A Comprehensive Review On Herbal Drug Standardization

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ABSTRACT

Now days more importance has been given to use medicinal plant products in healthcare system. It fulfills worldwide need of alternative medicine so traditional systems of medicine becomes more and more popular. It involves Proper combination of modern scientific techniques and traditional knowledge. The quality of herbal products is checked through stability testing studies which depends on various factors, such as temperature, humidity, light, oxygen, moisture, other ingredient, microbial contamination, trace metal contamination, leaching from the container, etc. Therefore such studies involves various types of evaluation such as chemical, physical, microbiological, therapeutic and toxicological studies can serve as an important tool in stability studies. Standardization of herbal drugs means confirmation of its identity, Quality and purity. The present study includes review of various standardization parameters with their effectivity towards the quality herbal drugs. Present article also overviews various well-designed methodologies, techniques such as Chromatography, Spectroscopic tech. for the standardization of herbal raw materials and herbal formulations.

Keywords: Herbal drug, Standardization of herbal formulation, well-designed methodologies, Stability studies.

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INTRODUCTION

Herbal drug:
Finished label products that contain active ingredients such as aerial and underground parts of plant or other plants material or combinations thereof, whether in the crude state or as plant preparations.
The term “herbal drugs” denoted by means of plant or part of plants that have been converted into phytopharmaceuticals by simply means of processes involving collection or harvesting, drying and storage. Phytomedicine and phytopharmaceuticals sold as over the counter (OTC) products in modern dosage forms such as tablets, capsules and liquids for oral use.

Herbal drugs are of two types:
Single/ crude drug
Multiple herbal formulations

Single /crude drugs:
All mainly whole, fragment or cut plant, plant parts usually dried forms, but sometimes fresh. It also includes algae, fungi and lichen.

Multiple herbal formulations:
Formulations are obtained by subjecting herbal ingredients to various manufacturing process such as extraction, distillation, expression, fractions, partition, chromatography and formulations.

Figure 1: Multiple herbal formulations

Standardization of Herbal drug:
“Standardization is a system to ensure that every packet of medicine that is being sold has the correct amount and will induce its therapeutic effect (Chaudhry, 1992).” Standardization of herbal formulations is essential in order to assess of quality drugs, based on the concentration of their
active principle, physical, chemical, physcio-chemical standardization and in vitro, in-vivo parameters[1]. Natural products have been our single most successful source of medicines. Each plant is like factory capable of synthesizing unlimited no of highly complex and unusual chemical substance whose structure covered otherwise escape the imagination forever. It is necessary to maintain reproducible efficacy and safety of phyto pharmaceutical therefore if phytopharmaceutical have to regard as rational drug should be standardized and pharmaceutical quality must be approved.

**Advantages of herbal drugs**

1. Low cost of production.
2. They may have fewer side effects.
3. Effective with chronic condition.
4. Wide spread availability.

**Disadvantages of herbal drugs**

1. Lack of dosage instruction.
2. Poison risk associated with wild herbs.
3. Can interact with other drugs.
4. Inappropriate for many condition.
5. Some are not safe to use.

**STANDARDIZATION OF HERBAL MEDICINES – CONCEPT AND SCOPE:**

Generally, all medicines, whether they are synthetic or of plant origin, should fulfil the basic requirements of being safe and effective (EMEA, 2005; WHO, 2002c, 1998c, 1996, 1991a,b, 1990, 1988). The term “herbal drugs” denotes plants or plant parts that have been converted into phytopharmaceuticals by means of simple processes involving harvesting, drying, and storage (EMEA, 1998). Hence they are capable of variation. This variability is also caused by differences in growth, geographical location, and time of harvesting.

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 herbal medicine. Hence standardization is a tool in the quality control process.

Several problems not applicable to synthetic drugs often influence the quality of herbal drugs. For instance:
1. Herbal drugs are usually mixtures of many constituents.
2. The active principle(s) is (are), in most cases unknown.
3. Selective analytical methods or reference compounds may not be available commercially.
4. Plant materials are chemically and naturally variable.
5. Chemo-varieties and chemo cultivars exist.
6. The source and quality of the raw material are variable.

