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Review Article

Modern Approach to Herbal Drug Standardisation

Harshal Pawar[1], Pravin Jadhav[2], Surekha Jadhav[2] , Sweta singh[2], Megha Salunkhe[2]

Department of Quality Assurance, Dr. L. H. Hiranandani College of Pharmacy, Ulhasnagar, India.

ABSTRACT
Herbal medicines mark up a significant fundamental of the predisposition toward alternative medicine. Herbal medicine is pleasant ever more popular in today's world as people seek out natural remedies. Herbal medicines have been used since the emergence of civilization to maintain health and to treat various diseases. To compete with the emergent pharmaceutical market, there is an importance to use and scientifically authenticate more medicinally useful herbal products. Herbal medicines are not a simple assignment since many factors impact the biological efficacy and reproducible therapeutic effect. Standardized herbal products of consistent quality and containing well-defined constituents are required for reliable clinical trials and to provide consistent beneficial therapeutic effects. Pharmacological properties of an herbal formulation depend on phytochemical constituents present therein. Development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of market/bioactive compounds and other major constituents, is a major challenge to scientists. Now a day's various spectroscopic and chromatographic techniques are used for authentication and standardization of herbal drugs. The objective of the present study is to discuss some of the recent and advanced techniques used in the standardization of herbal drugs.

Key words: Herbal Medicine, Standardization, Authentication, Chromatography, Spectroscopy.

1. INTRODUCTION

Since ancient times herbal drugs are of great significance for treating various diseases. Although a great advancement is seen in modern medicine in recent decades, plants have important contribution in all over the world in traditional system which was used for treating disease from many centuries[1]. 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 are use of therapeutic herbs to prevent and treat diseases and ailments or to support health and healing. These are drugs or preparation made from a plant or plants and use for any of such purpose. Herbal drugs are chief fundamental in traditional medicinal system such as Ayurveda, homeopathic, neuropathic and other medicinal systems[3]. The toxic side effects of the drugs of modern medicine and the lack of medicines for many chronic diseases have led to re-emergence of the herbal medicines, with possible treatment for many health problems[1]. Usage of herbal drug is increasing because of the side effects seen in allopathic system as compared to herbal medicinal system[1]. According to WHO, 80% of world population are tending towards usage of herbal drugs for major health care. In herbal medicine the therapeutic effect varies as the phytochemical constituent varies which is due to distinction in geographical region, time of collection, environmental factors, etc[1]. Adding to this variability is the fact that in herbal medicine several plants may be used together to express same preparation[4]. For this purpose it is very important that a system of standardization is established for every plant medicine in the market because the scope of variation in different batches of medicine is enormous[5, 6]. In case of herbal medicine toxicity study will require to reveal the contribution of toxicity itself. In accessing the toxicity the dose preferred is very important[7]. Toxicity may also occur as a result of adulteration in herbalmedicines[8]. This may occur with contamination of toxic plants or molds due to improper selection or storage[1].In order to get constant composition of herbal preparation, adequate analytical methods have to be applied such as spectroscopic analysis, thin layer chromatography, high performance liquid chromatography, high performance thin layer chromatography and DNA Fingerprinting[9].

2. STANDARDIZATION

As the use of herbal medicine is drastically increased assurance of safety, quality and efficacy of the herbal drug is very important issue. Maintaining the quality of herbal

drug is a challenging task and this includes a strict set of many processes which help to maintain consistency of the drug within the specified limits. Standardization refers to the body of information and control necessary for the drug material of reasonable consistency. Standardization is a process of ensures predefined amount of quantity, quality, safety and efficacy of the drug. Standardization has some limitations that only isolated compounds are considered and ignoring the whole constituent of the herb which may have some synergistic and buffering activities to reduce the side-effects. Several pharmacopoeias provide standards for herbal medicines to get the consistent quality of the drug. Standardization of crude drug material is done by authentication:- stage of collection, parts of the plant collected, identity like photo-morphology, microscopically and histological analysis(characteristic of cell walls, cell contents, starch grains, calcium oxalate crystals, Trichomes, Fibers, Vessels. etc.), leaf constants:-palisade ratio, vein islets number, vein termination, Stomatal number and Stomatal index. Other histological tests are Trichomes, Stomata, Quantitative microscopy, Taxonomical identity, foreign matter, organoleptic evaluation, ash values and extractive values, moisture content determination, chromatographically and spectroscopical evaluation, heavy metal determination, pesticide residue, microbial contamination, radioactive contamination[10].

3. NEED OF STANDARDIZATION:

As herbal medicinal drugs is complex mixture originating from biological sources so there is a need for assurance a constant and adequate quality of drug.

- There is always chance of contamination of herbal crude drug with the other plant material rather than the actual crude drug.
- In case of highly potent and costly drug adulteration is the major problem.
- There is a variation in phytoconstituent content due to change in number of factors such as environmental, geographical region, etc.
- There is also possibility of variation in plant constituent due to variation in species and strains.
- The therapeutic active constituents are many times unknown.
- The method of harvesting, collecting, drying and storing have effect on the crude drug.
- Basically plant material is chemically and naturally variable, so they should be standardized for getting a constant chemical profile, biological or therapeutic effect.

