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Review Article

# TRADITIONAL AND MODERN APPROACHES FOR STANDARDIZATION OF HERBAL DRUGS: A REVIEW

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**ABSTRACT**

Nearly 80% of the world population use herbal medicine and World Health Organisation (WHO) also encourages, recommends and promotes the inclusion of herbal drags in national health care programmes because such drags are easily available at a price within the reach of common man and as such are time tested and thus considered to be safer than modem synthetic drags. Herbal drug is a main constituent in traditional medicine and a common constituent in ayurvedic, homeopathic, naturopathic and other medicine systems. Since the herbal formulations are of mainly plant origin, they are susceptible to contamination from different sources, detoriation and variations of chemical composition. Therefore to ensure the safety and efficacy of herbal medicines, standardization and development of quality control protocols for herbal medicines is extremely important. For the identification of medicinal plants and their constituents, WHO guidelines provide the fingerprinting methods to meet the global standards of quality control of the herbal formulations. Standardization in itself involves many parameters like gross morphology, microscopy, physical parameters, chemical fingerprinting, chromatographic fingerprinting, spectroscopic fingerprinting, DNA marker finger printing etc. The standardization of herbal drugs may give acceptance by worldwide moreover it improves the therapeutic efficacy and safety of the drugs. The present review article includes physical, chemical and various analytical methods of analysis as well as modern approaches employed in the standardization of herbal drugs/formulations.

**Keywords :**-World Health Organization, Standardization, Ayurvedic, Quality control protocol, Spectroscopic fingerprinting.

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## INTRODUCTION

Herbal medicines formed the basis of health care throughout the world since the ancient time of mankind and are still widely used. The recognition of clinical, pharmaceutical and economic value of herbs is still growing in each country of the world. Medicinal plants are important for pharmacological research and drug development. Medicinal plants also provides leads materials for the synthesis of drugs or as models for pharmacologically active compounds. Exploitation and exportation of the important of medicinal plants and its active constituents requires strict regulation, together with international cooperation and coordination for their conservation so as to ensure their availability for the future [1].

Since ancient times herbal drugs are of great significance for treating various diseases. Regular advancement is seen in modern medicine in recent decades, plants have important contribution in all over

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the world in traditional system which was used for treating disease from many centuries. The herbal drugs define as whole or plant parts, algae, and fungi in unprocessed state usually in dried form but sometimes fresh[2]. Herbal drugs referred as plants materials or herbalism, involves the use of whole plants or parts of plants, to treat injuries or illnesses [3]. Herbal drugs are use of therapeutic herbs to prevent and treat diseases and ailments or to support health and healing [4]. These are drugs or preparations made from a plant or plants and used for any of such purposes. Herbal drugs are the oldest form of health care known to mankind. Herbal drugs are chief fundamental in traditional medicinal system such as Ayurveda, homeopathic, neuropathic and other medicinal systems [5].

World Health Organization (WHO) has explain herbal drugs as complete, labeled medicinal products that have vigorous ingredients, aerial or secretive parts of the plant or other plant material or combinations. As per WHO estimation 80% of the world populations currently use herbal drugs for major healthcare [6].

The use of herbal drugs due to toxicity and side effects of allopathic medicines, has led to rapid increase in the number of herbal drug manufacturers. For the past few decades, herbal drugs have been more and more consumed by the people with no prescription. Seeds, leaves, stems, bark, roots, flowers, and extracts of all of these have been used in herbal drugs over the millennia of their use. Herbal products have reached extensive adequacy as beneficial agents like antimicrobial, antidiabetic, antifertility, antiageing, antiarthritic, sedative, antidepressant, antianxiety, antispasmodic, analgesic, anti-inflammatory, anti-HIV, vasodilatory, hepatoprotective, treatment of cirrhosis, asthma, acne, impotence, menopause, migraine, gall stones, chronic fatigue, Alzheimer's disease and memory enhancing activities.

**Classification of Herbal Medicine**

Based on their origin evolution and the forms of current usage-

- Category 1: Indigenous herbal medicines.
- Category 2: Herbal medicines in systems
- Category 3: Modified herbal medicines
- Category 4: Imported products with a herbal medicine base

**Importance of standardization in current scenario**

Herbal formulations show the number of problems when quality control aspect is considered. This is because of nature of the herbal ingredients and different secondary metabolites present therein. It is also due to variation in the chemical profile of the herbal due to intrinsic and extrinsic factors (growing, harvesting, storage and drying processes) [7,8,9].

There are two main important reasons for interest of development of standardization and quality aspect of the herbal products. Firstly, the use of medicinal plants, as such as phyto-medicines, dietary supplements, food and beverage ingredients and traditional medicines. Secondary, natural product continues remain as important source of new drug discovery. Quality is the sum of variable characteristics that significantly impact upon a product. For herbal medicines, such variable characteristics include the origin of the herb, botanical identity, purity, potency, stability and content of the marker compounds. Apart from these, good agriculture practices (GAP) and good manufacturing practices (GMP) are also important and directly assess the quality of the herbal products [10,11].

