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A Review on Chromatographic Techniques in Standardization of Herbal Products

[1]Milind Gajendra Jagdale, [2]Mohit Hemraj Patil, [3]Sampat Kella Padvi, [4]Nishant Nirmal Sharma, [5]Dnyanesh Govinda Vanjari

[1]Student, Herbal Department, Smt. Sharadchandrika Suresh Patil College Of Pharmacy Chopda.
[2]Student, Herbal Department, Smt. Sharadchandrika Suresh Patil College Of Pharmacy Chopda.
[3]Student, Herbal Department, Smt. Sharadchandrika Suresh Patil College Of Pharmacy Chopda.
[4]Student, Herbal Department, Smt. Sharadchandrika Suresh Patil College Of Pharmacy Chopda.
[5]Student, Herbal Department, Smt. Sharadchandrika Suresh Patil College Of Pharmacy Chopda.

ABSTRACT:

The world is witnessing an unprecedented growth in the usage of herbal products. India is a mother hub for natural herbs based science. Herbal drug technology is used for converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is important. For global harmonization WHO specific guidelines for the assessment of the safety, efficacy and quality of herbal medicines are of utmost importance. Standardization of drug means confirmation of its identity, quality and purity throughout all phases of its cycle.In order to prove constant composition of herbal preparations, adequate analytical methods the have to be applied such as photometric analysis, thin layer chromatography [TLC], high performance liquid chromatography [HPLC], and gas chromatography [GC], DNA Fingerprinting.

Keyword: Chromatographic technique, TLC, HPLC, HPTLC, UHPLC, GC

Introduction:

In recent years, there has been great demand for plant derived products in developed countries. These products are increasingly being sought out as medicinal products, nutraceuticals and cosmetics. There are around 6000 herbal manufacturers in India. According to an estimate of the World Health Organization (WHO), about 80% of the world population still uses herbs and other traditional medicines for their primary health care needs. Herbal medicine products are dietary supplements that people take to improve their health and are sold as tablets, capsules, powders, teas, extracts and fresh or dried plants. Herbals are traditionally considered harmless and increasingly being consumed by people without prescription. However, due to the complexity of natural products, there can be significant variations in their composition, quality, and potency. This can pose a challenge for healthcare professionals and consumers who rely on these products for their therapeutic effects. Standardization of herbal products is a process that aims to ensure consistency and quality in the composition, manufacturing, and labelling of herbal products. It involves establishing and maintaining specific standards for the identity, purity, and potency of the active ingredients in herbal products. Herbal products are widely perceived as being safe by patients because they are considering natural. Most medications before being offered to consumers undergo rigorous evidence-based clinical testing; this is not necessarily true for Consumers regularly herbs. Consumers regularly use these products without the knowledge of their healthcare professionals. Due to their long historical clinical use and reliable therapeutic efficacy, traditional Indian medicine attract and increase global attention, and many big pharmaceutical companies are using traditional Indian medicine as an excellent pool for discovering natural bioactive compounds.Marker compound means chemical constituents within a medicinal that can be used to verify its potency or identity. For sometimes, the marker compounds may be described as active ingredients or chemicals that confirm the correct botanical identity of the starting material. It is very difficult to identify correct marker compounds for all traditional medicinals, because some medicinals have unknown active constituents and others have multiple active constituents. A chromatographic fingerprint of a herbal medicine is a chromatographic pattern of the extract of some common chemical components of pharmacologically active and/or chemical characteristics. By using chromatographic fingerprints, the authentication and identification of herbal medicines can be accurately conducted even if the amount and/or concentration of the chemically characteristic constituents is not exactly the same for different samples of drug. Hence it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic component of the herbal drug. WHO has provided some terms related to herbal drugs, according to their definitions. Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products. In some countries herbal medicines may contain, by tradition, natural organic or inorganic active ingredients that are not of plant origin (e.g. animal and mineral materials).

Standardization

Standardization of herbal medicines is the process of prescribing a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, efficacy. safety and reproducibility. It is the process of developing and agreeing upon technical standards. Specific standards are worked out by experimentation and observations, which would lead to the process of prescribing a set of characteristics exhibited by the particular medicines. Hence standardization is a tool in the quality control process. American Herbal Product association defines: "Standardization refers to the body of information and control necessary to product material of reasonable consistency. This achieved through minimizing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing processes. "Standardization" expression is used to describe all measures, which are taken during the manufacturing process and quality control leading to a reproducible quality. It also encompasses the entire field of study from birth of a plant to its clinical application. It also means adjusting the herbal drug preparation to a defined content of a constituent or a respectively by adding excipients or by mixing herbal drugs or herbal drug preparations". "Evaluation" of a drug means confirmation of its identity and determination of its quality and purity and detection of its nature of adulteration". [**Kartik Chandra Patra, *et.al*, 2010]**

Need of Quality control and standardization of herbal products can be summarized as follows-

1. When traditional medicines were developed technology and concept of standardization was quite different.
2. During past thousand years dynamic process of evolution may have changed the identity of plant material.
3. Due to commercialization, supply of genuine raw material has become a challenge.
4. Properties of botanicals may have undergone change due to time and environmental factors". **•Standardization of herbal formulation**

Standardization of herbal formulation requires implementation of Good Manufacturing Practices (GMP) (WHO guideline, 1996) In addition, study of various parameters such as pharmacodynamics, pharmacokinetics, dosage, stability, self-life, toxicity evaluation, chemical profiling of the herbal formulations is considered essential. Heavy metals contamination, Good Agricultural Practices (GAP) in herbal drug standardization are equally important.

CHROMATOGRAPHY

Chromatography is the science which studies the separation of molecules based on differences in their structure and/or composition. In general, chromatography involves moving a preparation of the materials to be separated, "the "test preparation", over a stationary support. The molecules in the test preparation will have different interactions with the stationary support leading to separation of similar molecules. Test molecules which display tighter interactions with the support will tend to move more slowly through the support than those molecules with weaker interactions. In this way, different types of molecules can be separated from each other as they move over the support material. Chromatographic separations can be carried out using a variety of supports, including immobilized silica on glass plates (thin layer chromatography), very sensitive High Performance Thin Layer Chromatography (HPTLC), volatile gases (gas chromatography), paper (paper chromatography), and liquids which may incorporate hydrophilic, insoluble molecules (liquid chromatography). High performance thin layer chromatography (HPTLC) is a valuable quality assessment tool for the evaluation of botanical materials. It allows for the analysis of a broad number of compounds both efficiently and cost effectively.