The methods of harvesting, drying, storage, transportation, and processing (for example, mode of extraction and polarity of the extracting solvent, instability of constituents, etc.) also affect herbal quality. At present no official standards are available for herbal preparations. Those manufacturers, who are currently doing some testing for their formulations, have their own parameters, many of which are very preliminary in nature. Presently it is very difficult to identify the presences of all the ingredients as claimed in a formulation. Hence the first important task is to evolve such parameter by which the presence of the entire ingredient can be identified, various chromatographic and spectrophotometric methods and evaluation of physicochemical properties can be tried to evolve pattern for identifying the presence of different ingredient. Wherever possible these methods can be applied for quantitative estimation of bioactive group of compounds like alkaloids, flavonoids, polyphenolic components or estimation of particular compound (Wani, 2007).

**Need of Standardization of Herbal drug:**

Modern system of medicine is based on sound experimental data, toxicity studies and human clinical studies. But, Pharmacopoeial standards on raw material / finished products are not available. cGMP for herbal industry are not well defined nor are the barest minimum standards of medicinal plant products maintained or regulated. The lack of quality standards has resulted in mild to serious adverse effects ranging from hepatic toxicity to death. Hence, herbal ingredients require tools for determining identity, purity and quality and tools have to be technically sufficient, rapid and cost effective with GMP requirements. World health organization has set specific guidelines for the assessment of safety, efficacy and quality of herbal medicines. Standardization of herbal drug is not an easy task as numerous factors influence the bio efficacy, reproducible therapeutic effect. In order to obtain quality oriented herbal product care should be taken right from the proper identification of plants, season, area of collection, their extraction and purification and rationalizing the combination in case of polyherbal drugs.
METHODS OF STANDERDIZATION OF HERBAL DRUGS: [4-7]

METHODS:

Figure 2: Methods of herbal standardization

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:
Macro and microscopic examination:
For identification of right variety and search of adulterants.

Foreign organic matter:
This involves removal of matter other than source plant to get the drug in pure form.

Ash values:
These are criteria to judge the identity and purity of crude drug – Total ash, sulphated ash, water soluble ash and acid insoluble ash etc.

Moisture content:
Checking moisture content helps reduce errors in the estimation of the actual weight of drug material. Low moisture suggests better stability against degradation of product.

Extractive values:
These are indicative weights of the extractable chemical constituents of crude drug under different solvents environment.

Crude fibre:
This helps to determine the woody material component, and it is a criterion for judging purity.

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.

Chromatographic examination:
Include identification of crude drug based on the use of major chemical constituents as markers.

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.

The processes mentioned above involves wide array of scientific investigations, which include physical, chemical and biological evaluation employing various analytical methods and tools. The specific aims of such investigation in assuring herbal quality are as varied as the processes employed.
**Organoleptic or macroscopic evaluation:**
Organic evaluation of drugs by means of organs of sense (skin, eye, tongue, nose, and ear) or microscopic evaluation which include evaluation of drugs by color, odor, taste, size, shape, and special feature, like touch, texture, etc. It is the technique of qualitative evaluation based on the study of morphological and sensory profile of whole drugs.

The fractured surfaces in cinchona, quillia, and cascara barks and quassia wood are important characteristics. Aromatic odor of umbelliferous fruits and sweet taste of liquorices are the examples of this type of evaluation where odor of drugs depends upon the type and quality of odorous principles (volatile oils) present.

**Figure 3: Organoleptic evaluation of herbal drugs**

**Microscopic evaluation:**
It involves detailed examination of the drugs and it can be used to identify the organized drugs by their known histological characters. It is mostly used for qualitative evaluation of organized crude
drugs in entire and powder forms with help of microscopic. Using microscope detecting various cellular tissues, trichomes, stomata, starch granules, calcium oxalate crystals and aleuronic grains are some of important parameters which play important role in identification of certain crude drugs standardization. Starch and hemicelluloses is identified by blue color with iodine solution. All lignified tissues give pink strain with phloroglucinol and HCl etc. mucilage is stained pink with ruthenium red can be used to distinguish cellular structure. Microscopic evaluation also includes study of constituents in the powdered drug by the use of chemical reagents. Quantitative aspects of microscopy includes study of stomata number and index, palisade ratio, vein-islet number, size of starch grains, length of fibres etc which plays a very important role in the identification of drug.

Figure 4: Microscopic evaluation of herbal drugs
Chemical evaluation:
Most of drugs have definite chemical constituents to which their biological or pharmacological activity is attributed. Qualitative chemical test are used to identify certain drug or to test their purity. Isolation, purification, identification of active constituents is based on chemical methods of evaluation.