4. WHO GUIDELINES FOR HERBAL DRUG STANDARDIZATION [2]:

4.1 Identity of the drug

Botanical evaluation, sensory characters, foreign organic matter, macroscopical, histological, histochemical evaluation, quantitative measurements etc.

4.2 Physicochemical character of the drug

Physical and chemical identity, Chromatographic fingerprints, ash values, extractive values, moisture content, volatile oil and alkaloid assays, quantitative estimation protocols etc.

4.3 Pharmacological parameters

Biological activity profiles, bitterness values, hemolytic index, astringency, swelling factor, foaming index etc.

4.4 Toxicity details

Pesticide residues, heavy metals, microbial contamination like total viable count, pathogens like *E.coli, Salmonella, P.aeroginosa, S. aureus, Enterobacteria* etc.

4.5 Microbial and radioactive contamination

5. STANDARDIZATION METHODS

Standardization method should take into consideration all aspects that will contribute to quality of herbal drug which include correct identification of sample, organoleptic evaluation, pharmacognostic evaluation ,volatile matter, quantitative evaluation (ash value, extractive value) phyto-chemical evaluation, test for presence of Xenobiotics, microbial load testing, biological testing[1].From all the above phytochemical profile is of special importance. Since it directly results in bearing on the activity of herbal drug. Phytochemical standard encompasses all possible information generated which regard to chemical constituents present in herbal drug.

5.1 Authentication

It is the first an 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[7].

5.2 Organoleptic Evaluation

In these morphological features such as size, shape, odor, taste, and color are compared with standards. Organoleptic evaluation gives the basic idea about the crude drug quality. E.g. the color of senna leaf an idea about the content of sinuside present in it i.e. the dark color indicates high amount color in it. i.e. the dark color of leaf indicates high amount of sinusides. Although this method is very

good but there is need for more sophisticated technique for standardization.

5.3 Microscopic Evaluation

Evaluation of crude drug with help of microscope is done basically to identify the difference between genuine drug and adulterants. For this purpose TS, LS, RLS, RTS of intact crude drug is taken or the powder of crude drug is evaluated under microscope.

5.4 Chemical Evaluation

This type of evaluation involves various chemical testes for qualitative and quantitative evaluation of drug material. It involves some general tests for identification of chemical constituents such as Molisch test, Millions test, Biurets test, etc. and some specific chemical test like Born trager test (for anthraquinones), vitalis morin test (for terpene alkaloids). It also involves some quantitative determination test like total tannin content, total alkaloid content etc.

5.5 Physical Evaluation

5.5.1 Physical constant

The specific melting point or boiling point is a characteristic of specific. This property is helpful in detection of adulterant in crude drug sample. If the adulterant is present in genuine drug which changes its melting point or boiling point or physical constant[11].

5.5.2 Moisture content

Determination of moisture content is an important issue regarding its stability. As the presence of moisture in crude drug may accelerate enzymatic reaction and microbial growth which detoriates the drug, hence it is important issue for drug's stability.

It is determined by various methods:

1. Loss on drying
2. Karl Fisher reagent method
3. Azeotropic distillation method
4. Halogen balance

5.5.3 Ash value

This is inorganic impurities after incineration of the material. This is done to determine inorganic content of material. Ash value is of following types:

a) Sulphated ash
b) Water soluble ash
c) Acid insoluble ash
d) Total ash

5.6 Biological Evaluation

It is used to check the therapeutic activity, potency and therapeutic window of the drug. Also the lethal dose is determined. The three techniques that are used are as follows[11]:

5.6.1 Animal model

Animals are used to check the therapeutic activity of the drug like mice, sheep, horse, rabbit, dog, Guinea pigs, peagons, and etc.

5.6.2 Living tissues

By using tissue culture techniques, the activity and potency of the drug is determined by Morphological and histological evaluations, the effect are determined.

5.6.3 Microbial assays

In crude drug there is always possibility of microbial contamination due to its natural origin. So there is need to establishment of specific standard related to microbial load. As the presence of micro-organisms which leads to various side effect and toxicity.

There are various methods for detection of microbial contamination:

a) Cup plate method

In this method the crude drug is evaluated for presence of microorganism by using solid agar plate method and the presence of microbes is determined.

b) Turbidometry method

It uses liquid nutrient medium for checking the presence of microbes. This is done by spectroscopic method in which the concentration of microbes is determined by checking transmission using UV light.

5.7 Instrumental method

In this method of standardization various types of sophisticated instruments are used. This method provides various benefits like less sample requirement, faster and easier method of detection.

This method involves following methods:

5.7.1 Chromatographic method

This is done for qualitative determination of the drug. The constituents are separated according to is chemical nature. There are many methods used for this purpose like:

- TLC (Thin layer chromatography)
- HPLC (high performance liquid chromatography)
- GAS Chromatography

1) Thin layer chromatography

TLC is the simple, low cost versatile and popular technique. It is used for qualitative determination of drug. This method provides rapid analysis with minimum sample clean up requirement. In the TLC method the solute undergoes distribution between two phase i.e. stationary and mobile

phase. Stationary phase is acting through adsorption and mobile phase runs through TLC plate. