrogen phosphate buffer (pH 3.0 adjusted using orthophosphoric acid) in a ratio of 65:35 v/v, with a flow rate of 1 ml/min and UV detection at 255 nm. The retention time of berberine, quercetin, and piperine were found to be 2.7, 3.0 and 6.3 min respectively. Linearity for berberine, quercetin, and piperine were found in the range of 12-28 µg/ml. All

calibration curve showed good linear correlation coefficients (r^2> 0.999) within the tested ranges. Mean percent recoveries for berberine, quercetin, and piperine were found to be within the acceptance limits (98-120%). The percent relative standard deviation (% RSD) for precision was found to be less than 2% which indicates method is precise. The LOD and LOQ for berberine were found to be 0.0037 µg/ml and 0.012 µg/ml, 0.023 µg/ml and 0.077 µg/ml for quercetin and 0.019 µg/ml and 0.064 µg/ml for piperine, which indicates that the method is sensitive.[29]

9. Patel S and Parmar V (2018) has developed a sensitive **HPTLC** method for simultaneous determination of **piperine and 6-gingerol** from ayurvedic formulation **(Trikatu Churna)**. Chromatographic separation was achieved on aluminium plates precoated with silica gel G60 F_{254} as the stationary phase and hexane:ethyl acetate:toluene:diethyl ether (4.5:5.5:1.0:0.5) as the mobile phase. The densitometric evaluation was carried out at 282 nm. The R_f value of piperine and 6-gingerol was found to be 0.45±0.02 and 0.60±0.02, correspondingly. The response in terms of peak area was linear over the concentration range of 100-500 ng/spot for piperine and 6-gingerol, individually, with the regression coefficient values greater than 0.99 for both the drugs. The limits of detection were found to be 7.65 ng/spot and 8.83 ng/spot for piperine and 6-gingerol, respectively. The %RSD for intra and inter day precision was found to be less than 2%. The % recoveries were found out to be 97.32–103.80 % for piperine and 97.91– 102.20 % for 6-gingerol.[30]

10. Dongre N, Sharma P, Shrivastava B, Dubey P (2017) has developed **UV Spectrophotometric** method for determination of **piperine** in **Navayasa Churna.** Three batches of Navayasa Churna were prepared in laboratory and then piperine is extracted from it and compared it against standard piperine solution in UV-Visible Spectrophotometer (Shimadzu, UV-1700, Pharmaspec). The concentration of piperine present in raw material was found to be 2.981±0.38%w/w in Piper nigrum and 0.981±0.047%w/w in Piper longum, respectively in three laboratory batch of Navayasa churna was 0.223±0.34, 0.219±0.42, 0.215±0.43%(w/w) respectively with mean value 0.219±0.903%(w/w). Correlation coefficient was found to be 0.9933. The percent relative standard deviation (% RSD) for precision was found to be 0.351. The accuracy was found to be 99.2%. The piperine content of all three batches is found to be in close proximities with each other.[31]

11. Warule P, Patel V, Gosavi S (2017) has developed a rapid, simple, selective and precise **UV-Visible spectrophotometric** method for the determination of **curcumin** in ayurvedic polyherbal formulation **Haridrakhand**. The intake formulation was subjected for maceration process using methanol as a solvent for the extraction of curcumin as a one of the ingredients in turmeric powder. The spectrophotometric detection of curcumin was carried out at an absorption maximum of 421nm using methanol as a solvent. The linearity range was found to be 1-25µg/ml with a correlation coefficient of 0.993. Accuracy of the proposed method was determined by the recovery studies, and %recovery (100.47%) obtained indicates that the method is accurate. The method was found to be precise as %RSD values for interday and intraday was found to be 1.77% and 1.88% respectively. The method was also found to be rugged and robust as the % RSD values were found to be 1.41% and 2.01% respectively. The limit of detection and limit of quantification of the proposed method was found to be 1.29 and 4.3 µg/ml respectively.[32]

12. Kharat S, Namdeo A, Mehta P (2017) has developed a simple and precise **HPTLC** methods for the simultaneous estimation of two anti-inflammatory drugs **(curcumin and galangin)**. The method was tailored to analyze both drugs in their commercial dosage form **(capsules)** with no interference from ingredients. Chromatographic separation was performed over precoated TLC plates (60 F_{254}, 20 cm × 10 cm, 250m thickness, Merck, Darmstadt, Germany) via a linear ascending technique using mobile phase consisting of n-hexane: ethyl acetate: acetic acid: methanol (7:2:0.5:0.5, v/v/v). Detection and quantification were achieved at 404 nm through spectro-densitometric analysis. The calibration curves were linear with the limits of 80–450 and 200–1200 ng/spot for CU and GA, respectively, with correlation coefficients (r^2)>0.9998. The limits of detection were 18.31 and 40.50 ng/spot for CU and GA, respectively. Mean percent recoveries for curcumin and galangin were found to be within the acceptance limits. The percent relative standard deviation (%RSD) for precision was found to be less than 2% which indicates method is precise.[33]

13. Ranjana, Mishra A, Mishra A, Gupta R (2016) has developed **TLC and HPTLC** method to quantitatively estimate **Gallic acid and β-Sitosterol** in selected Polyherbal formulation **(Capsule)**. Chromatography was performed on TLC plate coated with 0.2mm layer of silica gel, Toluene: ethyl acetate: formic acid (5:5:1 v/v) for Gallic acid and Toluene: Ethyl acetate: Glacial acetic acid (6:2:0.1) solvent used for β-Sitosterol as mobile phase. Densitometric scanning was then performed with win CATS software at λmax=254nm for Gallic acid and 273nm for β-Sitosterol. TLC detection for gallic acid was done by keeping plate in Iodine vapour chamber and detection was done by spraying with anisaldehyde - sulphuric acid reagent for β-Sitosterol. The Gallic acid was found as 9.78µg/ mg and 12.79µg/ mg in S1and S2 respectively and β- sitosterol 4.18µg/ mg and 3.81µg/mg in S1and S2 respectively. Concentration range observed was 200–1000µg. Correlation coefficient was found to be 0.979 and 0.903 respectively. The %RSD values for the parameters carried out were found to be less than 2%. LOD and LOQ for Gallic acid found to be 4.71µg/ml and 14.29µg/ml respectively and the LOD and LOQ for β- Sitosterol found to be 4.90µg/ml and 14.87µg/ml respectively.[34]

14. Sheikh Z, Shakeel S, Gul S, Zahoor A, Khan S, Zaidi F, Khan U (2015) has developed a **HPTLC** method for quantitative estimation of biomarkers **gallic acid and berberine** in polyherbal formulation **Entoban syrup**. Analysis was performed on 20 cm × 10 cm HPTLC silica gel G60 F_{254} plates with fluorescent indicator. Toluene: ethyl acetate: formic acid: methanol 12:9:4:0.5 (v/v/v/v) for gallic acid and ethanol: water: formic acid 90:9:1 (v/v/v) for berberine, used as a mobile phase respectively. The R_f values (0.58) for gallic acid and (0.76) for berberine in both sample and reference standard were found comparable under UV light at 273 nm and 366 nm respectively.[35]

15. Khanvilkar V, Patil L, Kadam V (2014) has developed **HPTLC** method for standardization of **Chitrakadi Vati** by detection and quantification of markers **piperine and plumbagin** simultaneously from in-house and marketed formulations. Piperine in Piper nigrum and Piper longum; Plumbagin in Plumbago zeylanica are active components in the formulation and can be considered as marker compounds. The separation was performed on TLC aluminium plates precoated with silica gel 60 F_{254}, good separation was achieved in the mobile phase of toluene: ethyl acetate: formic acid (7.5:2.5:0.5 v/v/v) and densitometry determination of piperine (0.39 ± 0.02) and plumbagin (0.70 ± 0.02) was carried out at single wavelength scanning at 280 nm.

Piperine and plumbagin were satisfactorily resolved with R_f values at 0.39 ± 0.02 and 0.70 ± 0.02 respectively. Piperine and plumbagin showed linear response in the concentration range of 200-700 ng/spot and 200-800 ng/spot, respectively. Correlation coefficient was found to be 0.9919 and 0.995 respectively. The percent relative standard deviation (% RSD) for precision was found to be less than 2%. The LOD and LOQ were found to be 33.39, 101.19ng/spot for piperine and 36.10, 109.4ng/spot for plumbagin respectively. Percentage recovery study was found to be within limit.[36]

16. **Parameswaran S and Koshti S (2014)** has developed a simple, specific and precise **HPTLC** method for quantification of **piperine and diosgenin** in **Goksuradi guggulu**. The extraction and estimation of these compounds in a laboratory prepared sample of Goksuradi guggulu and two of its marketed formulations. The compounds were chromatographed on precoated silica gel G 60 F_{254} plates in the mobile phase comprising of toluene: hexane: ethyl acetate (6.8:0.2:3). The calibration plot was found to be linear in the range of 0.2-1µg spot-1 for piperine and 1.0 -3.0 µg spot-1 for diosgenin. The correlation coefficient (r^2) was 0.9979 and 0.9915 for piperine and diosgenin respectively. Mean recovery (% w/w) for piperine in all the formulations was in the range of 97.61 - 98.90 and for diosgenin, it was 93.76-94.33.[37]

17. **Ramaswamy S, Kuppuswamy G, Priyanka Dwarampudi L, Kadiyala M, Menta L and Kannan E (2014)** has developed a method for simultaneous estimation of **curcumin and piperine** by reverse phase Ultrafast liquid chromatographic **(RP-UFLC)** method in **various food products, herbal medicines and nutraceuticals**. The chromatographic separation was performed on a C8 column (250 x 4.6 mm, 5µ i.d.) stationary phase using a mobile phase of 25mM potassium dihydrogen ortho phosphate buffer (pH 3.5) and acetonitrile (30: 70 v/v) at a flow rate of l ml/min at detection wave length of 280nm. The calibration curve was plotted in the concentration range of 0-2200ng/ml and found to be linear for both curcumin (r^2=0.996) and piperine (r^2=0.999). Mean percent recoveries for curcumin and piperine were found to be within the acceptance limits. The percent relative standard deviation (% RSD) for precision was found to be less than 2% which indicates method is precise. The LOD and LOQ for curcumin and piperine were found to be 6 ng/ml and 60 ng/ml respectively.[38]

18. **Patel S, Vyas N (2012)** has developed **spectrofluorimetric** method for the analysis of **piperine** in ayurvedic formulation **(Trikatu Churna).** The fluorescent nature of piperine proved to be of immense value in the development of the spectrofluorimetric method. The excitation and emission wavelength for piperine were 339nm and 450nm respectively. This study was carried out with Perkin Elmer LS 55 Fluorescence Spectrometer to determine levels of fluorescence in piperine. Methanolic solution of piperine showed intense blue fluorescence when observed under UV light therefore spectrofluorometric method were used. The linearity was found to be in the range of 10 - 60 ng/mL. The correlation coefficients (r^2) were 0.9958, respectively. The percentage recovery was found to be in the range of 91.928-94.365%. The percent relative standard deviation (% RSD) for precision was found to be less than 2%. LOD and LOQ was found to be 1.031 ng/ml and 3.127 ng/ml.[39]

19. **Patil V, Kurhade S, Devdhe S, Kale S, Wakte P (2012)** has developed a new, simple and rapid **HPTLC** method for quantitative estimation of **Gallic acid (GA)** from **Triphala Churna extract**. By aqueous cold maceration technique, the Triphala churna (TC) was extracted and yield was found to be 2.1%. Gallic acid was chromatographed on silica gel 60 F_{254} TLC plate using Ethyl acetate – Methanol – Formic acid (8: 2: 1 v/v/v) as mobile phase and quantified by densitometric scanning at 280 nm. The method was found to give compact spots for the Methanolic extract of Gallic acid (MEGA) shown R_f 0.77 ±0.01which has found to be similar to that of standard GA R_f 0.78 ±0.01. The linear regression analysis data for standard GA spots showed good linear relationship with r^2 = 0.9994 in the concentration range 100-400 ng/spot. The content of Gallic acid was found to be 10.9 % from the extract by linear regression analysis data. The minimum detectable amount of GA from MEGA was found to be 0.80 ng/spot, whereas the limit of quantitation was found to be 2.42 ng/spot. The recovery was 104.5, 103.7 and 106.1% for MEGA at 80% 100% and 120% levels. Assay results show excellent label claim of 102 ± 1.5 % for MEGA. The percent relative standard deviation (% RSD) for precision was found to be less than 2%.[40]

20. **Deodhar P, Naresh Kumar K, Gunesh G, Mukkanti K, Chandra Sekhar Ch (2012)** has developed a rapid and simple simultaneous stability indicating method for determination of **Gallic acid and Glycyrrhizic Acid** in **cough syrup** formulation by **HPLC**. Instrument used was Hitachi HPLC (Elite Lachrome) with PDA detector (L-2455), quaternary gradient pump (L-2130), with auto injector (L-2200), the validation was carried out with Inertsil ODS 3V (4.6 x 250 mm), 5µ particle size. The mobile phase used was 0.1% Orthophosphoric Acid in water: Acetonitrile (v/v) through gradient elution at 251 nm. The calibration curve was linear for Gallic acid and Glycyrrhizic acid with correlation coefficient of 0.999 for Gallic acid and Glycyrrhizic acid. The percent relative standard deviation (% RSD) for precision was found to be less than 2%. Accuracy was found to be 99.84% and 99.85% respectively.[41]

21. **Vyas N, Gamit K, Khan M, Panchal S and Pundarikakshudu K (2011)** has developed **HPTLC** method for simultaneous estimation of **curcumin and piperine** in their **crude powder mixture and ayurvedic formulation** which contains both. The separation was performed on TLC aluminum plates precoated with silica gel G60 $F_{254.}$ Good separation was achieved in mobile phase of chloroform: methanol (9.6:0.4 v/v) at R_f= 0.57 and 0.82 for curcumin and piperine respectively. Densitometric scanning of both compounds were carried out at 373nm. The relationship between the concentration and peak response was linear within the range of 300 to 600 ng/spot for curcumin and 200 to 500 ng/spot for piperine with correlation coefficient of 0.9998 and 0.9986 respectively. The mean % recovery at three different levels of curcumin was found to be within acceptable limit. The percent relative standard deviation (% RSD) for precision was found to be less than 2%. LOD and LOQ was found to be 103.91 ng and 314.88 ng for curcumin and 66.09 ng and 218.12 ng for piperine respectively.[42]

22. **Kondawar M, Kamble K, Mali D (2011)** has developed a **HPTLC- densitometric** method for analysis of these markers i.e **gallic acid and ascorbic acid** in **Triphala Churna**. Water was selected as a solvent for preparing standard solutions. Quantitative estimation of gallic acid and ascorbic acid was performed separately on aluminum backed silica gel 60 F_{254} TLC plates (10 cm x 10 cm plate size, layer thickness 0.2 mm, E-Merck, Darmstadt, Germany). Ascorbic acid shows R_f value of 0.74 ± 0.1 using ethanol: glacial acetic acid: toluene (5.5:1:1.5) and gallic acid showed R_f value of 0.54 ±0.1, using ethyl acetate: toluene: acetone (4.5:4:1) as mobile phase. The polynomial regression data of ascorbic acid and gallic acid were interpreted separately for

its linearity at 500-3500 µg/ml with r^2=0.9986 and 0.9931 respectively. Percentage recovery [fo3r] ascorbic acid and gallic acid were found to be within range of 98.5631-101.916% for all three marketed products. The values of LOD and LOQ were found to be 25.248µg/ml and 76.510 µg/ml, 13.129 µg/ml and 39.388 µg/ml for ascorbic acid and gallic acid respectively. The percent relative standard deviation (% RSD) for precision was found to be less than 2%.[43]

