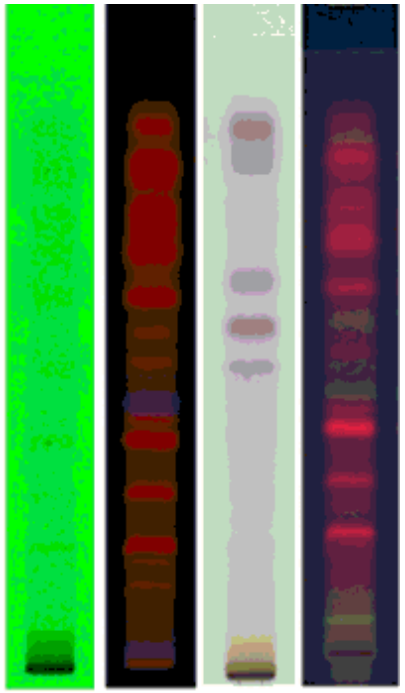
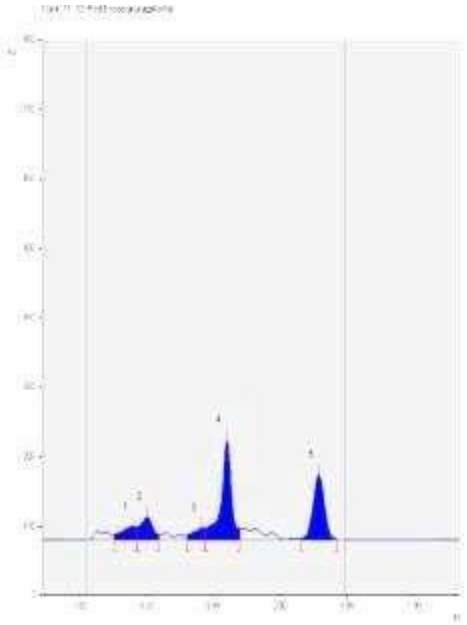
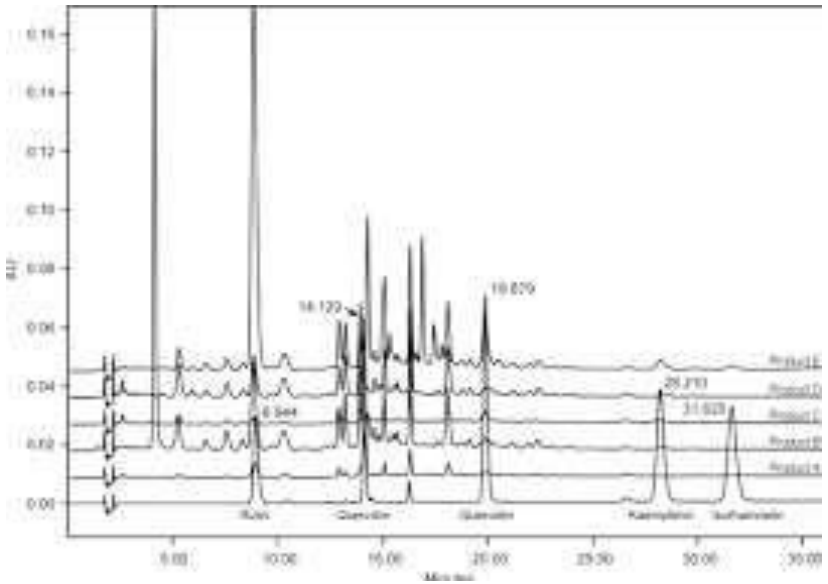
Stability testing of PHYTO-PHARMACEUTICALS HPTLC And HPLC FINGERPRINTING







Stabilitytestingofphyto-pharmaceuticals

Phyto-pharmaceutical drug is defined as purified and standardized fraction with defined minimum four bioactive or phytochemical compounds (qualitatively and quantitatively assessed) of an extract of a medicinal plant or its part, for internal or external use of human beings or animals for diagnosis, treatment, mitigation, or prevention of any disease or disorder but does not include administration by parenteral route.

WhyPPh?

Introduction of Phytopharmaceuticals would promote innovations and development of new drugs from Botanicals in a scientific way, and would give boost to research in drug development at National Research Laboratories as well as Pharmaceutical Research Labs in India.