- Evaluation test of resins: acid value, sulphated ash
- Evaluation test of balsams: acid value, saponification value, bester values.
- Evaluation test of volatile oils: acetyl and ester values
- The qualitative chemical tests are useful in identification of chemical constituents and detection of adulteration.

Physical evaluation:
Physical constants are sometimes taken into consideration to evaluate certain drugs. These include moisture content, specific gravity, optical rotation, refractive, melting point, viscosity and solubility in different solvents. All these physical properties are useful in identification and detecting of constituents present in plants.

Biological evaluation:
Some drugs have specific biological and pharmacological activity which is utilized for their evaluation. Actually this activity is due to specific type of constituents present in the plant extract. For evaluation the experiments were carried out on both intact and isolation organs of living animals. With the help of bioassays, strength of drug in its preparation can be evaluated.

Chromatography techniques:
❖ TLC (Thin layer chromatography):
TLC was the most common, versatile methods of choice for herbal analysis before instrumental chromatography methods like gas chromatography and HPLC were established. Even now a day’s TLC is still frequently used for the analysis of herbal medicines since various pharmacopeias such as Indian herbal pharmacopeia, Ayurvedic pharmacopeias, American herbal pharmacopeias, and Chinese drugs monographs. Rather TLC is used as an easier method of initial screening with a semi-qualitative evaluation together with other chromatography techniques as there is relative less change in the simple TLC separation of herbal medicines the with instrumental chromatography. TLC is a technique in which solute undergoes distribution between two phases a stationary phase acting through adsorption and mobile phase in the form of liquid. The adsorbent is relatively thin, uniform layer of drug finely powdered material apply to glass, plastic, metal sheet/plate. Glass
plates are the mostly commonly used. Separation may also be achieved on the basis of partition /a combination of partition and adsorptions depending upon the particular support its use with different solvent. Identification can be effected by observation of spots of identical Rf value and equal magnitude obtained, respectively with an unknown and a reference sample chromatography on the sample plate. A visual comparison of the size and the intensity of spots usually serve for semi-quantitative estimation. TLC has advantages of many folded possibilities of detecting in analysis herbal medicines. In addition TLC is rather simple and can be employed for multiple sample analysis. For each plate more than 30 spots of sample can be studied. CA MAG video stored system and TLC QA-UV methods it’s is possible to get useful qualitative and quantitative information from the developed TLC plates. For example the four sample of cordyceps sinensis from that joint product of china and Japan co-operation has more valuable medicinal effect compared to other as they contain the most effective component “cordycepine” more over with the help of imagine analysis and digitized technique developed in computer science, evaluation of similarities between different samples is also possible. TLC is being employed extensively for the following reasons:

- It enables rapid analysis of herbal extracts with minimum sample clean-up requirement.
- It provides qualitative and semi-quantitative information of the resolved compounds.

In TLC fingerprinting, the data that can be recorded using a high-performance TLC (HPTLC) scanner which includes information like 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 fingerprinting was done on the methanolic extract of Sitopaladi churna for determination of piperine using Silica Gel G plate and Toluene: Ethyl acetate: Formic acid (5:3.5:0.5 v/v/v) as mobile phase. Retention factor of piperine was found to be 0.69 (shown by peak 7) at 342 nm.
Figure 5: Thin-layer chromatography (TLC) fingerprint profiles of guggul (oleogum resin of Commiphora wightii). a Under ultraviolet (UV) 254 nm; b under UV 366 nm; after derivatization with anisaldehyde-sulphuric acid reagent - c under UV 366 nm; d in natural light. Tracks 1 and 2, sample; track 3, E-guggulsterone standard; track 4 Z-guggulsterone standard

Electrophoretic method:
Capillary electrophoresis was introduced in early 1980s as a powerful analytical and separation technique and has been developed almost explosively. It allows an efficient way to document the purity/complexity of a sample and can handle virtually every kind of charged sample components ranging from simple inorganic ions to DNA. Thus, there was an obvious increase of electrophoretic methods, especially capillary electrophoresis, used in the analysis of herbal medicines in last decades. The more or less explosive development of capillary electrophoresis since its introduction has to a great extent paralleled that of liquid chromatography. Most of the used techniques are capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE) and capillary isoelectric focusing (CIEF). CE is promising for the separation and analysis of active ingredients in herbal medicines, since it needs only small amount of standard and can analyzed samples rapidly with good separation ability. Also, it is a good tool for producing the chemical fingerprint prints of the herbal medicines, since it has similar technical characteristics of liquid chromatography. Recently, several studies dealing with herbal medicines have been reported and
two kinds of medicinal compounds, i.e. alkaloids and flavonoids, have been studied extensively. In general, CE is a versatile and powerful separation tool with high separation efficiency and selectivity when analyzing mixtures of low-molecular-mass components. However, the fast development in capillary electrophoresis causes improvement of resolution and throughout rather than reproducibility and absolute precision. On successful approach to improve the reproducibility of the both mobility and integral data has been on internal standards. But many papers were published unfortunately revealed the limited images on the real possibilities of CE in the field of finger print herbal medicines.