23. Singh N, Kumar P, Gupta D, Singh S and Singh V (2011) has developed a simple, rapid and precise **spectrophotometric** method for the estimation of **piperine** in Ayurvedic formulation **Chitrakadi Vati**. UV absorbance was recorded using Varian UV-spectrophotometer with Carry-100 software. Three marketed preparations of Chitrakadi Vati containing black pepper (Piper nigrum) and pippali (Piper longum) from different manufacturer (CV-1, CV-2 and CV-3) were taken in this study to estimate the % w/w of piperine. The % w/w content of piperine in the marketed preparations of Chitrakadi Vati CV-1, CV-2 and CV-3 were found to be 0.1032%, 0.0852% and 0.0898 % w/w respectively. The linearity concentration range between 2-20 µg/ml. Correlation coefficient was found to be 0.9956. Recovery studies were carried out by standard addition method and the average percentage recovery of the three samples CV-1, CV-2 and CV-3 were found to be 98.51%, 99.12% and 98.92% respectively.[44]

24. Gupta V and Jain U (2011) has developed a new, simple, rapid, sensitive, precise and economic **spectrophotometric** method in ultraviolet region for the determination of **piperine** in market and laboratory herbal formulation of **Sitopaladi churna**. Piperine obeys Beer Lambert'law in concentration range 10-50µg/ml at the λmax 342.5 nm. The correlation coefficient (r^2) value is 0.9961. The concentration of piperine present in raw material was found to be 1.45±0.341 %w/w in piper longum fruit. The concentration of piperine in different batches of STPLC (laboratory batches STPLC-I, STPLCII and STPLC-III) was found to be 0.31±0.008%, 0.34±0.002 %,0.32±0.003 % and in marketed formulations STPLC-A, STPLC-B, and STPLC-C was found to be 0.70±0.002 %, 0.72±0.005 %, 0.75±0.006 % respectively. The recovery study was performed at three levels by adding known amount of piperine with preanalysed sample of piperine in Sitopaladi churna (STPLC). The experiment was repeated Six times at both level and result shows 99.74%, 99.63%, and 99.72% recovery of piperine at all the level with mean value 99.69%. The % relative standard deviation(%RSD) value was found to be 0.57, 0.21, and 0.04, with mean 0.27 respectively.[45]

25. Gharate M and Kasture V (2011) has developed a simple, precise, accurate **RP-HPLC** method for the quantitative estimation of **atropine** in **Kankasava** polyherbal branded formulations. The separation was achieved with a column RP C- 18 (250 mm X 4.6 mm X 5 micron) using mobile phase mixture of Methanol &10 mmol dihydrogen phosphate buffer (the pH -2.5 adjusted with orthophosphoric acid) in a ratio of 50:50 v/v at a flow rate of 1 ml/min, &analysis was screened with UV detector at 254 nm. The retention time for standard atropine sulphate was found to be 4.0667 minutes. Calibration curve was linear over concentration range 20-100 µg/ml. Linearity was found to be r^2 =0.998. The percentage of RSD for precision and accuracy of the method was found to be less than 2%. The percentage recovery of atropine was found to be within limit. LOD and LOQ was found to be 6.16 µg/ ml and 18.67 µg /ml respectively.[46]