Methods Of Standardization Of Herbal Drugs

According to WHO (1996a and b, 1992), Standardization and quality control of herbals is the Process involved in the physicochemical evaluation Of crude drug covering aspects, such as selection and Handling of crude material, safety, efficacy and Stability assessment of finished product, Documentation of safety and risk based on Experience, provision of product information to Consumer and product promotion. Attention is Normally paid to such quality indices such as:

Morphology and organoleptic evaluation: In case of whole drug morphological characters are Important for differentiating purpose. It mainly Includes colour, odour, taste, shape, size etc. Detail Characteristics includes fractures, texture, venation etc.

Microscopic and histologic evaluation: These are Valuable in both whole as well as powdered drug. It Mainly includes study of characteristics like Parenchyma, trichomes, calcium oxalate crystals, Vascular bundle arrangements, stomata, fibres etc.

Quantitative microscopic study: Microscopic Determination such as vein islet number, stomatal Index, stomatal number, vein termination number, Size of fibres, palisade ratio. Such study helps in Differentiation of closely allied species.

Physical evaluation: study of various physical Parameters like moisture content, solubility, Viscosity, refractive index, melting point, optical Rotation, ash values, extractives and foreign organic Matter. size of fibres, palisade ratio. Such study helps in differentiation of closely allied species. Study of various physical parameters like moisture content, solubility, viscosity, refractive index, melting point, optical rotation, ash values, extractives and foreign organic matter.

Qualitative chemical evaluation: This covers identification and characterization of crude drug with respect to phytochemical constituent. It employs different analytical technique to detect and isolate the active constituents. Phytochemical screening techniques involve botanical identification, extraction with suitable solvents, purification, and characterization of the active constituents of pharmaceutical importance.

Quantitative chemical evaluation: To estimate the amount of the major classes of constituents.

Toxicological studies: This helps to determine the pesticide residues, potentially toxic elements, safety studies in animals like LD50 and Microbial assay to establish the absence or presence of potentially harmful microorganisms.

Microbiological parameters: It includes the full content of viable, total mould count, total coliforms count. Limiters can be used as a quantitative tool or semiquantitative to determine and control the amount of impurities, such as reagents used in the extraction of various herbs, impurities ships directly from the manufacturing and solvents etc.

CONVECTIONAL METHOD: This covers Identification and characterization of crude drug with Respect to phytochemical constituent. It employs Different analytical technique to detect and isolate the Active constituents. Phytochemical screening Techniques involve botanical identification, extraction with suitable solvents, purification, and Characterization of the active constituents of Pharmaceutical importance.

Pesticide residues Even though there are no serious reports of toxicity due to the presence of pesticides and fumigants, it is important that herbs and herbal products are free of these chemicals or at least are controlled for the absence of unsafe levels. Herbal drugs are liable to contain pesticide residues, which accumulate from agricultural practices, such as spraying, treatment of soils during cultivation, and administering of fumigants during storage.

Analytical Methods- It helps in determining identity, quality and relative potency. The most important step in the development of analytical methods for botanical and herbal preparations is sample preparation. The basic operation includes steps such as pre- washing, drying of plant materials or freeze drying and grinding, to obtain a homogenous sample and often improving the kinetics of extraction of the constituents. In the pharmacopoeial monographs, method such as sonication, heating under reflux, Soxhlet extraction, and others are commonly used.

Chromatogarphy-Separation of individual components from the herbal mixture is the key step to enable identification and bioactivity evaluation. Chromatography is a powerful analytical method suitable for the separation and quantitative determination of a considerable number of compounds, even from a complex matrix. These include paper chromatography (PC), thinlayer chromatography (TLC), gas chromatography (GC), HPLC, and capillary electrophoresis (CE).

CHROMATOGRAPHY TECHNIQUE IN STANDARDIZATIONOF HERBAL PRODUCTS

A simple chromatographic technique such as TLC may provide valuable additional information to establish the identity of the plant material. This is especially important for those species that contain different active constituents. Qualitative and quantitative information can be gathered concerning the presence or absence of metabolites or breakdown of products (AOAC, 2005). TLC fingerprinting is of key importance for herbal drugs made up of essential oils, resins, and gums, which are complex mixtures of constituents that no longer have any organic structure. It is a powerful and relatively rapid solution to distinguish between chemical classes, where macroscopy and microscopy may fail. The instruments for UV-Visible determinations are easy to operate, and validation procedures are straightforward but at the same time precise. Although measurements are made rapidly, sample preparation can be time consuming and works well only for less complex samples, and those compounds with absorbance in the UV-Visible region. HPLC is the preferred method for quantitative analysis of more complex mixtures. Though the separation of volatile components such as essential and fatty oils can be achieved with HPLC, it is best performed by GC or GCMS. The quantitative determination of constituents has been made easy by recent developments in analytical instrumentation. Recent advances in the isolation, purification, and structure elucidation of naturally occurring metabolites have made it possible to establish appropriate strategies for the determination and analysis of quality and the process of standardization of herbal preparations. Classification of plants and organisms by their chemical constituents is referred to as chemotaxonomy. TLC, HPLC, GC, quantitative TLC (QTLC), and high performance TLC (HPTLC) can determine the homogeneity of a plant extract. Over-pressured layer chromatography (OPLC), infrared and UV-Visible spectrometry, MS, GC, liquid chromatography (LC) used alone, or in combinations such as GC-MS and LC-MS, and nuclear magnetic resonance (NMR), electrophoretic techniques, especially by hyphenated chromatographic techniques, are powerful tools, often used for standardization and to control the quality of both the raw material and the finished product. The results from these sophisticated techniques provide a chemical fingerprint as to the nature of chemicals or impurities present in the plant or extract (WHO, 2002). Based on the concept of photo equivalence, the chromatographic fingerprints of herbal medicines can be used to

address the issue of quality control. Methods based on information theory, similarity estimation, chemical pattern recognition, spectral correlative chromatograms (SCC), multivariate resolution, the combination of chromatographic fingerprints and chemometric evaluation for evaluating fingerprints are all powerful tools for quality control of herbal products.