Keeping the trade of herbal products, the maintaining quality of these products is a challenging task. To meet this challenge, standardization and quality assessment of herbal products as per international norms will be inevitable. Standardization of natural products is a complex task due to their heterogeneous composition, which is in the form of whole plant. To ensure reproducible quality of herbal products, proper control of starting material is utmost essential.

**Standardization**

Numerous today's medicines are directly or indirectly derived from higher plants. Safety and efficacy should be the basic requirements for all medicines, whether they are derived synthetically or of plant origin. Standardization of herbal medicines is the process of prescribing a set of standards or inherent characteristics, constant parameters, and definitive qualitative and quantitative values that carry assurance of quality, efficacy, safety, and reproducibility. Quality of raw materials, good agricultural practices, and good manufacturing practices play fundamental roles in guaranteeing the quality and stability of herbal preparations. 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 herbal medicine. Hence, standardization is a tool used in the quality control process [12]. Standardization is an imperative stride in establishing a quality assurance plan for production and manufacturing thereby, curtailing batch to batch variation and reassuring acceptability, safety, quality and efficacy of the polyherbal formulations [13,14]. The validation of plant based drugs and recognition of adulterants from authentic curative herbs are important for both pharmaceutical industries and

community health. Technological advancements which take place in the processes of isolation, purification and structural elucidation of natural compounds have made it probable to generate appropriate strategies for the analysis of quality and standardization of plant based medicines. An appliance of highly oriented hyphenated techniques provides a definite tool in herbal investigations. A variety of sophisticated methods such as spectrophotometric, chromatographic, polarography, electrophoresis, and the use of molecular biomarkers in fingerprints are presently employed in standardization of plant based medicines[15].

**WHO Guidelines for Quality Standardized Herbs and Herbal formulations** [16]

World Health Organization (WHO) has certain standards for herbal drugs. It involves the following parameters.

**Crude drug identification**

Botanical characters, sensory evaluation, foreign organic matter, microscopic, histological, histochemical assessment, quantitative measurements

**Physicochemical parameter of the drug**

Physical and chemical identity, fingerprints chromatography, ash values, extractive values, moisture content, volatile oil and alkaloids tests, quantitative estimation protocols,

**Pharmacological parameters**

Estimation of biological activity, the values of bitterness, astringency hemolytic index, a factor swelling, foaming index,

**Toxicity**

Detail-toxicity pesticides residues, heavy metals, microbial contamination as viable count total, pathogens such as E. coli, Salmonalla, P. aeroginosa, S. aureus, Enterobacteriaceae,

**Microbial and radioactive contamination**

Total viable count, Pathogens, Aflatoxins, Radioactive contamination.

The objectives of these guidelines are to provide:

- Guiding principles for assessing the quality in relation to the safety of herbal medicines with specific reference to contaminants and residues.
- Model criteria for use in identifying possible contaminants and residues.
- Examples of methods and techniques
- Examples of practical procedures for controlling the quality of finished products.

**Protocols for standardization of herbal drugs**

Most of the regulatory guidelines and pharmacopoeias suggest macroscopic and microscopic evaluation and chemical profiling of the botanical materials for quality control and standardization With respect to this, Department of AYUSH Govt. of India gave some parameters for Drug Development, Standardization & Quality of Ayurveda, Siddha and Unani drugs, which include five protocols as follows Protocol-I (Standardization of Single Plant Material), Protocol-II (SOP of Preparation of Extracts), Protocol-III (Standardization of Plant Extract), Protocol-IV (SOP of Finished Product) and protocol-V (Standardization of Formulations) [17,18]. In order to assure a consistent and acceptable quality herbal product, care should be taken right from the identification and authentication of herbal raw materials to the verification process of final product. The following parameters are recommended (Table 1).

**Authentication**

It is the first and important step of standardization. Each and every step has to be authenticated i.e. area of collection of drug, parts of plant collection, the regional situation as morphological, botanical identity, microscopic and histological analysis. To ensure and enhance the quality of herbal medicines, the Government of India has notified Good Manufacturing Practices under Schedule 'T' of the Drugs and Cosmetics Act 1940 which also ensures raw materials used in the manufacture of drugs are authentic, of prescribed quality and are free from contamination.

**Macroscopic evaluation**

For convenience to description of macroscopic characters may be divided into four headings

a) Shape and size
b) Colour and external marking
c) Fracture and internal colour
d) Odour and taste. .

Organoleptic evaluation of crude drugs refers to the evaluation of a drug by colour, odour, taste, size and shape, occasionally the sound or snap of fracture and special features including touch, texture, etc. The fractured surface in cinchona, quillia and cascara barks and quassia wood are important characteristics. Aromatic odour of umbellifrous fruits and sweet taste of liquric are the example of this evaluation. The ovoid tears of gum acacia ribbon shaped characterizes of tragacanth disc shaped structure of nux vomica concial shape of aconite quills of cinchona.

**Microscopic Evaluation**

Microscopy inspection of the herbal drug is

valuable both powder and crude drugs. The types of certain factors such as epidermal parenchyma, stomata, trichomes, fibers, vessels and calcium oxalate crystals, help identification of drugs. In quantitative microscopy determination such as veinislet number, veinlet termination numder, palisade ratio, stomatal number, stomatal index and determination of size of trichomes, fibers, and vessels help in the identification of the herbal drugs.