Different approaches to product development

Selection of approach: •Random approach & •Ethnomedical approach

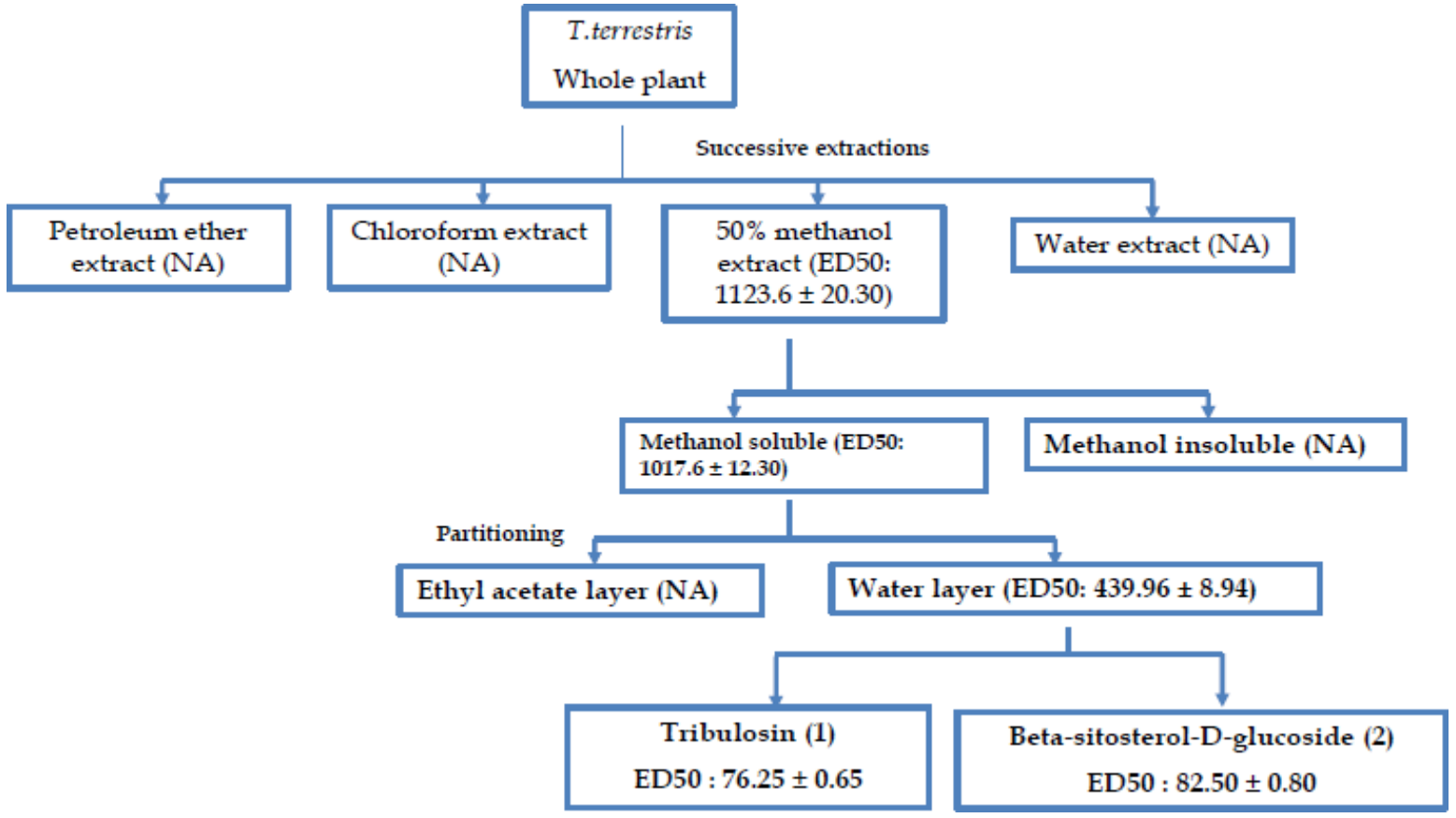
•"There is little evidence that working with plants used in traditional medicine greatly enhances chance of finding biological activity" (Alan L. Harvey, An introduction to drugs from natural products, in Drugs from natural products, Ellis Horwood, 1993, ISBN 0-93-096546-4).

•"Ethnodirected collection is a more efficient means of drug discovery than random plant screens" (Donald F Slish et al. Ethnobotany in the search for vasoactive herbal medicines, J of E. 1999, 66, 159-165.)

Developmentofstandardizedphytopharmaceuticals

1. Selection of plants & preparation of their extracts
2. Standardization of relevant in-vitro mechanism based bioassays
3. Screening of extracts
4. Bio-activity guided fractionation for isolation, identification and characterization of active principle(s)
5. Optimization of the extraction procedure
6. Standardized ingredient/extract
7. Development of final dosage form
8. Validation of safety and efficacy

Bio-activityguidedfractionation



Standardization of phytopharmaceuticals

Standardized extract

Indian Pharmacopoeia, 2014: Standardized extract means an extract adjusted within an acceptable tolerance to a given content of biomarker or chemical / analytical marker. Standardization may be achieved by adjusting the extracts with approved inert material or by blending one or more batches of extracts.

USP Dietary Supplements Compendium, 2012, states that standardized ingredients contain a defined amount of a particular chemical constituent or group(s) of constituents known as marker compound(s). A complete definition of standardization includes the information and controls needed to produce a material of predetermined and defined consistency.

European Pharmacopoeia - Standardisation means the adjusting of the herbal drug preparation to a defined content of a constituent or a group of substances with known therapeutic activity, respectively by adding excipients or by blending herbal drugs or herbal preparations (CPMP/QWP/2819/00)

Regulatory requirements

• Description of product and documentation of prior human experience

Description of botanical raw materials used and known active constituents or chemical constituents Prior human experience.

• Quality control

Botanical raw materials

Botanical drug substance and drug product Identity, chemical characterization, manufacturing processes, biological assay, specifications, stability, current good manufacturing practices, and environmental assessment.

• Evidence to ensure therapeutic consistency : Botanical raw material control ; Quality control by chemical test(s) and manufacturing control; Biological assay o Clinical data: Dose-response data and multiple batch clinical data.

In Indian regulations, the major class of Ayurveda, Siddha, or Unani (ASU) drugs included are:[7] a. Classical ASU drugs as mentioned in the authoritative books of ASU system drugs, which are manufactured and named in accordance with the formulations described in the authoritative texts. For this category, issue of license to manufacture is based on citation in authoritative books and published literature, unless the drug is meant for a new indication when proof of effectiveness is required. b. Patent or proprietary medicine makes use of ingredients referred in the formulations of authoritative texts but with intellectual intervention, innovation, or invention to manufacture products different from the classical medicine. For this category issue of a license to manufacture requires proof of effectiveness, based on the pilot study as per relevant protocol for ASU drugs.

In Schedule Y, the newly added Appendix I B describes data to be submitted along with the application to conduct clinical trial or import or manufacture of a phytopharmaceutical drug in the country.[8] The regulatory requirements for NDA for the phytopharmaceutical drug include standard requirements for a new drug-safety and pharmacological information, human studies, and confirmatory clinical trials. For phytopharmaceutical drug, there is a lot of stress on:

- Available information on the plant, formulation and route of administration, dosages, therapeutic class for which it is indicated and the claims to be made for the phytopharmaceutical, and supportive information from published literature on safety and efficacy and human or clinical pharmacology information
- Data generated on:

Identification, authentication, and source of the plant used for extraction and fractionation; Process for extraction and subsequent fractionation and purification; Formulation details of phytopharmaceutical drug

Manufacturing process of formulation; Stability data.