Gas Chromatography:

Gas chromatography also known as gas liquid chromatography, Its is a technique for separation of mixtures of mixtures into components by a process which depends on the redistribution of the components between a stationary phase or the support material in the form of a liquid, solid or combination of both and a gaseous mobile phase. It is well-known that many pharmacologically active components in herbal medicines are volatile chemical compounds. Thus, the analysis of volatile compounds by gas chromatography is very important in the analysis of herbal medicines. The GC analysis of the volatile oils has a number of advantages. Firstly, the GC of the volatile oil gives a reasonable “finger print” which can be used to identify the plant. The composition and relative concentration of the organic compounds in the volatile oil are the 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 straight forward and can be standardized and the components can be readily identified using the GC-MS analysis. The relatively quantities of the components can be used to monitor or assess certain characteristics of the herbal medicines. Changes in composition of the volatile oil may also be used as indicators of oxidation, enzymatic changes or microbial fermentation. The advantages of GC clearly lie in its high sensitivity of detection for almost all the volatile chemical compounds. Thus, over the past decades, GC is a most popular and useful analytical tool in research field of herbal medicines. Especially, with the use of hyphenated GC-MS instrument, reliable information on the identity of the compounds is available as well.

However, the most serious disadvantages of GC are that it is not convenient for its analysis of the samples of the polar and nonvolatile compounds. For this, it is necessary to use tedious sample work-up which may include derivatization. Therefore, the liquid chromatography becomes another necessary tool for us to apply the comprehensive analysis of herbal medicines.
The first fully automated on-line GC-IR system was developed by Scott et al. Each eluted solute was adsorbed in a cooled packed tube, and then thermally regenerated into an infrared vapor cell. Subsequent to the IR spectrum being obtained, a small sample of the vapor was drawn from the IR cell into a low-resolution mass spectrometer and the mass spectrum was also be taken.

As some of the bioactive constituents of herbal medicines are volatile, GC analysis can often be used for authentication and quality control. The high selectivity of capillary columns enables separation of many volatile compounds simultaneously within comparatively short times. However, the most serious disadvantage of GC is that this method is not convenient for the analysis of samples which are thermo labile and non-volatile.

The identification and quantification of chemical constituents present in poly herbal oil formulation (Megni) was done by GC for determination of Eugenol using DB-5 fused silica capillary column and helium as a carrier gas. The retention time was found to be 8.63 min (Fig. 6).

![Figure 6 GC chromatogram of Eugenol (Rt = 8.63 min)](image)

- **High-Performance Liquid Chromatography (HPLC):**

High performance liquid chromatography is also known as high pressure liquid chromatography in which the stationary phases consists of small particle (3-50μm) packing contained in a column with a small bore (2-5μm), one end of which is attached to a source of pressurized liquid eluent (mobile phase). The three forms of high performance liquid chromatography most often used are
ion exchange, partition and adsorption. HPLC is a popular method for the analysis of herbal medicines. Because it is easy to learn and use and is not limited by the volatile or stability of the sample compound. In general, HPLC can be used to analyze almost all the compounds in the herbal medicines. Reversed-phase (RP) columns may be most popular columns used in the analytical separation of herbal medicines. It is necessary to notice that the optimal separation condition for the HPLC involves many factors, such as the different compositions of the mobile phase, their pH adjustment, pump pressures, etc. Thus, a good experiment design for the optimal separation seems in general necessary. In order to obtain better separation, some new techniques have been recently developed in research field of liquid chromatography. These are micellar electro kinetic capillary chromatography (MECC), high speed counter current chromatography (HSCCC), low pressure size exclusion chromatography (SEC), reversed-phase ion pairing HPLC (RPIPC-HPLC) and strong anion exchange HPLC (SAX-HPLC). They will provide new opportunities for good separation for some specific extracts of some herbal medicines. On the other hand, the advantages of HPLC lie on its versatility for the analysis of the chemical compounds in herbal medicines, however. The commonly used detector in HPLC, say single wavelength UV detector, seems to be unable to fulfill the task, since lots of chemical compounds in herbal medicines are non chromophoric compounds. Consequently, a mark increases in the use of HPLC analysis couples with evaporative light scattering detection (ELSD) in a recent decade demonstrated that ELSD is an excellent detection method for the analysis of non-chromophoric compounds.