Thin Layer Chromatography:

A method of separation or identification of a mixture of components into individual component by using finely divided adsorbent solid [liquid] spread over a glass plate and liquid as mobile phase.

Principle:

- It is based on the principle of adsorption chromatography or partition chromatography combination of both.
- Depending on adsorbent its treatment and nature of solvents employed.
- The components with more affinity towards stationary phase travels slower.
- Components with less affinity towards stationary phase travels faster.

In the phytochemical evaluation of herbal drugs, TLC is being Employed extensively for the following reasons: **[Christopher Johnson Mwankuna, *et.al* , 2022]**

1. It enables rapid analysis of herbal extracts with minimum sample clean up requirement.
2. It provides qualitative and semi quantitative information of the resolved compounds.
3. It enables the quantification of chemical constituents. Fingerprinting using HPLC and GLC is also carried out in specific cases.

RETENTION FACTOR:

$$R_f = \frac{\text{Distance moved by the analyte}}{\text{Distance covered by the mobile phase}}$$

Table 1: Example of analytes evaluated by TLC

Sr. No.	**ANALYTES**	**TLC System parameters**
1	Harhra' (Terminaliachebula and Gallic acid)	Stationary phase: Silica gel Mobile phase :Toluene – ethyl acetate – formic acid, 5:5:1
2	Azadirachta indica, Catharanthus roseus and Momordica charntia	Stationary phase: Silica gel Mobile phase: Dichloro methane–methanol, 2:8
3	Mushroom extracts	Stationary phase: Silica gel Mobile phase: Dichloromethane – ethyl acetate-methanol, 3:1:1
4	Strychnos nux vomica	Stationary phase: Silica gel Mobile phase: Chloroform–ethyl acetate– diethyl amine, 0.5:8.5:1
5	Constituents from the fruit of Piper chaba (Piperine, piperamine, Piperlonnguminine, and methyl piperate)	Stationary phase: Silica gel Mobile phase: n-hexane-ethylacetate, 1:1
6	Quinones	Stationary phase: Silica gel 60 Mobile phase: dichloromethane-n-hexane, 8:2

In TLC fingerprinting, the data that can be recorded using a high performance TLC (HPTLC) scanner includes the chromatogram, retardation factor (Rf) values, the color of the separated bands, their absorption spectra, λ max and shoulder inflection/s of all the resolved bands. All of these, together with the profiles on derivatization with different reagents, represent the TLC fingerprint profile of the sample. The information so generated has a potential application in the identification of an authentic drug, in excluding the adulterants and in maintaining the quality and consistency of the drug. TLC was the common method of choice for herbal analysis before instrumental chromatography methods like GC and HPLC were established. Even nowadays, TLC is still frequently used for the analysis of herbal medicines since various pharmacopoeias such as American Herbal Pharmacopoeia (AHP) , Chinese drug monographs and analysis, Pharmacopoeia of the People's Republic of China etc. still use TLC to provide first characteristic fingerprints of herbs . Rather, TLC is used as an easier method of initial screening with a semi quantitative evaluation together with other chromatographic technique.

Example-

Densitometric Thin-Layer Chromatographic determination of Aescin in a herbal medicinal product containing Aesculus and Vitis dry extracts: [P]

"Aescin", the saponin mixture obtained from the seeds of Aesculus hippocastanum L. , is widely used in the treatment of peripheral vascular disorders. This mixture of saponins has been reported to show anti-inflammatory activity and structural requirements of aescins for this activity were obtained by *Matsuda et al* by investigating the effects of the pure saponins on acute inflammation in experimental animal models. In view of the potential commercialisation of capsules containing Vitis vinifera L.extract in combination with the Aesculus extract to treat peripheral insufficiency and haemorrhoids, a method for quality control on the saponin content in both the extract and the HMP capsules needed to be developed.

Experimental -

Chemicals and reagents: Aescin (According to the German Pharmacopoeia (DAB), Aescin RN: purity: 97.6% calculated on the dried material; water content: 4.83%) was obtained from Fluka (Sigma, Bornem, Belgium). Distilled water (RiOs) prepared with a Millipore water purification system (Millipore, Bedford, MA, USA) was used. Acetic acid (p.a.) was purchased from Merck (Darmstadt, Germany), methanol (HPLC grade) and butanol (99%) from Acros Organics NV (Geel, Belgium). Detection reagent: 0.5 ml p-anisaldehyde (Sigma) was successively mixed with 10 ml 100% glacial acetic acid (Merck), 85 ml methanol and 5 ml sulfuric acid (98–97% p.a.; Merck). The HMP capsules (250 mg of A. hippocastanum dry extract, 120 mg of V. vinifera dry extract; excipients: 34 mg cellulose microcrystalline, 8 mg colloidal silicium dioxide, 8 mg hydrogenated cotton seed oil), both dry extracts and excipients were from Biover (Brugge, Belgium).

Method –

- **Preparation of sample solutions:** About 80 mg of the powder was accurately weighed in a 25.0 ml volumetric flask and filled up with water. The obtained solution was sonicated in an ultrasonic bath for 5 min. After cooling down to room temperature, 3.0 ml of this solution was transferred on a 500 mg SPE C18ec cartridge (Macherey-Nagel, Filter Service, Eupen, Belgium) previously washed with two volumes methanol and two volumes water. The SPE cartridge was successively washed with two volumes water and two volumes methanol 20% v/v. The saponins were eluted with 18 ml methanol and diluted to 20.0 ml with the same solvent. Each sample was analyzed (full sample preparation) in duplicate.

- **Preparation of the aescin standard solution:** About 20.0 mg aescin was accurately weighed in a volumetric flask of 50.0 ml, dissolved in methanol and filled up to 50.0 ml with the same solvent. 4.0 ml of this solution was diluted to 10.0 ml with methanol.

- **Equipment and chromatographic procedure:**One, two, three and four microliters of the standard solution and 4l of the test solution were spotted in duplicate (15 mm from the edge, 4 mm width) with a Desaga TLC-applicator AS 30 on a HPTLC-plate silica gel 60 F254 (0.2 mm layer thickness, 10 cm × 20 cm) from Merck. The plate was developed to 5 cm in a development chamber presaturated with the upper layer of a mixture acetic acid–water–butanol (10:40:50, v/v/v), dried, sprayed and heated (100–105 ◦C) for about 5 min. The TLCplate was covered with a glass plate and the remission absorption was measured at 535 nm by means of a Desaga CD 60 TLC scanner. The saponin content was calculated as aescin using the data-pair technique.

Fig. 2. Picture of the TLC plate of the analysis of samples of the HMP capsules as well as of *Aesculus* dry extract subjected to thermal, acidic, and alkaline stress conditions.

Overview of the linearity data of aescin

	Aescin
Correlation coefficient	0.9987
Slope ± standard error	1200.0 ± 19.3
Intercept ± standard error	16.7 ± 9.8
Confidence interval (95%)	−5.2 to 38.7
F_{LOF} (F_{crit} = 5.41)	17.0
QC (QC_{crit} = 2.50)	2.30
Range (µg/spot)	0.160–0.800
Number of standards (duplo)	6

High Performance Thin Layer Chromatography [HPTLC]:

It is also known under the synonym planar chromatography is a modern, powerful analytical technique with separation power, performance and reproducibility superior to classic TLC.

It is an automated and sophisticated form of TLC. HPTLC is the most simple separation technique today available to the analyst.

HPTLC technique is widely employed in pharmaceutical industry in process development, identification and detection of adulterants in herbal product and helps in identification of pesticide content, mycotoxins and in quality control of herbs and health Food. It has been well reported that several samples can be run simultaneously by use of a smaller quantity of mobile phase than in HPLC. It has also been reported that mobile phases of pH 8 and above can be used for HPTLC. Another advantage of HPTLC is the repeated detection (scanning) of the chromatogram with the same or different conditions. Consequently, HPTLC has been investigated for simultaneous assay of several components in a multicomponent formulation. With this technique, authentication of various species of plant is possible, as well as the evaluation of stability and consistency of their preparations from different manufactures. Various workers have developed HPTLC method for phytoconstituents in crude drugs or herbal formulations suchas bergenin, catechine and gallic acid in Bergenia cilliata and Bergenia lingulate **[Rakesh S. Shivatare, *et.al* , 2013].**

There are several advantages of using HPTLC for the analysis of compounds as compared to other techniques, like HPLC, spectrophotometry, titrimetry, etc.Some of the advantages of HPTLC are:

- The separation process is easy to follow especially with colored compounds.
- Ability to analyze crude samples containing multicomponents.
- Several samples can be separated parallel to each other on the same plate resulting in a high output, time saving, and a rapid low-cost analysis.
- Choice of solvents for the HPTLC development is wide as the mobile phases are fully evaporated before the detection step.
- Two-dimensional separations are easy to perform. Stability during chromatography should be tested using two-dimensional development.
- Specific and sensitive colour reagents can be used to detect separated spots (Dragendroff reagent/Kedde reagent).
- HPTLC can combine and consequently be used for different modes of evaluation, allowing identification of compounds having different light absorption characteristics or different colours.
- Contact detection allows radiolabelled compounds to be monitored and microbial activity in spots to be assessed.
- HPTLC method may help to minimizes exposure risk of toxic organic effluents and significantly reduces its disposal problems, consequently, reducing environment pollution.

Table 2: Generally used Mobile phase in detection of some chemical compound:

Sr No.	Chemical Compounds	Mobile Phase
1	Polar Compounds Anthraglycosides, Arbutin, Alkaloids, Cardiac Glycosides, Bitter Principles, Flavonoids, Saponin	Ethyl Acetate: Methanol: Water [100:13.5:10]
2	Lipophilic Compounds, Essential oils, Terpenes, Coumarin, Napthoquinons, Velpotriate	Toluene: Ethyl Acetate [93:7
3	Alkaloids	Toluene: Ethyl Acetate: Diethyl Amine [70:20:10]
4	Flavonoids	Ethyl Acetate: Formic Acid: Glacial Acetic Acid :Water [100:11:11:26]
5	Saponin	Chloroform: Glacial Acetic Acid: Methanol: Water [64:32:12:8]
6	Coumarin	Diethyl Ether: Toluene [1:1] Saturated with 10% Acetic Acid
7	Bitter Drug	Ethyl Acetate: Methanol: Water [77:15:8]
8	Cardiac Glycosides	Ethyl Acetate: Methanol: Water [100:13.5:10] OR [81:11:8]
9	Essential Oil	Toluene: Ethyl Acetate [93:7]
10	Pungent Testing	Toluene: Ethyl Acetate [70:30]
11	Terpenes	Chloroform: Methanol: Water [65:25:4]
12	Triterpenes	Ethyl Acetate: Toluene: Formic Acid[50:50:15] Toluene: Chloroform: Ethanol [40:40:10]

Sample Preparation and Application

A good solvent system is one that moves all components of the mixture off the baseline, but does not put anything on the solvent front. The peaks of interest should be resolved between Rf 0.15 and 0.85. The elution power of the mobile phase depends on a property called eluent strength which is related

to the polarity of the mobile phase components. 23 The more nonpolar the compound, the faster it will elute (or the less time it will remain on the stationary phase) and the more polar the compound the slower it will elute (or more time on the stationary phase).

Table 3: The following chart is helpful in predicting the order of elution.:

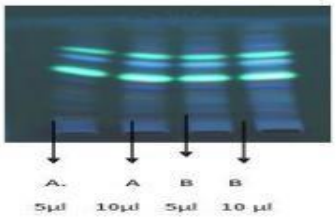
S. No	Solvent	Eluent Strength
1	N- Pentane	0.00
2	Hexane	0.01
3	Cyclohexane	0.04
4	Carbon tetrachloride	0.18
5	Toluene	0.29
6	Chloroform	0.40
7	Methylene Chloride	0.42
8	Tetrahydrofuran	0.45
9	Acetone	0.56
10	Ethyl Acetate	0.58
11	Aniline	0.62
12	Acetonitrile	0.65
13	Ethanol	0.88
14	Methanol	0.95
15	Acetic Acid	Large

Example [F,H,V]

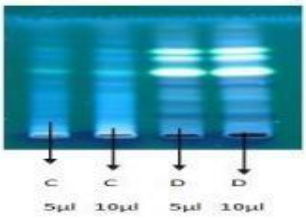
High Performance Thin Layer Chromatography Now a days HPTLC is a very important versatile separation technique in the standardisation of herbal of extract1. It is mainly used for the isolation and identification of herbal extract and its constituents. So, in the present study an attempt was taken to separate and identify the phytocomponents of herbal extract mixture and it was compared with individual extract development of chromatogram. A rectangle twin trough glass chamber was used in the experiment. To avoid insufficient chamber saturation and the undesirable edge effect, a smooth filter paper was placed in the glass chamber and was placed in the glass chamber and was allowed to be soaked in the developing solvent.