•***British Herbal Pharmacopoeia*** **(1996) 4th edition, British Herbal Medicines Association, West Yorks, England.**

•*European Pharmacopoeia* (1997) 3rd edition, Council of Europe, Strasbourg.

•*Martindale: the Extra Pharmacopoeia* 91996) 31st edition, Pharmaceutical Press, London.

•*The British Pharmaceutical Codex*, Pharmaceutical Press, London.

•*The British Pharmacopoeia* (1998), Her Majesty's Stationery Office, London.

•*The United States Pharmacopeia and National Formulary* USP24/NF19 (2000) USP Convention Inc, Rockville, Maryland.

•***Pharmacopoeia of the People's Republic of China*** **(1997), Vol 1.**

Note that this list does not contain any Indian Pharmacopoeia. The pharmacopoeia commissions should insist on the inclusion of IP and API in this list.

InUSA

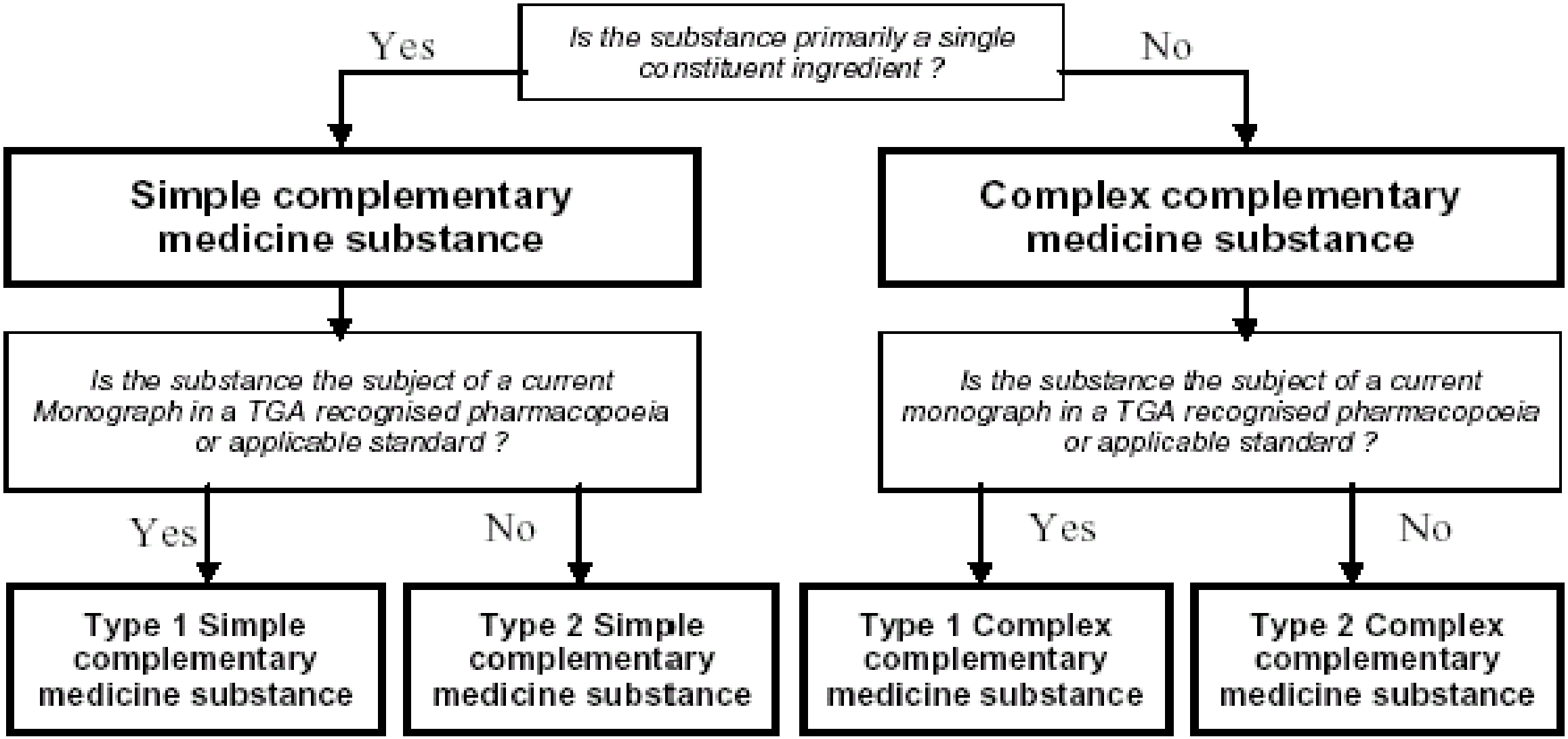
•A food ingredient can be developed into a medicine but not vice versa. For example - a standardized extract of ginger can be converted into a phytomedicine for motion sickness.

•Accordingly, the US-FDA permits up gradation of a Dietary Supplement to a botanical drug but not vice versa.

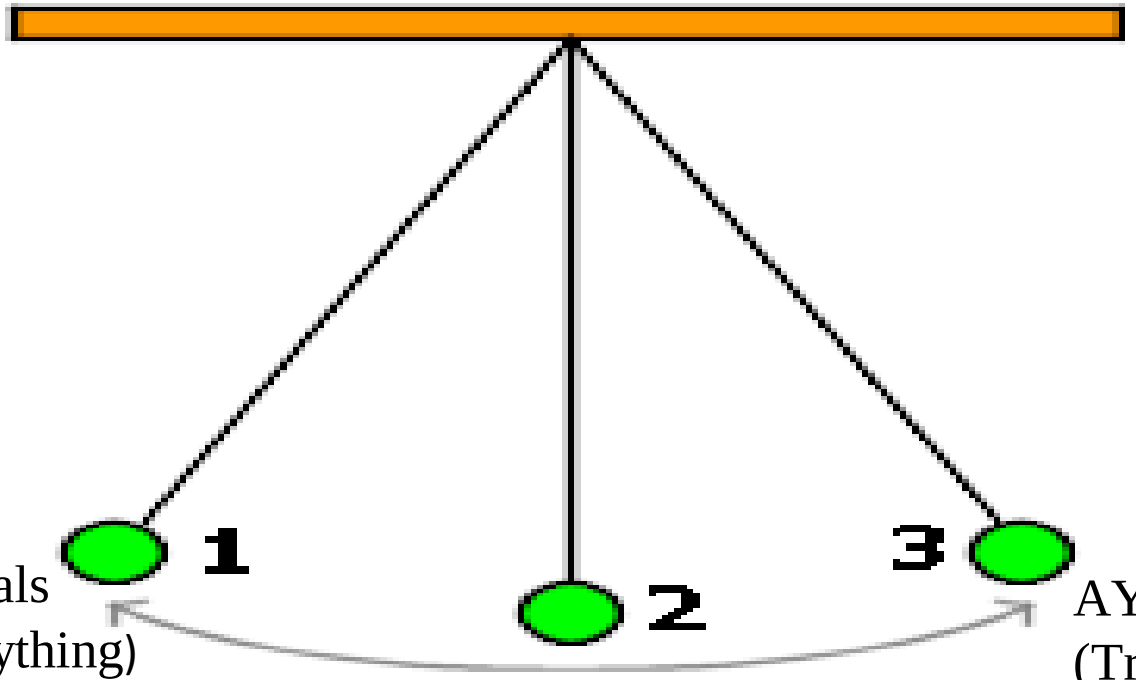
•A product is regulated as a dietary supplement or a botanical drug based on the manner it is presented in the market, intention of the manufacturer, nature of claims made, etc. Being a drug or a food is not an inherent property of a substance.

•Similarly, if a product is made using a medicinal plant having traditional use, should the regulation force the manufacturer to obtain manufacturing license for it as an Ayurvedic medicine or Phytopharmaceutical or a functional food?

Australian Regulatory Guidelines for ComplimentaryMedicinesPartIII



Phytopharmaceutical regulation



Conventional pharmaceuticals (Suspect everything)

AYUSH medicines (Trust everything)

Phytopharmaceutical (a balanced approach)

Some Important Definitions of Chromatographic Fingerprinting

- According to State Drug Administration of China (2000) - "A chromatographic fingerprint is a chromatographic pattern of chemical characteristic constituents present in the specific extract".
- According to Reich E & Schibli A, (2007) - An optimized fingerprint of a plant species allows as toobtain a whole spectrum of information about the sample, understand "Total Ingredient Patterns" of that plant species and serves as "Chemical Signature" of particular herbal entity. This fingerprint is established, which, within limits, is representative of a given plant species.

1. State Drug Administraion (2000). *Chin Tradit Pat Med;* 22:825.
2. Ciesia L (2012). *Chrom. Res. Int;* Vol. 2012: 1-9.
3. Reich E and Schibli A (2007). High Performance Thin Layer Chromatography for the Analysis of Medicinal Plants; published by Thieme Medical Publishers, Inc, The Americans.

- **Fingerprinting**

"A pattern or an impression which is highly specific enough to become useful as characteristic identifier for that particular entity."

- **Chromatogram Fingerprinting**

In such cases, the entire chromatogram is the fingerprint and the analysis must fulfil some requirements: (a) a high peak capacity, since all components in the sample are potentially relevant; (b) retention-time stability and (c) detector stability, as recording the chromatograms may take considerable time; (d) a wide dynamic range, because both major and minor components are important and (e) the use of multivariate-analysis techniques (for example partial least squares or principal components regression), to correlate fingerprints with the product specifications and characteristics.