This new detector provides a possibility for the direct HPLC analysis of many pharmacologically active components in herbal medicines, since the response of ELDS depends only on the size, shape, and number of the finger prints of the herbal medicines. Moreover, the qualitative analysis or structure elucidation of the chemical components in herbal drugs by simple HPLC is not possible, as they rely on the application of techniques using hyphenated HPLC, such as HPLC-IR, HPLC-MS, HPLC-NMR, for the analysis of herbal medicines.

**HPLC-DAD:**

It has become a common technique in most analytical laboratories in the world now. With the additional UV spectral information, the qualitative analysis of complex samples in herbal medicines turns out to be much easier than before. For instance, checking peak purity and comparing with the available standard spectrum of the known compound to the one in the investigated sample. Especially, with the introduction of electrospray mass spectrometry, the coupling of liquid chromatography and mass spectrometry has opened the new way to widely and
routinely applied to the analysis of herbal medicines. HPLC chromatography finger prints can be the applied for documentation of complete herbal extracts with more information and on-line qualitative analysis becomes possible. In last decades, the increasing usage of LC-MS and HPLC-DAD in the analysis of herbal medicines is quite obvious. Several good reviews have been published for the analysis of the bioactive chemical compounds in plants and medicines, in which the technique used most in HPLC, especially the hyphenated HPLC technique. Moreover, combined HPLC-DAD-MS technique take advantage of chromatography as a separation method and both DAD and MS as an identification method. DAD and MS can provide on-line UV and MS information for each individual peak in a chromatography. With the help of this hyphenation, in most cases, one could identify the chromatography peaks directly online by compression with literature data. Recently, the hyphenation between HPLC and NMR also available, which might become a vital and an attractive analytical tool for the analysis of herbal medicines. In fact the tendency of the hyphenation or multi-hyphenation of the chromatography with the common used four spectroscopic detectors. Kankasava is a polyherbal formulation prepared with Kanaka and other ingredients which is used in chronic bronchitis, asthmatic cough and breathlessness. A simple, precise, accurate RP- HPLC method was developed for the quantitative estimation of atropine using column RP C-18 (250mm×4.6mm×5 micron) and mobile phase which is mixture of methanol and 10 mM dihydrogen phosphate buffer in a ratio of 50:50 v/v at a flow rate of 1 ml/min, and analysis was screened with UV detector at 254 nm. The retention time for standard atropine sulphate was found to be 4.0667 minutes (Fig. 7).

Figure 7 HPLC chromatogram for atropine sulphate (Rt =4.0667 min)
Chromatographic fingerprinting:
Chromatographic fingerprinting is the most powerful approach for the quality control of herbal medicines. Chromatographic fingerprint of Herbal Medicine is a chromatographic pattern produced from extract of some common chemical components which may be pharmacologically active or have some chemical characteristics. This chromatographic profile should be featured by the fundamental attributions of - integrity and -fuzziness or - sameness and - differences so as to chemically represent the herbal medicines investigated. This suggest that chromatographic fingerprint can successfully demonstrate both sameness and differences between various samples and the authentication and identification of herbal medicines can be accurately conducted even if the number and/or concentration of chemically characteristic constituents are not very similar in different samples of herbal medicine. Thus chromatographic fingerprint should be considered to evaluate the quality of herbal medicines globally; considering multiple constituents present in the herbal medicines. This technique can be employed for identification and authentication as well as for determination of various adulterants and contaminants and for standardization purpose. In contrast to macroscopic, microscopic and other molecular biological methods this technique is not restricted to raw herbs, but can also be applied to pharmaceutical preparations. Chromatographic fingerprinting can be carried out using techniques such as thin layer chromatography (TLC), High performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), Gas chromatography (GC) and other hyphenated techniques.