Procedure:

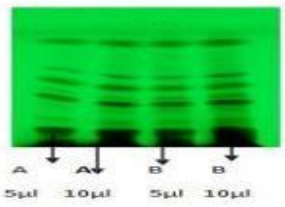
The plate was dipped in a saturated chromatographic chamber containing the solvent system and was allowed to elute up to 8 cm and was air dried. The result for HPTLC analysis of Terminalia belerica & Herbal extract mixture were shown:



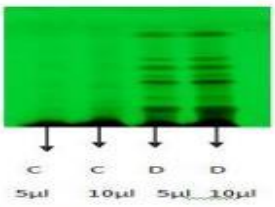
A. *Terminalia belerica*
B. Herbal extract mixture



C. *Solanum xanthocarpum*
D. Herbal extract mixture



A. *Terminalia belerica*
B. Herbal extract mixture



C. *Solanum xanthocarpum*
D. Herbal extract mixture

- HPTLC of Aqueous extract of Solanum xanthocarpum and Herbal Extract Mixture (5µl at 254nm).

HPTLC of Aqueous extract of Solanum xanthocarpum and Herbal Extract Mixture [5µl at 366nm].

The plants were extracted with solvents of increasing polarity. The herbal extract was standardised by physicochemical analysis. The results provide a protocol for identification and authentification of drugs in the herbal extract mixture and standardisation of bioactive constituents. The presented finger print and chromatogram of HPTLC analysis of herbal extract mixture provided a data for identification and standardisation of bioactive constituents. In conclusion, this present work supports the earlier claims of plant for the treatment of diabetes.

This study also provides evidence that aqueous extract of plants Terminalia belerica : Solanum xanthocarpum (70:30) have both physical and Phytochemical data are proof for standardization of herbal extract mixture.

High-Performance Liquid Chromatography (HPLC):

HPLC is a robust analytical technique mainly used for the qualitative analysis of non-volatile classes of compounds such as phenolics, terpenoids, and alkaloids. It is highly efficient and provides rapid and better analytical separation with higher sample loading capacity. The application of liquid chromatography is the qualitative or quantitative estimation of a particular composition of samples obtained from natural sources. The results of the qualitative analysis are evaluated based on the consistency in retention time of reference standards and the compounds in the analyzed sample. Quantitative estimation is done based on the standard curve generated after reference standards are injected at different concentration levels. Sample derivatization is not required before analysis in this method The column is a major component of HPLC. The column contains the particles used as stationary phase. The stationary phases usually have a particle size ranging between 3 and 50 µm packing contained in a column with 2–5 mm bore size. Most of the separations are carried out on reversed-phase (RP) columns. RP columns used in HPLC are more desirable and widely used for the analysis of multiple phytoconstituents. Routine HPLC methods use RP octadecyl silica columns for phenolic compounds. Silica-based C18 columns in RP-HPLC contain aliphatic C18 ligands, free silanols, water and mobile phase modifier.

PRINCIPLE: The basic principle of HPLC in normal phase and reverse phase mode is adsorption. The sample is introduced into HPLC column,different components of the sample move according to their affinities towards the stationary phase.

- ✞ Components having high affinity gets adsorb on stationary phase while components having less affinity towards stationary phase travels faster down the column.
- ✞ As no two components have the same adsorption and affinity towards stationary phase the components get separated by this method.

A detection unit (e.g. UV detector) recognizes the analytes after leaving the column. The signals are converted and recorded by a data management system (computer software) and then shown in a chromatogram. After passing the detector unit, the mobile phase can be subjected to additional detector units, a fraction collection unit or to the waste. In general, a HPLC system contains the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and a data processing unit. The solvent (eluent) is delivered by the pump at high pressure and constant speed through the system. To keep the drift and noise of the detector signal as low as possible, a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by the injection valve.

Table 4: Types of HPLC Techniques:

Characteristic	Normal Phase	Reverse Phase
Stationary Phase	Polar [silica gel]	Non Polar [C_{18}]
Mobile Phase	Non Polar	Polar
Mechanism	Adsorption	Partition
Compound eluted first	Non Polar	Polar

HPLC is a widely used technology for analysing medicinal herbs since it is simple to learn and use and is not limited by the volatility or stability of the sample. Reversed-phase (RP) columns are the most commonly used columns in the analytical separation of herbal medicines. HPLC– DAD is now used in almost all analytical laboratories throughout the world. The qualitative examination of complicated samples in herbal medicines becomes considerably easier than before with the addition of UV spectral information. The use of LC–MS and HPLC–DAD in the analysis of herbal medicines has clearly increased in recent decades. Several good reviews on the investigation of bioactive chemical components in plants and herbal medicines have been published, with HPLC, specially hyphenated HPLC techniques, being the most commonly used approach. Thanks to hyphenation techniques, which allowed one to identify the chromatographic peaks directly on-line by correlation with reported literature or with standard compounds, in most cases the LC–DAD–MS became a successful way for the rapid identification of constituents in herbals, and it can be used to avoid the time-consuming isolation of all compounds to be identified.