**Determination of foreign matter**

Herbal drugs should be made from the stated part of the plant and be devoid of other parts of the same plant or other plants. They should be entirely free from moulds or insects, including excreta and visible contaminant such as sand and stones, poisonous and harmful foreign matter and chemical residues. Animal matter such as insects and "invisible" microbial contaminants, which can produce toxins, are also among the potential contaminants of herbal medicines [20]. Weigh a sample of plant material, taking the quantity indicated above unless other-wise specified in the test procedures for the plant material concerned. Spread it in a thin layer and separated the foreign organic matter into groups either by visual inspection, using a magnifying lens (6x or 10x). After complete separation foreign organic matters was weighed and calculate the content of each group in grams per 100 g of air-dried sample.

**Ash values**

Ash value is used to determine the quality and purity of the drug and to establish its identity. Ash contains inorganic radicals lie phosphates, carbonates, and silicates of sodium, potassium, magnesium, calcium, etc. These are present in definite amount in a particular crude drug, hence quantitative determination in terms of various ash values helps in their standardization. To determine ash content the plant material is burnt and the residual ash is measured as total and acid-insoluble ash. Total ash is the measure of the total amount of material left after burning and includes ash derived from the part of the plant itself and acid-insoluble ash. The latter is the residue obtained after boiling the total ash with dilute hydrochloric acid, and burning the remaining insoluble matter. The second procedure measures the amount of silica present, especially in the form of sand and siliceous earth [38].

**Determination of extractive value**

The determination of water soluble or ethanol soluble extractive matter is used as a means of evaluating drugs. Extractive value determined the amount of active constituents in a given amount of medicinal plant material when extracted with solvent. It is employed for that material for which no chemical or biological assay method exist. It can be determined as alcohol soluble extractive and/or water soluble extractive.

**Loss on drying (LOD)/ Moisture content**

Moisture is an expected component of crude herbal drug, which must be eliminated as far as practicable. Drying of crude drug is important during collection of drug and is also important for preservation, preventing hydrolytic degradation of active constituents and for easy size reduction of crude drug. Excess moisture or insufficient drying is responsible for spoilage of drug due to growth of microbes. There for drying process should reduce the moisture content of drug below the critical level. It is determined by various methods viz. Loss on drying, Karl Fisher reagent method, Azeotropic distillation method, Halogen balance etc.

**Foaming index**

The saponins are high molecular weight containing phyto-constituents having the detergent or soap like property. Many herbal drugs contain saponins that can cause persistent foam when shaken with water. The foaming ability of herbal drugs and their extracts is measured in terms of foaming index. Saponins give persistent foam when shaken with water. Hence, plant material/extract containing saponins is evaluated by measuring the foaming ability in terms of foaming index

**Swelling index**

Most of herbal drugs are of specific therapeutic properties or pharmaceutical utility because of their swelling properties, especially gums and those containing an appreciable amount of mucilage, pectin or hemicelluloses. The swelling index is the volume in ml taken up by the swelling of 1 g of plant material under specified conditions. Its determination is based on the addition of water or a swelling agent as specified in the test procedure for each individual plant materials. It gives an idea about the mucilage content of the drug; hence it is useful in the evaluation of crude drugs containing mucilage.

**Determination of 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. Samples of herbal material are extracted by a standard procedure, impurities are removed by partition and/or adsorption, and individual pesticides are measured by different analytical methods. Some simple procedures have been published by the WHO [22] and the European Pharmacopoeia has laid down general limits for pesticide residues in medicine (Table 2) [23].

**Determination of Heavy Metals**

Contamination by toxic metals can either be accidental or intentional. Contamination by heavy metals such as mercury, lead, copper, cadmium, and arsenic in herbal remedies can be attributed to many causes, including environmental pollution, and can pose clinically relevant dangers for the health of the user and should therefore be limited. A simple, straightforward determination of heavy metals can be found in many pharmacopeias and is based on color reactions with special reagents such as thioacetamide or diethyldithiocarbamate, and the amount present is estimated by comparison with a standard. Instrumental analyses have to be employed when the metals are present in trace quantities. The potential intake of the toxic metal can be estimated on the basis of the level of its presence in the product and the recommended or estimated dosage of the product. This potential exposure can then be put into a toxicological perspective by comparison with the so-called Provisional Tolerable Weekly Intake values (PTWI) for toxic metals, which have been established by the Food and Agriculture Organization of the World Health Organization (FAO-WHO) (Table 3) [24,25].

**Determination of Microbial Contaminants and Aflatoxins**

Medicinal plants may be associated with a broad variety of microbial contaminants, represented by bacteria, fungi, and viruses. Risk assessment of the microbial load of medicinal plants has therefore become an important subject in the establishment of modern Hazard Analysis and Critical Control Point schemes. Herbal drugs normally carry a number of bacteria and molds, often originating in the soil. Poor methods of harvesting, cleaning, drying, handling, and storage may also cause additional contamination, as may be the case with *Escherichia coli* or *Salmonella* spp. While a large range of bacteria and fungi are from naturally occurring microflora, aerobic spore-forming bacteria frequently predominate. Laboratory procedures investigating microbial contaminations are laid down in the well-known pharmacopeias, as well as in the WHO guideline. Limit values can also be found in the sources mentioned. In general, a complete procedure consists of determining the total aerobic microbial count, the total fungal count, and the total Entero-bacteriaceae count, together with tests for the presence of *Escherichia coli*, *Staphylococcus aureus*, *Shigella*, and *Pseudomonas aeruginosa*and *Salmonella* spp. The European Pharmacopoeia also specifies that *E. coli* and *Salmonella* spp. should be absent from herbal preparations (Table 4) [26].

**Chromatographic methods of standardization**

The standardization of herbal drugs 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. Chromatography and spectroscopic methods of analysis especially by hyphenation of chromatography with various spectroscopic 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 [44].

**Thin layer chromatography (TLC)**

Thin-layer chromatography is, at present, the most popular method in the authentication of traditional herbal medicines. TLC is used as an easier method of initial screening with a semi quantitative evaluation together with other chromatographic techniques. It provides visible, UV images or fluorescent ones. Compared with the column chromatography, it has an additional colour parameter. This method identifies several samples at the same time. In TLC fingerprinting, the data that can be recorded using a high-performance TLC 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 has the advantages of many-fold possibilities of detection in analyzing herbal medicines. TLC is being employed extensively for the following reasons i.e. it enables rapid analysis of herbal extracts with minimum sample clean-up requirement, second, It provides qualitative and semi quantitative information of the

resolved compounds. It enables the quantification of chemical constituents [27]. Micro-emulsion TLC development leads to increased separation efficiency and signal enhancement and resulting in an improved sensitivity. Other accepted modifications in herbal analysis include TLC densitometry, two-dimensional TLC and coupled-layer planar chromatography (graft-TLC) [28].

**High performance thin layer chromatography (HPTLC)**

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 foods [29]. HPTLC is the common fingerprint mainly used to analyze the compounds which is having low or moderate polarities. It has been well reported that several samples can be run simultaneously by use of a smaller quantity of mobile phase than in HPLC [30]. 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 multi-component formulation [31]. With this technique, authentication of various species of plant possible, as well as the evaluation of stability and consistency of their preparations from different manufactures.

**High performance liquid chromatography (HPLC)**

HPLC is a popular method for the analysis of herbal medicines because it is easy to learn and use. Moreover is not limited by the volatility or stability of the sample compound. In general, HPLC can be used to analyze almost all the compounds in the herbal medicines. The applicability of HPLC is much wider than that of GC. Equipped with different mobile phases and detectors, HPLC can detect the majority of organic compounds. Preparative and analytical HPLC are widely used in pharmaceutical industry for isolating and purification of herbal compounds. There are basically two types of preparative HPLC: low pressure HPLC (typically under 5 bar) and high pressure HPLC (pressure >20 bar). The important parameters to be considered are resolution, sensitivity and fast analysis time in analytical HPLC whereas both the degree of solute purity as well as the amount of compound that can be produced per unit time i.e. throughput or recovery in preparative HPLC. In preparative HPLC (pressure >20 bar), larger stainless steel columns and packing materials particle size 10-30 μm are needed [32]. Reversed phase columns are the most popular columns used in the analytical separation of herbal medicines. In order to obtain better separation, some new techniques are developed in research field of liquid chromatography like Micellarelectrokinetic capillary chromatography, High speed counter current chromatography, Low pressure size exclusion chromatography, Reversed phase ion-pairing HPLC and strong anion exchange HPLC. One of the main advantages of HPLC is the possibility to make hyphenation with different detectors such as ultraviolet for UV absorbing compounds [33], and diode array detector for herbal fingerprinting [34], evaporative light scattering detector [35] and chemo-luminescence detectors for non-UV absorbing compounds, NMR for metabolomic profiling [36] and mass spectrometry for identification of the separated compounds [37].

**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 8000psi 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 mm 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 [38]. In comparison to HPLC, UHPLC analyses reported a decreased analysis time by a factor upto eight without loss of information [39]. The results obtained not only showed decreased analysis time but also proved a great enhancement in selectivity compared to conventional HPLC analysis.

**Gas chromatography (GC)**

GC is a well established analytical technique commonly used for the characterization, quantization and identification of volatile compounds. The powerful separation efficiency and sensitive detection make GC a useful tool for the analysis of essential oils [40]. Despite its advantages, GC analysis of herbal products is usually limited to the essentials oils because of possible degradation of thermolabile compounds and the requirement of volatile compounds makes GC unsuitable for many herbal compounds [41]. 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 [42]. The GC analysis of the volatile oils has a number of advantages. Firstly, the GC of the volatile oil gives a reasonable "fingerprint" which can be used to identify the plant. The composition and relative concentration of the organic compounds in the volatile oil are characteristic of the particular plant and the presence of impurities in the volatile oil can be readily detected. Secondly, the extraction of the volatile oil is relatively straightforward and can be standardized and the components can be readily identified using GC–MS analysis [43]. The non-volatile substances can be also analyzed by GC with precolumn derivative technology, by pyrolysis GC or by flash GC. A number of detectors are used in gas chromatography. The most common are the flame ionization detector (FID) and the thermal conductivity detector (TCD). Both are sensitive to a wide range of components, and both work over a wide range of concentrations.