26. Rubesh Kumar S, Ram Kishan J, Venkateshwar Roa K.N, Duganath N and Kumanan R (2010) has developed **UV/Visible spectrophotometric** method for the simultaneous estimation of **curcuminoids and ascorbic acid** in commercially marketed ayurvedic polyherbal formulation **Nisha Amalaki vatti (tablet)**. Spectroscopic analysis was carried out using Elico SL-197 UV/Vis-Double beam spectrophotometer with Spectra treaties software. The linearity concentration ranges were found to be from 10-50 µg/mL for curcuminoids and ascorbic acid respectively. Correlation coefficient was found to be 0.999 for both. Curcuminoids has absorbance maxima at about 447nm and Ascorbic acid maxima at about 247nm in methanol. The percent relative standard deviation (% RSD) for precision was found to be less than 2%. Percentage recovery study was found to be within limit.[47]

27. Sunita S, Menon S and Singh A (2010) has developed a simple, rapid and precise **reverse-phase high performance liquid chromatographic** method for the quantitative determination of **piperine** from Ayurvedic Polyherbal formulations **Arkavati, Krvyadras and Marichyadi taila**. Chromatographic analysis was carried out on cosmosil C18 column (150mm x 4.6mm, 5µm particle) with a mobile phase of methanol: water in the volume ratio of 70:30 at a flow rate of 1.0 mL min-1. Quantitation was performed using a PDA-detector at 342 nm. Linear response for piperine was obtained over a range of 200 to 3000 ng mL-1. The correlation coefficient is 0.9997. The concentration of piperine in Arkavati was 17.00 mg/gm, in Krvyadras was 3.70 mg/gm and in Marichyadi taila was 0.058mg/ml. RSD values were less than 2%, indicating the method to be precise and reproducible. Recovery studies were carried out by standard addition method and the average percentage recovery of arkavati, krvyadras and marichyadi taila were found to be 99.17%, 99.94% and 99.54% respectively.[48]

28. Jain V, Saraf S and Saraf S (2007) has developed method for **spectrophotometric** determination of **piperine** from the **fruits of pippali, marica and Trikatu churna** at absorption maxima 342.5 nm. The concentration of piperine present in raw material was found to be 3.6 ± 0.31 % (w/w) in marica and 1.4 ± 0.27 % (w/w) in pippali, respectively and in three identical laboratory batch of Trikatu churna name TK-I, TK-II, TK-III, was 1.66 ± 0.39, 1.71 ± 0.42, 1.69 ± 0.43 % (w/w), respectively with mean value 1.69 ± 0.41 % (w/w). The linearity concentration ranges between 2-20 µg/mL. Correlation coefficient was found to be 0.9989. The recovery study was performed at two levels by adding known amount of piperine with preanalyzed sample of piperine in Trikatu churna. The experiment was repeated six times at both level and result shows 99.34 ± 0.26 and 99.11 ± 0.24 % recovery of piperine at both the level with mean value 99.23 ± 0.25 %. The relative standard deviation (RSD %) value was found to be 0.409 and 0.292 with mean 0.351.[49]

IV. SUMMARY AND CONCLUSION

➢ From the review of various literature, it was concluded that different analytical methods were used as HPTLC, UV, RP-HPLC, UPLC and Fluorimetry for the analysis of biomarkers like piperine, gallic acid, curcumin, galangin, quercetin etc. in different polyherbal formulations. HPTLC technique is low cost, fast, precise and accurate. UV method was found to be simple, sensitive, accurate, linear, precise, rapid, economical, specific, robust, rugged and reproducible. RP-HPLC method was found to be novel, simple, rapid, accurate and precise. Spectro fluorimetry provides a faster and cost-effective method. RP-UPLC method was found to be sensitive, specific and rapid. These methods were validated by determination of parameters like accuracy, linearity, precision,

limit of detection, and limit of quantification as well as robustness and ruggedness according to ICH guidelines. Above methods can be used for qualitative and quantitative control for routine analysis of biomarker in polyherbal formulation. These methods can be used for controlling quality control parameters in industries.

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