WHO GUIDELINES:[3,4]
The following WHO technical guidelines have been published:

- WHO guidelines on assessing quality and safety of herbal medicines with reference to contaminants and residues.
- WHO GACP monograph for Artemisia Annua L.
- WHO draft guidelines for the selection of substances for quality control of herbal medicines (outline and key technical issues discussed at two WHO working group meetings in 2004 and 2005).
- WHO GMP: Updated supplementary guidelines for manufacture of herbal medicines.
- Support to national capacity building on quality control of herbal medicines.
- WHO Interregional Training Workshop on GACP and GMP for Herbal Medicines, held in China, September 2005.
Guidelines for the Assessment of Herbal Medicines (WHO, 1991) and Quality Control Methods for Medicinal Plant Materials (WHO, 1998)

FACTORS AFFECTING ON HERBAL DRUG STANDARDIZATION: [7-10]

Microscopic Evaluation:
Quality control of herbal drugs has traditionally been based on the appearance and today microscopic evaluation is indispensable in the initial identification of herbs, as well as, in identifying small fragments of crude or powdered herbs, and detection of foreign matter and adulterants. A primary visual evaluation, which seldom needs more than a simple magnifying lens, can be used to ensure that the plant is of the required species, and that the right part of the plant is being used. At other times, microscopic analysis is needed to determine the correct species and/or that the correct part of the species is present. For instance, pollen morphology may be used in the case of flowers to identify the species, and the presence of certain microscopic structures such as leaf stomata can be used to identify the plant part used. Although this may seem obvious, it is of prime importance, especially when different parts of the same plant are to be used for different treatments. Stinging nettle (Urtica urens) is a classic example where the aerial parts are used to treat rheumatism, while the roots are applied for benign prostate hyperplasia.

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 matters such as insects and “invisible” microbial contaminants, which can produce toxins, are also among the potential contaminants of herbal medicines. Macroscopic examination can easily be employed to determine the presence of foreign matter, although, microscopy is indispensable in certain special cases (for example, starch deliberately added to “dilute” the plant material). Furthermore, when foreign matter consists, for example, of a chemical residue, TLC is often needed to detect the contaminants.

Ash Content:
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.
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. 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). A simple, straightforward determination of heavy metals can be found in many pharmacopoeias and is based on colour reactions with special reagents such as thioacetamide or diethylidithiocarbamate, and the amount present is estimated by comparison with a standard (WHO, 1988a). Instrumental analyses have to be employed when the metals are present in trace quantities, in admixture, or when the analyses have to be quantitative. Generally, the main methods commonly used are atomic absorption spectrophotometry (AAS), inductively coupled plasma (ICP) and neutron activation analysis (NAA).

Microbial contaminants and aflatoxins:
Medicinal plants may be associated with a broad variety of microbial contaminants, represented by bacteria, fungi, and viruses. Inevitably, this microbiological background depends on several environmental factors and exerts an important impact on the overall quality of herbal products and preparations. 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 (HACCP) 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 that frequently predominate. Laboratory procedures investigating microbial contaminations are laid down in the well-known pharmacopoeias, as well as, in the WHO guidelines [20]. Limit values can also be found in the sources mentioned. Generally, a complete procedure consists of determining the total aerobic microbial count, the total fungal count, and the total Enterobacteriaceae count, together with tests for the presence of Escherichia coli, Staphylococcus aureus, Shigella, and Pseudomonas aeruginosa and Salmonella sp. The European Pharmacopoeia also specifies that E. coli and Salmonella spp. should be absent from herbal preparations.
Radioactive contamination:

Dangerous contamination, however, may be the consequence of nuclear accident. The WHO, in close cooperation with several other international organizations, has developed guidelines in the event of a wide spread contamination by radionuclide resulting from major nuclear accidents. These publications emphasize that the health risk, in general, due to radioactive contamination from naturally occurring radio nuclides is not a real concern, but those arising from major nuclear accidents such as the nuclear accident in Chernobyl and Fukushima may be serious and depend on the specific radionuclide, the level of contamination, and the quantity of the contaminant consumed. Taking into account the quantity of herbal medicine normally consumed by an individual, is unlikely to be a health risk. Therefore, at present, no limits are proposed for radioactive contamination

Validation:

The validation of herbal products is a major public health concern both in developed and resource-poor countries, where fakers selling adulterated herbal medicines are common. In this regard, there is no control by the government agencies, despite the existence of certain guidelines in some individual countries and those outlined by the WHO. If the herbal products are marketed as therapeutic agents, and irrespective of whether the products really have any positive effects to cure and reduce the severity of the disease, it is necessary to ensure scientific validation and periodic monitoring of the quality and efficacy by drug control administrators. It is feasible that the introduction of scientific validation would control the production of impure or adulterated herbal products and would eventually ensure their rational use. This could also lead to the regulation of the industry so that only qualified physicians and health providers are allowed to prescribe the medication. Several of the principal pharmacopoeias contain monographs outlining standards for herbal drugs. The major advantage of an official monograph published in a pharmacopoeia is that standards are defined and available, and that the analytical procedures used are fully validated. This is of major importance, since validation can be a rather time-consuming process.