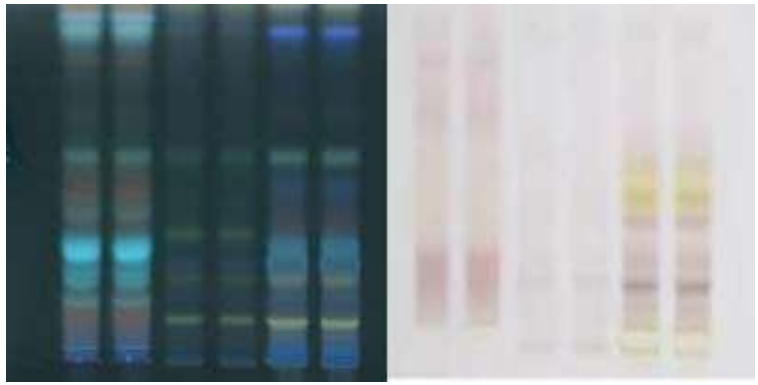
- **HPTLC and HPLC fingerprinting**

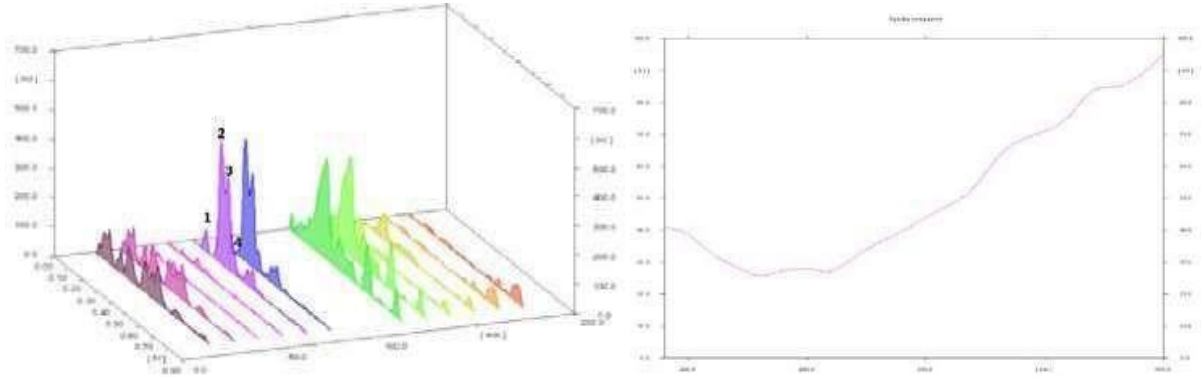
"It is a pattern on TLC plate (or a densitogram of that pattern) of separated compounds, generated according to their highly specific R_F– values, presented in sample, specific enough to become an authentic statement or bio-chemical marker (in the case of biological samples) for that sample and capable enough to provide unique, specific and characteristic identity. HPLC is a highly powerful, sensitive, and versatile chromatographic technique for separation, purification, and characterization of chemical compounds, especially of natural product origin.

Here TLC is refered as qualitative and semi-quantitative purpose and HPTLC is refered as quantitative analysis purpose with high sample throughput.

Purpose of HPTLC analysis

In the High performance thin layer chromatography (HPTLC) we try to study the separation on a inert stationary plate (stationary phase) and allow molecules of a carrier solution (M.P.) that able to dissolve the components of sample and provide a adjustable position of separated sample by varying solvent strength.





Procedures	Its application
Plate material	Rewashing HPTLC plates (silica gel 60 F 254) in methanol.
Sample application	With the help of Linomat, Automatic TLC sampler
Preparation and storage of developing solvents	The developing solvents are preparing by measuring the require volume of each component separately and transferring them into a solvent bottle of appropriate size. The bottle is close with lid and shakes to ensure proper mixing of the content. Developing solvents should be clear solution.
Developments	Plates are developed in a saturated Twin Trough Chamber
Derivatization	Spraying or dipping may accomplish transfer of reagent for derivatization of samples on a HPTLC plate. Dipping is preferred method. If derivatization includes heating, a plate heater is used.
Documentation of plates	Each developed plates is documented with an electronic documentation system under UV 254 nm (Short-wave UV light), UV 366 nm (Long-waver UV light) and white light. All images are labeled and listed in the project work sheet.
Labeling	Each plate images are given an identification number (ID), which will be written in pencil in the top right corner. The ID includes Project number, dash, Year, month, day, dash and a consecutive number each day. [Example:- A091-060322-02 and for image label is A091-060322-02-A254]
Quantitative evaluation	It is performed with the TLC Scanner 3 using WinCATS software. The analysis files are labeled to reflect the plate ID.
Documentation of work	All work performed is documented in a project worksheet.

Importance of HPTLC fingerprinting for botanicals

- The checklist of botanical/ medicinal plants alongwith their scientific validation is still biased. The traditional methods are poor, time consuming and less scientific, so there is a need to used emerging technological knowledge and sophisticated analytical methods. Before HPTLC the botanical analysis is restricted to wet chemical reaction analysis and morphological studies only.

- HPTLC provide a deep inside in to the plant's compound profile and their chemistry.

- There is no substitution of qualitative visual results of HPTLC for botanicals.

- As a comparative with HPLC/GC and other advanced chromatographic/molecular techniques, the HPTLC fingerprinting is cheap, fast and solvent saving,

- Sensitivity and accuracy is always a matter (as compare with hplc) but HPTLC is advanced enough for providing a reliable authentic biochemical integrity statement for botanicals.

Before starting -

(Theoretical concepts of Chromatography)

-chromatographyhighlydependsonaffinity.

- Mobile phase – the molecules, that moves with flow (on TLC plate/in column).
- Stationary phase – the molecules, that remains immobile.

Controller of chromatography

- In gas chromatography – temperature is a major factor.
- In liquid chromatography – degrees of solubility is major factor.
- In thin layer chromatography – highly depends at the mercy of capillaryforces.

Major Controller ofTLC

- Adsorption. - Partition. - Complex Formation.

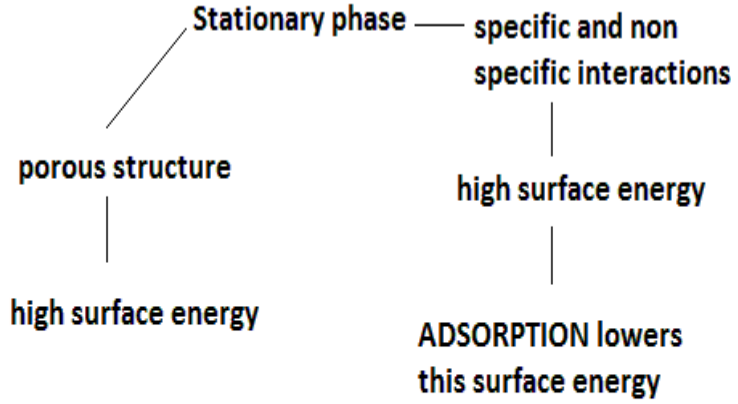
- Adsorption Phenomenon – Two models for explanation-
  - Competition model (Snyder & Soczewinski).

    (Deals with displacement of the molecules)

  - Solvent model (Scott & Kucera).