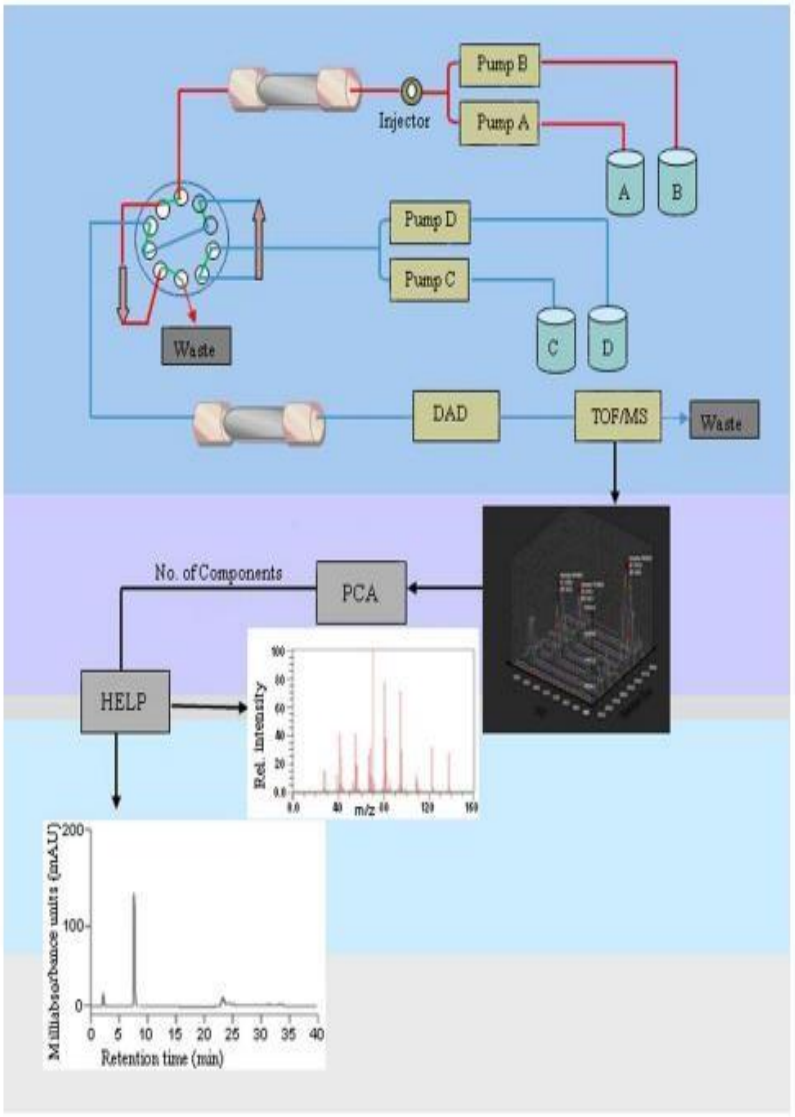


Fig. 2 3D data obtained from 2D LC/MS (HPLC–HPLC–TOF/MS) and resolution of data by employing PCA and HELP.

Example.

Table 5: Example of analytes evaluated by HPLC

Herbal drug extract	Active compounds	Column	Mobile phase	Flow rate	Gradient	Detector	Stop time	Inj vol.
Atropa Belladona	Atropine	4.6 x 75 mm Zorbax Eclipse XDBC18, 3.5 µm	A = 0.05M KH2P04in water (pH = 3), B = acetonitrile	1.0 ml/min	At 0 min 10 % B At 20 min 60 % B At 23 min 60 % B At 25 min 10 % B	UV[diode array detector 210 nm/16 (ref. 360 nm/100), standard cell]	25 min	5 µl
Cortex Cinchonae	Quinidine Quinine	4 x 125 mm Purospher RP18, 5 µm	A = 0.05M KH2P04in water (pH = 3), B = acetonitrile	0.8ml/min	At 0 min 4 % B At 25 min 10 % B At 45 min 30 % B At 46 min 60 % B At 49 min 60 % B At 50 min 4 % B	UV[diode array detector 210 nm/16 (ref. 360 nm/100), standard cell]	50 min	5 µl

Ephedra Sinica	Ephedrine Norephedrine	4.6 x 75 mm Zorbax SBC18, 3.5 µm	A = 0.025M KH2P04in water (pH = 3), B = acetonitrile	1.0 ml/min	At 0 min 2 % B At 10 min 10 % B At 15 min 80 % B At 18 min 80 % B At 20 min 2 % B	UV[diode array detector 210 nm/16 (ref. 360 nm/100), standard cell]	20 min	5 µl
Ginko Biloba	Quercetin Kaempferol	4 x 125 mm Hypersil ODS, 5 µm	A = 0.5 % H 3P04 in water, B = methanol	2.0 ml/min	At 0 min 38 % B At 12 min 48 % B At 17 min 100 % B At 20 min 38 % B	Diode array detector 370 nm/16 (ref. off), standard cell	20 min	10 µl
Rheum Palmatum	Rhein Emodin	4 x 125 mm Hypersil ODS, 5 µm	A = 0.05 M NH 4 0Ac in water (pH = 2.5), B = acetonitrile	1.0 ml/min	At 0 min 30 % B At 10 min 80 % B At 14 min 80 % B At 15 min 30 % B	Diode array detector 440 nm/16 (ref. off), standard cell	15 min	1 µl

Ultra-High Performance Liquid Chromatography (UHPLC):

In recent years, UHPLC has been emerging as a feasible technique for the quality control of herbal products. UHPLC can withstand a pressure of at most 8000 psi and it brings liquid chromatographic analysis to another level by hardware modifications of the conventional HPLC machinery. UHPLC makes it possible to perform high resolution separations superior to HPLC analysis by using solid phase particles of less than 2 µm in diameter to achieve superior sensitivity and resolution. Smaller particle size leads to higher separation efficiency and shorter columns size leads to shorter analysis time with little solvent consumption. Within a period of last few years, UHPLC fingerprints of herbal products were developed instead of conventional HPLC approach. In comparison to HPLC, UHPLC analyses reported a decreased analysis time by a factor up to eight without loss of information. The results obtained not only showed decreased analysis time but also proved a great enhancement in selectivity compared to conventional HPLC analysis.

Hydrophilic Interaction Chromatography (HILIC):

HILIC has gained attention in herbal fingerprinting because of good separation quality of hydrophilic compounds. Many of polar compounds of herbal medicines are extracted by using aqueous solution, which might be better separated by means of HILIC. HILIC was introduced as an alternative for normal-phase liquid chromatography (NPLC); HILIC enables the separation of polar compounds on polar stationary phases with aqueous mobile phases. It is based on the principle of partitioning between a water-enriched layer in the hydrophilic stationary phase and a relatively hydrophobic mobile phase usually containing 5–40% water in organic solvent. This technique is more eco-friendly as compared to NPLC because of the use of water and polar organic solvents as mobile phase. In addition, the polar compounds are more soluble in the mobile phase of HILIC. As HILIC is a relatively recent technique, few papers analyzing herbal products have been published yet. Most papers usually describe a methodology exploiting the orthogonal character of the HILIC and reversed-phase liquid chromatography (RPLC) methods for quality control.