By definition, validation is the process of proving that an analytical method is acceptable for its intended purpose for pharmaceutical methods. Guidelines from the United States Pharmacopeia (USPC, 1994 to 2001), the International Conference on Harmonization (ICH), and the US Food and Drug Administration (FDA) provide a framework for performing such validations. Generally, validation investigations must include studies on specificity, linearity, accuracy, precision, range, detection, and quantitative limits, depending on whether the analytical method used is qualitative or quantitative. Also, of utmost importance is the availability of standards. For macroscopic and
microscopic procedures in general this means that reliable reference samples of the plant must be available. A defined botanical source (e.g. voucher specimens) will normally solve this problem. Standards for chromatographic procedures are less easy to obtain. Characteristic plant constituents, either active or markers, are seldom available commercially. Sometimes an LC-MS approach can be referred to as a mode of characterization. Going one step further, after isolation of such a compound, elucidations to prove its definite structure will not be easy. The method often employed is to use readily available compounds that behave similarly in the chosen chromatographic systems, and to calculate retention values and/or times towards these compounds as a standard. Qualitative chemical examination is designed to detect and isolate the active ingredients. TLC and HPLC are the main analytical techniques commonly used. In cases when active ingredients are not known or too complex, the quality of plant extracts can be assessed by a “fingerprint” chromatogram.

**Labelling of herbal products:**
Quality of consumer information about the product is as important as the finished herbal product. Warnings on the packet or label will help to reduce the risk of inappropriate uses and adverse reactions. The primary source of information on herbal products is the product label. Currently, there is no organization or government body that certifies herb or a supplement as being labelled correctly. It has been found that herbal remedy labels often cannot be trusted to reveal what is in the container. Studies of herbal products have shown that consumers have less than a 50% chance of actually getting what is listed on the label, and published analyses of herbal supplements have found significant differences between what is listed on the label and what is in the bottle. The word “standardized” on a product label is no guarantee of higher product quality, since there is no legal definition of the word “standardized.” Consumers are often left on their own to decide what is safe and effective for them and the lack of consistent labelling on herbal products can be a source of consumer frustration. Certain information such as “the product has been manufactured according to Pharmacopoeia standards,” listing of active ingredients and amounts, directions such as serving quantity (dosage) and frequency of intake of the drug, must be in the label.

**RECENT APPROACHES IN STANDARDIZATION FOF HERBAL DRUG:**\(^{10-14}\)

**Chromatographic Fingerprinting and Marker Compound Analysis:**
A chromatographic fingerprint of an Herbal Medicine (HM) is a chromatographic pattern of the extract of some common chemical components of pharmacologically active and or chemical characteristics. This chromatographic profile should be featured by the fundamental attributions of “integrity” and “fuzziness” or “sameness” and “differences” so as to chemically represent the HM
investigated. It is suggested that with the help of chromatographic fingerprints obtained, the authentication and identification of herbal medicines can be accurately conducted (integrity) even if the amount and/or concentration of the chemically characteristic constituents are not exactly the same for different samples of this HM (hence, “fuzziness”) or, the chromatographic fingerprints could demonstrate both the “sameness” and “differences” between various samples successfully. Thus, we should globally consider multiple constituents in the HM extracts, and not individually consider only one and/or two marker components for evaluating the quality of the HM products. However, in any HM and its extract, there are hundreds of unknown components and many of them are in low amount. Moreover, there usually exists variability within the same herbal materials. Hence it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic components of the HM.

**Liquid Chromatography- Mass Spectroscopy (LC-MS):**

LC-MS has become method of choice in many stages of drug development (Lee, 1999). Recent advances includes electrospray, thermospray, and ion spray 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 (Bhutani, 2000).

**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 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.

**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 (Guo et al., 2006 and Teo et al., 2008). 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 quadrupole instruments. GC-MS instruments have been used for identification of hundreds of components that are present in natural and biological system (Sharma, 2009).

**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.

**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 (Matthew et al., 2006). 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 (Patil et al., 2010).