**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. 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 inorganic solvent. This technique is more eco-friendly as compared to normal-phase liquid chromatography 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 [27].

**Super critical fluid chromatography (SFC)** [44]

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 nonvolatile 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.

**Spectroscopic method** [45,46]

Rapid identification and characterization of known and new natural products directly from plant and marine sources without the necessity of isolation and purification can be achieved by various spectroscopy techniques.

**Ultraviolet Spectroscopy**

This is mainly used to establish the class and/or structural type to which the herbal drugs being investigated belongs. Such assignments are made because ultraviolet spectrum of a compound is not a characteristic of the whole molecule but only of the chromophoric system(s) present.The usual practice is to record the ultraviolet spectra of a very large number of different types of herbal drugs. Then, the data are analyzed and categorized with respect to structure correlation. Each groups of drug having a particular chromophoric system benzene, pyridine, indole, quinoline, etc. yields characteristic absorption maxima and extinction coefficients.Therefore, the comparison of these data with standard data may allow the identification of the exact nature of the aromatic or heterocyclic system in the herbal drugs. The part of the molecule containing the electrons involved in the electronic transition which gives rise to an absorption is called the chromophore. The wavelength of the maximum of the broad absorption is labelled$\lambda$max.

**Infrared spectroscopy**

In infrared spectroscopy method infrared radiation is passed of through a sample and measuring the amount of radiation transmitted or reflected by the sample. Infrared is divided in to three regions namely-near infrared, mid infrared far infrared. In standardization of herbal drugs, it is mainly used to ascertain the presence and sometimes the absence of particular functional group.The presence of aldehyde, ketone, alcohols, phenols, ester, amide, lactone, carboxylic acid, carbonyl groups and primary and secondary amines can rapidly be identified and distinguished by comparison of the observed frequencies with those reported for structural related compounds. One can also ascertain the presence of O-methyl, N- methyl and aromatic groups from the infra-red spectrum of an alkaloid but the quantitative analysis of such groups is best accomplished by Nuclear Magnetic Resonance spectroscopy.

**Nuclear Magnetic Resonance (NMR) Spectroscopy**

Radiation in the radio-frequency region is used to excite atoms, usually protons or carbon-13 atoms, so that their spins switch from being aligned with to being aligned against an applied magnetic field. The range of frequencies required for excitation and the complex splitting patterns produced are very characteristic of the chemical structure of the molecule. NMR has application in determination of impurities and minor components in mixtures because of ease, speed and specificity of analysis

**Mass spectroscopy (MS)**

Mass spectroscopy is concerned with electron ionization, subsequent fragmentation of molecules, determination of mass to charge ratio and relative abundances of ions which produced. This technique is quite useful because it gives quite useful information about the chemical components present in herbal drugs like the molecular weight, the empirical formula by accurate mass measurement of the molecular ion, and knowledge of the molecular structure by comparison of the fragmentation pattern with those of analogous system. Most of the success has been achieved in the case of polycyclic indole alkaloids because the indole nucleus of these substances gives rise to an abundant, stable molecular ion which subsequently undergoes decomposition by highly specific bond fusion involving the acyclic portion of the molecule containing the other nitrogen atom(s).

**Hyphenation of chromatography and spectroscopy Techniques**

Chromatographic separation techniques can be coupled to various detection techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR), infrared spectroscopy (IR) etc. These hyphenated techniques provide information about the structure of the compound present in chromatogram and thus provide higher sensitivity in comparison to conventional approaches.

**Gas chromatography-Mass spectroscopy (GC-MS)**

GC-MS instruments have been used for identification of hundreds of components that are present in natural and biological system. The combination of GC and MS gives an efficient separation and detection. GC-MS is 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. Nowadays, the analysts turn to gas chromatography as a powerful separation method and combined it with mass spectrometry to aid identification. The GC-MS technique, along with improved data handling tools-will immediately be relevant to the essential oil area [47]. A number of detectors are used in gas chromatography.

**Gas chromatography-flame ionization 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 (Sharma). Both are sensitive to a wide range of components, and both work over a wide range of concentrations. 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 [45].

**Gas Chromatography Fourier Transform Infrared spectrometry (GC-FTIR)**

Coupling capillary column gas chromatographs with Fourier Transform Infrared Spectrometer provides a potent means for separating and identifying the components of different mixtures [48].

**Liquid chromatography-mass spectrometry (LC-MS)**

Liquid chromatography-mass spectrometry is the technique which performs separation by liquid chromatography and mass analysis with the help of the mass spectrometry. With the help of HPLC impurities and degradation products can be separated and Mass Spectrometry allows us to obtain the molecular weight and identification of the same. LC-MS is highly selective and sensitive technique. LC-MS leads to detection and identification of chemicals in presence of other chemicals therefore it is called as specific. The flow rate of HPLC is around 1ml/min which is difficult to accommodate in mass spectrometry vacuum system also the diluents which is used has to be vaporized which leads to damage of the thermally labile compounds by excessive heating. In Pharmaceutical industry 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 [49].