    (Deals with retention mechanism as adsorption process

    Emphasizing sample-mobile phase interactions)

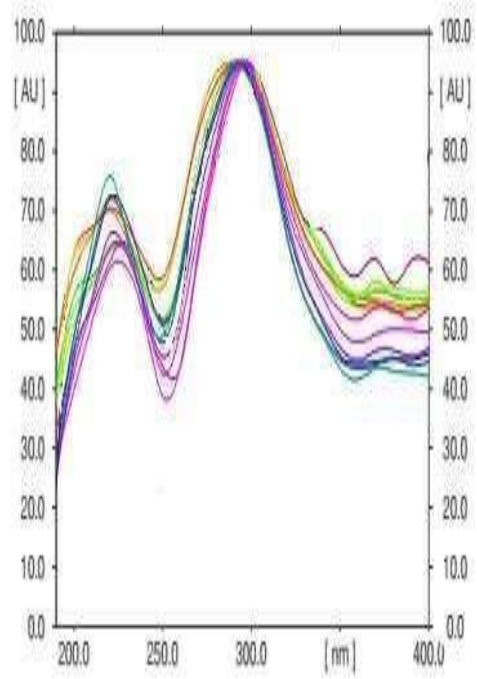


Degree of this retention depends on -
- Availability of active sites for interactions/attractions.
- number of functional group present.

- Partition phenomenon – **Utilizes the different solubility of a sample in two immiscible liquid phases. (concept explained by solvophobic concept of horvath).**

- Complex formation phenomenon – **Ligand exchange is also a retention mechanism. Major principle is that the reversible formation of co-ordination compounds between lewis acid – bases.**

Important factors of consideration for TLC studies



- **For TLC as an open system** - So there is a contact with air and water vapor.

  - Humidity = That water vapor.

  - Absolute humidity = Amount of water in gas phase/air (in meter *3).

  - Saturation humidity = Max. amount of humidity, air can contain (Temp.= Constant).

  - Relative humidity = Ratio of absolute humidity and saturation humidity.

- **For Mobile phase** –

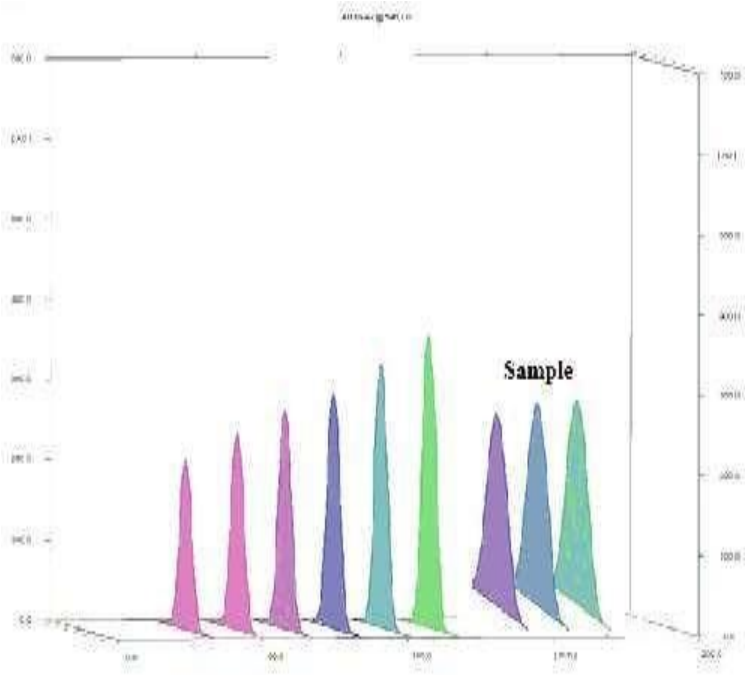
- Solvent Strength (Important for position of bands) – Solvent strength is proportional to RF value (1/retention).

- Polarity Index - It is the solute-solvent intermolecular attraction. If the polarity index is same then RF values will also be same (prism model).

- Selectivity (Important of separation and sequence of zones.) – It is about how the same solvent can react with structural differences in the sample components.

Facts on Mobile phase optimization for HPTLC fingerprinting of botanical

1. Similar substances = same R_F values.
2. Exchange the solvents or modify their rations, depends on experimental needs.
3. Change in Solvent strength, change the R_F values.
4. Change in the selectivity, change in the relative positions.
5. Same polarity index of solvents = same R_F.
6. Same selectivity so similar elution order of compounds.
7. Achievement of optimization of Mobile phase = R_F value between 0.3 to 0.5. (best = 0.3).
8. Models for theoretical understandings = prisma model and four solvent model.



Lets start (How to make a initial sample for HPTLC Plant Fingerprinting ?)

- **Sample preparation -**

- Drying – Shed drying for 15 – 21 days are enough.
- Grinding – Depends up on samples.
- You can use soxhlet (not for heat sensitive samples) and other advanced procedure, depends up on the experimental needs.
- Selection of solvent – Depends up on experimental needs. (usually methanol or water is used) .

- **Extraction/Fraction preparation -**

- Simply 1: 10 - sample : solvent (universal).
- Initially 500 mg powered sample in to 10 ml solvent.
- As comparative study, you can also make 5% HCl /solvent only /5% NH_3 modifications in the 500 mg in to 10 ml solvent sample.
- For fraction preparation use column chromatography from low polarity to high polaritysolvent.

Solvent selection

- The choice of solvent is influenced by what is intended with the extract, for this reason Successful determination of biologically active compounds largely dependent on type of solvent used in the extraction procedure.
- Properties of a good solvent in plant extraction includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate, maximum selectivity and best capacity for extraction in terms of coefficient of saturation of the product .
- The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of solvent in the bioassay process and the potential health hazard of the extractants.