Gas Chromatography (GC-MS):

GC equipment can be directly interfaced with rapid scan mass spectrometer of various types. GC and GC-MS are unanimously accepted methods for the analysis of volatile constituents of herbal medicines, due to their sensitivity, stability and high efficiency. Especially, the hyphenation with MS provides reliable information for the qualitative analysis of the complex constituents. The flow rate from capillary column is generally low enough that the column output can be fed directly into ionization chamber of MS. The simplest mass detector in GC is the Ion Trap Detector (ITD). In this instrument, ions are created from the eluted sample by electron impact or chemical ionization and stored in a radio frequency field; the trapped ions are then ejected from the storage area to an electron multiplier detector. The ejection is controlled so that scanning on the basis of mass-to-charge ratio is possible. The ions trap detector is remarkably compact and less expensive than quadrapole instruments. GC-MS instruments have been used for identification of hundreds of components that are present in natural and biological system. GC is a well-established analytical technique commonly used for the characterization, quantization and identification of volatile compounds. It can be used in many different fields such as pharmaceuticals, cosmetics and even environmental toxins. Since the samples have to be volatile, human breath, blood, saliva and other secretion containing large amounts of organic volatiles can be easily analyzed using GC. The powerful separation efficiency and sensitive detection make GC a useful tool for the analysis of essential oil. Despite its advantages, GC analysis of herbal products is usually limited to the essentials oils because of possible degradation of thermo-labile compounds and the requirement of volatile compounds makes GC unsuitable for many herbal compounds. GC can be used to determine the identity of natural products containing complex mixtures of similar compounds. For example, the geographic source of crude oil or natural gas can be determined by the fingerprint or relative distribution of major and trace compounds in each oil. Naturally produced oil such as food products and fragrances, can be identified by GCFID or GCMS. For example,

Sample	Carrier	Column	Injection	Detector
0.25 cc natural gas sample	Helium (8.6ml/min,60^{0}C)	HP-PLOT Q, (30 m X 0.53 mm X 40 µm)	Split mode (100ml/min)	TCD 250 ^{0}C

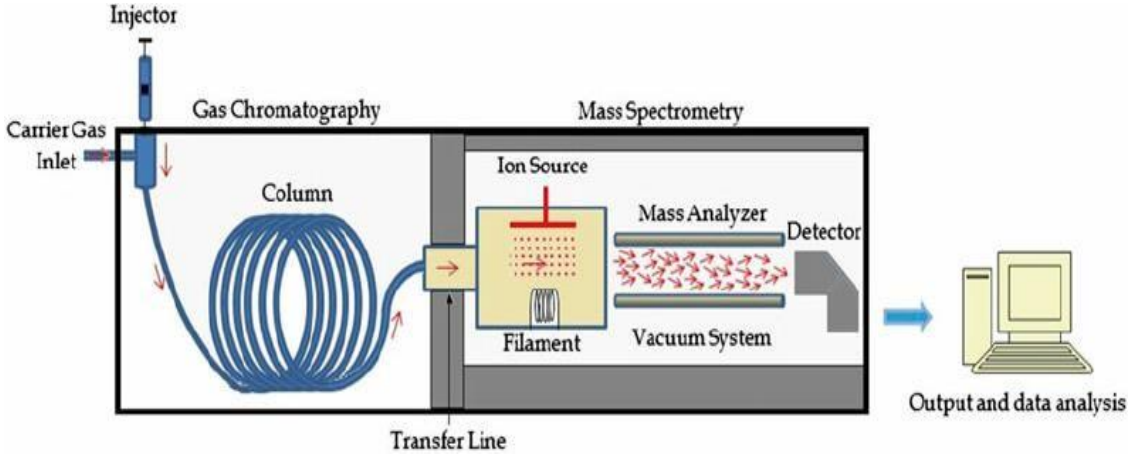


Fig. Schematic diagram of GC-MS

Gas Chromatography- Flame Inonization Detector [GC-FID]:

A number of detectors are used in gas chromatography. The most common are the flame ionization detector (FID) and the thermal conductivity detector (TCD). Coupling capillary column gas chromatographs with Fourier Transform Infrared Spectrometer provides a potent means for separating and identifying the components of different mixtures. Both are sensitive to a wide range of components, and both work over a wide range of concentrate.

While TCDs are essentially universal and can be used to detect any component other than the carrier gas (as long as their thermal conductivities are different from that of the carrier gas, at detector temperature), FIDs are sensitive primarily to hydrocarbons, and are more sensitive to them than TCD. However, an FID cannot detect water. Both detectors are also quite robust. Since TCD is non-destructive, it can be operated in-series before an FID (destructive), thus providing complementary detection of the same analytes.

Supercritical Fluid Chromatography (SFC):

Supercritical fluid chromatography is a hybrid of gas and liquid chromatography that combines some of the best features of each. SFC permits the separation and determination of a group of compounds that are not conveniently handled by either gas or liquid chromatography. SFC has been applied to a wide variety of materials including natural products, drugs, food and pesticide. These compounds are either non volatile or thermally labile so that GC procedures are inapplicable or contain no functional group that makes possible detection by the spectroscopic or electrochemical technique employed in LC.

Liquid Chromatography- Mass Spectroscopy (LC-MS)

LC-MS has become method of choice in many stages of drug development. Recent advances includes electrospray, thermospray, and ionspray ionization techniques which offer unique advantages of high detection sensitivity and specificity, liquid secondary ion mass spectroscopy, later laser mass spectroscopy with 600 MHz offers accurate determination of molecular weight proteins, peptides. Isotopes pattern can be detected by this technique.

Advantages of LC–MS method: The method offers various advantages over other chromatographic methods, which are described below:

a) Selectivity: Co-eluting peaks can be isolated by mass selectivity and are not constrained by chromatographic resolution.

b) Peak assignment: A molecular fingerprint for the compound under study is generated, which ensures correct peak assignment in the presence of complex matrices.

c) Molecular weight information: Confirmation and identification of both known and unknown compounds even with low concentrations of the sample.

d) Structural information: Controlled fragmentation pattern enables structural elucidation of a chemical compound.

e) Rapid method development: Providing easy identification of eluted analytes without retention time validation.

f) Sample matrix adaptability: Decreasing sample preparation time and hence less timeconsuming.

g) Quantitation: Quantitative and qualitative data can be obtained simultaneously with limited instrument optimization.

h) Cost effective: The capacity to multiplex several analytes within a single analytical run with minimal incremental cost. This method has the potential to simplify laboratory set-up (e.g. creation of test panels) and provides additional useful information (e.g. metabolite profiles).