**Liquid chromatography-nuclear magnetic resonance spectrometry (LC- NMR )** [50]

Liquid chromatography-nuclear magnetic resonance spectrometryis the hyphenated technique in which HPLC is combined with the NMR. This technique is widely used for the analysis of complex mixtures which contain unknown impurities, natural products and synthetic polymers. 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 LC-NMR 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.

**Others methods for standardization of herbal drugs**

**Thermal Analysis of Herbal Drugs**

Thermo gravimetric analysis (TGA), differential thermal analysis (DTA) or differential scanning calorimetry (DSC) have been employed to study any physical or chemical changes in various products including herbal drug and also used to study drug excipient compatibility [51].TGA may be operated under sub ambient conditions to analyze ethanol in herbal formulations such as asavas and arista [52].TGA and DTA analysis of mercury based Indian traditional metallic herbal drug Ras-sindoor indicated the presence of mercury sulphide based on a sharp peak at 3500 C which corresponded to melting temperature of mercury sulphide [53]. The optimized extraction obtained by distillation showed the presence of volatile oil in dry ginger as a component of volatile oil-β-cyclodextrin inclusion compound using DTA. DSC thermo gram data confirmed the formation of phospholipids complex with emodin (an anthraquinone) and naringen.

**Differential pulse polarography (DPP)**

Differential pulse polarography can be used to study trace amounts of chemicals with detection limits on the order of $10^{-8}$M. Some heavy metals, including Pb, Cd, Zn, Cu and Fe were successfully identified and determined in chamomile and calendulea flowers by DPP [54,55]. Accumulation of heavy metals, namely Pb, Cd, Cu and Zn was estimated in market a well as genuine samples of important herbal drugs of India viz., Alpiniagalanga, Artemesiaparviflora, Buteamonosperrma, Coleus forskohlii, Curcuma amada,Euphorbia prostrate, Leucasaspera, Malaxisaccuminata and Pueraria tuberose. The concentration of Pb and Cd was found beyond the WHO permissible limits in most samples [56]. Trace amounts of selenium in Chinese herbal medicines [57] and flavonoids in small amount of medicinal herb samples were determined by DPP [58]. A DPP method has been for the determination of total hypericin in phytotherapeutic preparations (drops, tablets and capsules) in various buffer systems over the pH range 3.5–10.0 [59].

**Chemometrics**

Chemometrics is the application of mathematical and statistical techniques to retrieve more information from the chromatographic data [60]. The International Chemometrics Society defines chemometrics as the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods [61]. Chemometrics is used in optimizing experimental procedures, extracting useful information from the chromatographic data and resolution of the mixture into linear components. Chemometrics is found to be a useful tool in estimating the quality of herbal drugs [62,63]. Keeping in view of the complexity of the chromatographic fingerprint and the irreproducibility of chromatographic and spectral instruments and experimental conditions, several chemometric techniques in herbal drug standardization such as principal component analysis (PCA), linear discriminate analysis (LDA), spectral correlative chromatography (SCC), information theory (IT), local least square (LLS), heuristic evolving latent projections (HELP) and orthogonal projection analysis (OPA) are employed to deal with the chromatographic fingerprint. The basic principles for this approach are variation determination of common peaks/ regions and similarity comparison with similarity index and linear correlation coefficient. To facilitate the data processing, software named

Computer Aided Similarity Evaluation (CASE) has been developed. All programs of chemometric algorithms for CASE are coded in METLAB5.3 based on windows. Data loading, removing, cutting, smoothing, compressing, background and retention time shift correction, normalization, peak identification and spectral matching, variation determination of common peaks/regions, similarity comparison, overly of sample classification and other data processes associated with the chromatographic fingerprint can be investigated with this software [64].

**DNA markers fingerprinting**

DNA-based techniques have been widely used for authentication of plant species of medicinal importance. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phyto-chemically indistinguishable.DNA as molecular markers have several advantages over typical phenotypic markers and are reliable for informative polymorphisms as the genetic composition is unique for each species and is not affected by age, physiological conditions as well as environmental factors [65] . DNA can be extracted from fresh or dried organic tissue of the botanical material hence the physical form of the sample for assessment does not restrict detection. Based on the specificity of the genotype of a system, a particular DNA profile being unique can be ascribed to a particular organism. Hence, various DNA markers based methods can be used for species characterization and adulteration detection in medicinal plants. In the past authentication by DNA profiling and their patents have been reviewed by several authors [66,67,68]. Various types of DNA-based molecular techniques [69] are utilized to evaluate DNA polymorphism. These are

- Hybridization-based methods,
- Polymerase chain reaction (PCR)-based methods and
- Sequencing-based methods.

**Hybridization-based methods**

Hybridization-based methods include restriction fragment length polymorphism [70] and variable number tandem repeats [71]. Labelled probes such as random genomic clones, cDNA clones, probes for micro satellite [72]and minisatellite [73] sequences are hybridized to filters containing DNA, which has been digested with restriction enzymes. Polymorphisms are detected by presence or absence of bands upon hybridization.

**Polymerase chain reaction (PCR)-based methods**

Polymerase chain reaction based markers involve in vitro amplification of particular DNA sequences or loci, with the help of specific or arbitrary oligonucleotide primers and the thermo stable DNA polymerase enzyme. PCR-based techniques where random primers are used, include random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP–PCR) [74]and DNA amplification fingerprinting (DAF). Inter simple sequence repeats (ISSRs) [75] polymorphism is a specific primer-based polymorphism detection system, where a terminally anchored primer specific to a particular simple sequence repeat (SSR) is used to amplify the DNA between two opposed SSRs of the same type. Polymorphism occurs whenever one genome is missing in one of the SSRs or has a deletion or insertion that modifies the distance between the repeats. A recent approach known as amplified fragment length polymorphism (AFLP) [76] is a technique that is based on the detection of genomic restriction fragments by PCR amplification. Adaptors are ligated to the ends of restriction fragments followed by amplification with adaptor-homologous primers. AFLP has the capacity to detect thousands of independent loci and can be used for DNAs of any origin or complexity.

**Sequencing-based markers**

DNA sequencing can also be used as a definitive means for identifying species. Variations due to transversion, insertion or deletion can be assessed directly and information on a defined locus can be obtained. Genetic variation occurs extensively at the single nucleotide level. Direct sequencing can efficiently identify such single nucleotide polymorphisms that usually depend on how closely related are the organisms being compared. Other sequencing based strategies include analysis of the variable internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA). The ITS region of 18s–26s rDNA has proved to be a useful sequence for phylogenetic studies in many angiosperm families. The level of ITS sequence variation suitable for phylogenetic analysis is found at various taxonomic levels within families, depending on the linkage. A number of researchers have also sequenced other regions of DNA such as trnK of chloroplast and spacer region of 5s rDNA as diagnostic tools for authentication [77].

**Metabolomic Techniques**

In the area of genomic sequencing, a newest field of functional genomics i.e. "Metabolomics" comes in the picture which is specifically focused on the biochemical complement of cells and tissues. Metabolomics is defined as a comprehensive

quantitative and qualitative analysis of all metabolites present in a specific cell, tissue, or organism. The metabolome represents all metabolites collection in a biological organism, which are mainly the products of its gene expression[78]. The metabolomes represent the life history of an individual organism, including age and environmental factors such as soil type, moisture content, temperature, and stress factors. The study involving the detailed analysis of these metabolomes is referred to as "metabolomics," which is a newly emerging area in natural product research in the postgenome era. The two major approaches in metabolomics are the targeted (a specific set of metabolites) and untargeted (all metabolites) the metabolite analyses. Targeted metabolite analysis or metabolite profiling targets a subset of metabolites in a sample, instead of a complete metabolome analysis, using a particular set of analytic techniques or hyphenated analytical techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) etc. Metabolite analysis armory have some modern analytical techniques including Fourier transform ion cyclotron mass spectrometry (FTMS), Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy, and nuclear magnetic resonance (NMR). MS is currently the most widely applied technology in metabolomics. Among a variety of MS techniques, GC-MS has long been used in metabolite profiling of human body fluids or plant extracts. The untargeted method, also known as the chemometric, involves spectral profiling combined with multivariate statistical analyses to identify spectral features within different set of samples[79].

The data processing challenges in this technique are quite unique and often require specialized (or expensive) data analysis software. A number of metabolomics databases, based on chemical/spectral data, or chemical and biological/ biochemical data, have recently been made are publicly available. The databases include the BioMagResBank (BMRB), the Madison Metabolomics Consortium Database (MMCD), MassBank.jp for high-field mass spectral data, the GolmMetabolome Database (GMD and BiGG provide sufficient spectral, chemical and biological insight into metabolic profiles are the future of development in computational metabolomics. Optimization of chromatographic and spectral method of analysis with better sensitivity instruments, with higher resolution capability and greater mass accuracy, will be essential for future improvements in metabolomics. A proper integration of information from genomics, proteomics, and metabolomics is expected to provide solid evidence-based scientific rationales for the development of modern phytomedicines and its authentication [80]. Metabolomics has been used for identification of active phyto-constituents from herbal medicine. Metabolomic approach was employed to identify the chemical constituents in Sophoraflavescens, which were further analyzed for their effect on Pregane X receptor activation and Cytochrome P3A regulation. The greater potential of metabolomics has been reported in the development of active secondary metabolites from medicinal plants as novel or improved phytotherapeutic agents [81]. The recent studies showed that NMR based metabolomics approach combined with orthogonal projections to latent structure discriminate analysis identified the purity of an herbal medicine [82].

**Table 1. Protocols for standardization of herbal drugs**

| | |
|---|---|
| **1. Authentication, macroscopic and microscopic studies** | it involved authentication on the basis of taxonomic, macroscopic and microscopic studies. |
| **2. Physical parameters** | Physical tests include organoleptic evaluation (sensory characters such as taste, appearance, odor, feel of the drug, etc.), viscosity, moisture content, pH, disintegration time, friability, hardness, flow ability, sedimentation, and ash value. |
| **3. Pesticide residue analysis** | Standard limits of pesticides have been set by WHO and FAO (Food and Agricultural Organization). Some common pesticides that cause harm to human beings, such as DDT, BHC, toxaphene, and aldrin, should be analyzed. |
| **4. Heavy metal analysis** | Toxic metals such as Cu, Zn, Mn, Fe, and particularly Cd, As, Pb and Hg should be analyzed. In the analysis of metals, their speciation is to be taken into consideration. |
| **5. Aflatoxin analysis** | Aflatoxins are group of toxic compounds produced by certain molds, especially *Aspergillusflavus*. Aflatoxin is the strongest known naturally occurring carcinogen. Among 18 different types of aflatoxins identified, major members are aflatoxin B1, B2, G1 and G2. |

| | |
|---|---|
| **6. Microbiological parameters** | Microbiological contamination can be measured according to methods described in the *Romanian Pharmacopoeia*, as well as in the *British Pharmacopoeia*. Microbiological analysis includes analysis of limits of *E. coli* and molds, total viable aerobic count, total enteriobacteria and their count, aflatoxin analysis. |
| **7. Chromatographic and spectroscopic evaluation** | Sophisticated modern techniques of standardization such as UV–vis spectrophotometry, TLC, HPTLC, HPLC, NMR, near infrared spectroscopy provide quantitative and semi-quantitative information about the main active constituents or marker compounds present in the crude drug or herbal products. Markers play an important role in fingerprinting of herbs. Quality of drug can also be assessed by chromatographic fingerprint. |

**Table 2. Limits for Pesticides residues**

| S.N. | Name of Pesticides/Insecticides | Limit as per FDA/ EP |
|---|---|---|
| **1.** | Quinolphos | 0.01 ppm |
| **2.** | DDE (Di-chloro-di-phenyl-di-chloro-ethylene) | 1.00 ppm |
| **3.** | Alderin | 0.05 ppm |
| **4.** | Dieldrin | 0.05 ppm |
| **5.** | DDT (Dichloro-Diphenyl-Trichloroethane) | 1.00 ppm |
| **6.** | DDD (Di-chloro-di-phenyl-di-chloro-ethane) | 1.00 ppm |
| **7.** | HCH (Hexachlorocyclohexane) | 0.30 ppm |
| **8.** | Malathion | 0.10 ppm |
| **9.** | Parathion | 0.30 ppm |

**Table 3. Limits for heavy/toxic metals**

| S. N. | Heavy/ Toxic Metals | As per WHO / FDA(Permissible limit) |
|---|---|---|
| **1.** | Lead | 10.0 ppm |
| **2.** | Cadmium | 30.0 ppm |
| **3.** | Mercury | 1.00 ppm |
| **4.** | Arsenic | 10.0 ppm |

**Table 4. WHO limit for number of micro-organisms per gram of material**

| Type of microorganism | Finished product | Raw material |
|---|---|---|
| E.coli | $10^1$ | $10^4$ |
| Salmonella | -- | -- |
| Total aerobic bacteria | $10^3$ | -- |
| Enterobacteria | $10^3$ | -- |

## CONCLUSIONS

The standardization of herbal drugs may give acceptance by worldwide moreover it improves the therapeutic efficacy and safety of the drugs. The standardization gives a clear picture about the intrinsic value of the drug i.e. the amount of medicinal principles and constituents present, presence or absence of adulterants etc. Most of the countries explain medicinal plants or its derived products in different ways and have adopted different legal procedure to licensing, dispensing, manufacturing and trading to ensure their safety, quality and efficacy. The increase uses of herbal drugs require the good method of standardization for their great acceptance. Latest technology and software used in computer has been providing reproducible information without any error. The major problem of quality assurance of herbal medicines has been solved to a great extent with the help of chromatographic and spectroscopic fingerprint analysis. Chromatographic and spectroscopic technologies are still two main methods for establishing the fingerprint, including TLC, HPLC, GC, CE, IR, NMR, as well as DNA fingerprinting. Nowadays, more and more hyphenated technologies are used to obtain much more information, such as GC-MS, HPLC-MS, and LC-NMR. Especially, the hyphenation of MS with HPLC or GC has been a very useful means to the chemical constituents' analysis, quality control and metabolite studies, etc. Chromatographic and spectral fingerprint profiling delivers precise and effective information regarding qualitative as well as quantitative

information of herbal drugs under investigation. With the global increase in the demand for plant-derived medicine as an alternative to synthetic medicine, there is a need to ensure the quality of the herbal drugs using modern analytical techniques, for therapeutic efficacy and safety. It is an urgent requirement from the scientists all over the world that their contribution towards the development of new techniques and instruments by using concept of the traditional and modern methods. New researches and techniques are still awaited in the field of standardization of herbal medicines.

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**CONFLICT OF INTEREST**

No interest

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