Lets start with Mobile Phase OptimizationExperiment

- **Level 1 –**

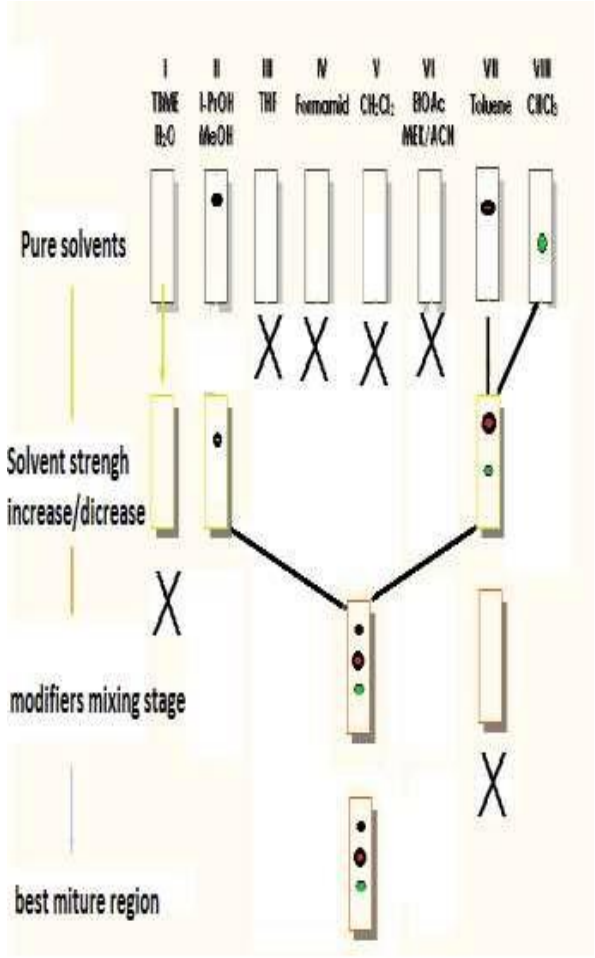
- 7 to 12 net solvents for 1-8 selectivity groups.

- You will get three results –

1 - Suitable RF value.

2 – Too much RF (above 0.7 or 0.8) = then exchange solvent. (remember same selectivity group).

3 – Too low RF (below 0.2) = exchange with high polarity solvents.



Mobile phase optimization scheme diagram

- **Level 2 –**

- Manage solvent strength

1 – Dilution with hexane for down the high RF (2).

2 – Polar modification with acids or ammonia for increase the lower RF(3).

- **Level 3 –**

- Two solvent combination of different selectivity groups. (the ratio = 1:1).

- Short cut shunt – solvent of high RF (2) and solvent of low RF (3) in the level 1, can be directly combine with each other and skip the level 2 and level 3.

[Ratio = 1:1 or 10% of high RF solvent (2) in to low RF solvent (3)], if results are good then go to the level 4 directly).

- **Level 4 –**

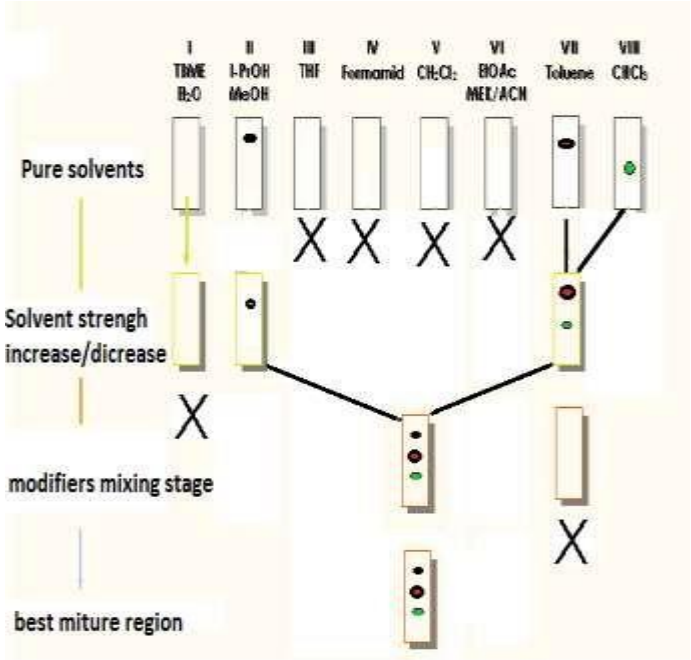
- Minor adjustment and fine tuning level.

1– Improve the band shape by the use of modifiers.

2– If tailings occurs then use 0.5% or 10% water. 3

– Miscible acid / bases can also be exchangeable.

4 – Finally 2- d chromatography for stability testing.



Mobile phase optimization scheme diagram

How to do HPTLC Plant Fingerprinting ?

Basic steps –

1. Firstly, Plate is checked before using it for sample applicator under the 254nm, whether it was giving fluorescence or not and mark the limit of run at 80 mm and direction by a HB – pencil.
2. Cut all 20 x 20 cm plate, in to the 10 x 20 cm or 10X10 cm plate with the use of a scissor depends on the experimental needs.
3. Working program is generated with the use of winCATS software in the computer.
4. Specific volume of sample is taken by the use of 100 µl or 500 µl Hamilton Syringe and applied on plate as the predefined 6mm or 8mm band length by the means of sample applicator.
5. After the completion of the sample applicator program the plate is subjected for drying with the use of a drier and then placed on to TLC plate heater for 10 minutes to remove any water or moisture content from the plate.

6. An optimized mobile phase is used for analytical demand of experiment.

7. This mobile phase is subjected to the development chamber.

8. A filter paper rinsed with mobile phase is also subjected in the chamber for a uniform vapor.

- saturation of the chamber prior adding of the sample applied plate.

9. Chamber saturation time is now optimized (basically 20 or 25 minutes).

10. the plate is placed in it, till the solvent front reached up to the distance of 80mm (previously marked).

11. After it, Densitometry evaluations can be carried out under 254nm (Deuterium lamp) and 366nm (Hg lamp) in the different – different files (.cna extension files for winCATS software) with the use of Scanner for peak height and peak area calculation. (some times spectra analysis can also be performed under 190nm-400nm).

12. In the last step the plate is derivatized with specific derivatizing agent for visual identification in the derivatizating chamber (specific time) and air dried.

13. Final images are quickly captured by the Plate Visualizer under visible white light and florescence (366 nm). Then densitometry scanning was performed at 540nm (W lamp) and 366 nm (Hg lamp).

14. After it, spectral analysis, R_F values calculations, peak areas calculations are analyzed for final interpretation and HPTLC Fingerprinting pattern profile generation.

References and books suggestions

- For HPTLC fingerprinting experimental and analytical purpose -

(**Reich, E., and Schibli, A. (2007).** *High Performance Thin Layer Chromatography for the Analysis of Medicinal Plants*; published by Thieme Medical Publishers, Inc, The Americans)

- For specific class of phytochemical study –

- Qualitative atlas study – (**Wagner, H., & Bladt, S. (1995).** *Plant Drug Analysis: A Thin Layer Chromatography Atlas*, 2nd edition published by Springer - Verlag, Berlin Heidelberg, NY. ISBN 3-540-58676-8)

- Quantitative and densitometrical analysis – (**Monika, W.H., Joseph, S., and Teresa, K. (2008).** *Thin layer chromatography in phytochemistry*. 1st edition published by CRC press Taylor & Francis Group. London).

Thank you

S.No.	Churna/extract	Constituents	Solvent system and Scanning wavelength	Standard
1.	**Ajmodadi churna (Methanol) [3]**	Piper species (*Piper longum* in both form root and fruit and Piper nigrum) [10]	Toluene: Ethyl acetate (07:03) at 336 nm.	Piperine
2.	**Amukkara choornam (toluene) [30]**	*Piper nigrum, Piper longum, Zingiber officinale, Amukkara (Withania somnifera), Elletaria cardamomum, Cinnamomum wightii, Syzygium aromaticum*	Toluene: Ethyl acetate (9:3 v/v) at 260 nm.	Not mentioned
3.	**Chaturjat churna (Methanol) [11]**	*Cinnamomum zeylanicum, Elettaria cardamomum, Cinnamomum tamala and Tribulus terrestris*	Toluene: Ethyl acetate (9:1) at 254 nm.	Not mentioned
4.	**Hingashtak Churna (methanol) [31]**	*Piper longum, Piper nigrum, Curcuma longa, Thymus vulgaris*	Toluene-ethyl acetate-methanol, 9:1:0.5 at 420, 333, and 277 nm	Curcumin, piperine, and thymol
5.	**Kuberaksha/Kantaki karanja patra Churna (methanol) [32]**	*Caesalpinia bonduc*	Ethyl acetate: methanol: water (100:13.5:10) at 254 nm.	Not mentioned
6.	**Laghugangadhar Churna (methanol) [1]**	*Cyperus rotundus, Symplocos racemosa, Woodfordia fruticosa, Aegle marmelos,*	Toluene: Ethyl acetate (90:10) at 254 and 366 nm.	Not mentioned
7.	**Nisamalaki churna (Methanol and Aqueous) [33]**	*Curcuma longa; Emblica Officinalis*	Chloroform-methanol (9.5:0.5 v/v), Ethanol-glacial acetic acid (9:1 v/v) at 500 nm	Curcumin; Ascorbic acid
8.	**Pancasma churna(Ethanol) [7]**	*Operculina turpethum; Terminalia chebula, Cyprus rotundus; Piper longum*	Toluene: ethyl acetate: Formic acid (5.0:3.5:1.0 v/v) at 366 nm.	Piperine and gallic acid
9.	**Panchaskar churna (methanol) [34,35]**	*Cassia angustifolia, Foeniculum vulgare, Terminalia chebula, Zingiber officinale, Anethum sowa , Rock salt* (Saindhava lavana).	Toluene: ethyl acetate at 260 nm	Not mentioned
10.	**Palas abijadi churna (methanol) [36]**	*Butea monosperma; Holarrhena antidysentrica,embelia ribes, Azadirachta indica, Swertia chirata*	Toluene: Ethyl acetate (90:10 v/v) at 260 nm.	Not mentioned
11.	**Panchkol Churna [37]**	*Piper longum, Piper nigrum, Cuminum cyminum, Plumbago zeylanica, Embelia ribes, Zingiber officinale*	Toluene: ethyl acetate (7:3) at 340, 420 nm.	Piperine, plumbagine, zingiberine
12.	**Triphla Churna (aqueous) [13,14]**	*Terminalia chebula, Terminalia belerica and Embellica officinalis*	Ethanol: glacial acetic acid: toluene (5.5:1:1.5) for ascorbic acid and Ethyl acetate: toluene: acetone (4.5:4:1) for gallic acid at 254 nm.	Ascorbic acid and gallic acid [13]
13.	**Trikatu Churna (methanol) [15]**	*Piper longum, Piper nigrum, Zingiber officinale*	Toulene: ethylacetate: glacial acetic acid (8:2:0.1 v/v/v) at 550 nm.	Piperine
14.	**Talishadi churna (methanol) [38]**	*Piper longum, Piper nigrum, Zingiber officinale, Elletaria cardamomum, Cinnamomum zeylanicum, Bambusa arundinacea,*	Toluene: ethyl acetate (9:3 v/v) at 260 nm.	Not mentioned
15.	**Vidanga churna (methanol) [39]**	*Embelia ribes*	Chloroform: ethyl acetate: formic acid (5:4:1 v/v/v) at 291 nm.	Embelin
16.	**Ashwagandha churna (methanol) [40,41]**	*Withania somnifera*	Toluene: ethyl acetate: formic acid at 540 nm.	Beta-sitosterol D glucoside