Liquid Chromatography- Nuclear Magnetic Resonance (LCNMR):

LC-NMR improves speed and sensitivity of detection and found useful in the areas of pharmacokinetics, toxicity studies, drug metabolism and drug discovery process. The combination of chromatographic separation technique with NMR spectroscopy is one of the most powerful and time saving method for the separation and structural elucidation of unknown compound and mixtures, especially for the structure elucidation of light and oxygen sensitive substances. The online LC-NMR technique allows the continuous registration of time changes as they appear in the chromatographic run automated data acquisition and processing in LCNMR improves speed and sensitivity of detection. The recent introduction of pulsed field gradient technique in high resolution NMR as well as three-dimensional technique improves application in structure elucidation and molecular weight information. These new hyphenated techniques are useful in the areas of pharmacokinetics, toxicity studies, drug metabolism and drug discovery process..

•Commercial production of herbal medicines and their trade are the fastest growing sector of industry today, due to increasing demand of medicinal plants; the supply line is adversely affected leading to the adulteration and substitution for genuine drugs.

1. Fluorescence quenching: When a plant extract is spotted on a fluorescent silica gel layer and exposed to UV light, it appears as a spot on a fluorescent background, thus causing quenching and is directly proportional to the concentration of the extract. The silica gel GF plate was used as an adsorbent for fluorescence quenching. Solvents took hexane toluene, ether, ethyl acetate, butanol, methanol and water.

2. Use of fingerprinting and marker compounds for identification and standardization of botanical drugs: Chemical and chromatographic techniques may be used to aid in identification of an herbal material or extract. Chromatographic technique such as HPLC, TLC, GC and capillary electrophoresis and spectroscopic methods such as IR, NMR and UV may also be used for fingerprinting. DNA fingerprinting has been widely used in many species, e.g. DNA fingerprinting of Panax species and their adulterants. Marker compounds may be used to help identify herbal materials, set specifications for raw materials, standardize botanical preparations during all aspects of manufacturing processes and obtain stability profiles.

3. Determination of stigmasterol, beta-sitosterol and stigmastanol in oral dosage forms using HPLC with evaporative light scattering detection: A validated and repeatable HPLC method with online evaporative light scattering was developed for the analysis of two sterols, stigmasterol, beta-sitosterol and a stanol found to be common in many herbal formulations and health care supplements. This method was used to assay commercially available products formulated as oral dosage forms purported to contain African potato and associated sterols and stanol.

4. Elemental analysis of herbal preparations for traditional medicines by neutron activation analysis with the kO standardization method: Medicinal herb preparations prescribed for specific treatment purposes were purchased from markets and were analysed by instrumental neutron activation analysis with kO standardization. 500- 700mg of each sample was palletized under a pressure of six tones and irradiated together with monitors for alpha and neutron flux ratio determination for about 6h in a thermal flux of 2.29 x 10 n/cm2 /s.

5. Liquid chromatography UV-determination and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry characterization of sitosterol and stigmasterol in soya bean oil: A narrow bore HPLC-UV method was developed for the analysis of two of the most abundant naturally occurring phytosterols in vegetable oils: sitosterol and stigmasterol. The method enabled detection of the compounds at a concentration of 0.42µ/ml and quantization at a concentration of 0.52 and 0.54µ/ml for sitosterol and stigmasterol, respectively.

6. Simultaneous determination of cinnamaldehyde, eugenol and paeonol in traditional Chinese medicinal preparations by capillary GC-FID: A capillary GC method was established for simultaneous determination of cinnamaldehyde (CNMD), eugenol (EL) and paeonol (PL) in two traditional Chinese herbal medicinal preparations, Wei Tong Ding tablet (WTDT) and Guifu Dihuang pill (GDHP). The assays were based on a programmed temperature GC in a 30m x 0.53mm capillary columns with nitrogen as the carrier and FID detector. Good linearity was obtained over ranges of 0.45-0.452mg/l CNMD, 0.31-0.625mg/l EL and 0.30-610mg/l PL, respectively.

7. HPTLC fingerprinting of marketed formulation containing Shankhpushpi: These are the important Ayurveda formulations used for perinatal care of mother and child health. Standardization of churnas was carried out by organoleptic study, phytochemical analysis; qualitative organic and inorganic analysis, thin layer chromatography, UV visible spectrophotometer and HPLC fingerprint studies. Qualitative organic analysis of both the churnas revealed the presence of alkaloids, steroids, phenols, tannins, glycosides, resins, saponins and flavonoids.

CONCLUSION

The Indian herbal industry is growing in a tremendous rate. More number of herbal products was arrived in the market. The safety and efficacy of herbal products were dependent upon the standardization of these herbal drugs. The traditional approach towards standardization was insufficient for current herbal market and hence there was need for more advanced techniques for standardization. There, basically two techniques used for standardization these were Chromatographic fingerprinting and DNA fingerprinting. The chromatographic fingerprinting was based on the chromatographic separation and identification of marker compound from other constituents. For these purpose TLC, HPTLC, HPLC, LC-MS, LC-NMR, GC-MS, GCFID and SFC methods was used. The other method used was DNA fingerprinting. As the DNA fingerprint of genome remain the same irrespective of the plant part

used while the phytochemical content will vary with the plant part used, physiology and environment, hence this was well established and highly precious method for standardization of herbal drug.

Quality control of herbal medicines aimed to ensure its quality, safety and efficacy. Chemical markers were pivotal in the current practice of quality control. Chemical markers should be used at various stages of the development and manufacturing of a herbal medicine, such as authentication and differentiation of species, collecting and harvesting, quality evaluation, stability assessment, diagnosis of intoxication and discovery of lead compounds. Lack of chemical markers remained a major problem for the quality control of herbal medicines. In many cases, we did not have sufficient chemical and pharmacological data of chemical markers. Furthermore, there were many technical challenges in the production of chemical markers. For example, temperature, light and solvents often cause degradation and/or transformation of purified components; isomers and conformations may also cause confusions of chemical markers.

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