**DNA FINGERPRINTING:**
DNA analysis has been proved as an important tool in herbal drug standardization. This technique is useful for the identification of phytochemically indistinguishable genuine drug from substituted or adulterated drug. It has been reported that DNA fingerprint genome remain the same irrespective of the plant part used while the phytochemical content will vary with the plant part used, physiology and environment (Shikha et al, 2009). Deoxyribonucleic acid (DNA) is the fundamental building component of all living cells. Our characteristics, traits and physical features are determined by the specific arrangement of DNA base-pair sequences in the cell. It is this distinct arrangement of adenine, guanine, thymine and cytosine (called DNA nucleotides) that regulates the production of specific proteins and enzymes via the Central Dogma Theory. Central Dogma theory can be defined as the fundamental theory of molecular biology that genetic information flows from DNA to RNA to proteins (Breithaupt, 2003). This concept of fingerprinting has been increasingly applied in the past few decades to determine the ancestry of plants, animals and other microorganisms. Genotypic characterization of plant species and strains is useful as most plants, though belonging to the same genus and species, may show considerable variation between strains. Additional motivation for using DNA fingerprinting on commercial herbal drugs is the availability of intact genomic DNA from plant samples after they are processed. Adulterants can be distinguished even in processed samples, enabling the authentication of the drug (Mihalov et al., 2000). The other useful application of DNA fingerprinting is the availability of intact genomic DNA specificity in commercial herbal drugs which helps in distinguishing adulterants even in processed samples.

**GENETIC MARKER:**

A *genetic marker* is a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait. It can be described as a variation, which may arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism SNP), or a long one, like mini satellites. Some commonly used types of genetic markers are

- RFLP (or Restriction fragment length polymorphism)
- AFLP (or Amplified fragment length polymorphism)
- RAPD (or Random amplification of polymorphic DNA)
- VNTR (or Variable number tandem repeat)
- Micro satellite polymorphism
- SNP (or Single nucleotide polymorphism)
STR (or Short tandem repeat)
SFP (or Single feature polymorphism)

They can be further categorized as dominant or codominant. Dominant markers allow for analyzing many loci at one time, e.g. RAPD. A primer amplifying a dominant marker could amplify at many loci in one sample of DNA with one PCR reaction. Co-dominant markers analyze one locus at a time. A primer amplifying a co-dominant marker would yield one targeted product (Raya et al., 2002).

ROLE OF GENETIC MARKER IN HERBAL DRUGTECHNOLOGY:

Genetic variation/genotyping:
It has been well documented that geographical conditions affect the active constituents of the medicinal plant and hence their activity profiles. Many researchers have studied geographical variation at the genetic level. Estimates of genetic diversity are also important in designing crop improvement programmes for management of germ plasm and evolving conservation strategies. RAPD-based molecular markers have been found to be useful in differentiating different accessions of neem collected from different geographical regions (Khanuja, 2002). Germplasm analysis to study genetic diversity is another important area in which a lot of efforts have been put in. Fingerprinting of crops like rice, wheat, chickpea, pigeon pea, pearl millet etc is being carried out extensively (Khanuja, 2002; Ramakrishna et al., 1994)

Authentication of medicinal plants
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 phytochemically indistinguishable (Srivastava et al., 2009). Dried fruit samples of Lycium barbarum were differentiated from its related species using RAPD markers. The RAPD technique has also been used for determining the components of a Chinese herbal prescription, yu-pingfeng san. In this study the presence of three herbs (Astragalus membanaceus (Fisch.) Bge, Ledebouriellaseseloides Wolff and Atractylodes macrocephala Koidz) in the formulation have been detected using a single RAPD primer

Detection of adulteration/substitution
Sequence characterized amplified region (SCAR), AP–PCR, RAPD and RFLP have been successfully applied for differentiation of these plants and to detect substitution by other closely related species. e.g. P. ginseng is often substituted by P. quinquefolius (American ginseng).

Medicinal plant breeding
ISSR–PCR has been found to be an efficient and reliable technique for the identification of zygotic plantlets in citrus interploid crosses. Molecular markers have been used as a tool to verify sexual and apomictic offspring of intraspecific crosses in Hypericum perforatum, a well-known anthelminthic and diuretic. An attempt has been made towards marker-assisted selection of fertile clones of garlic with the help of RAPD markers. RAPD markers have been successively used for selection of micropropagated plants of Piper longum for conservation.

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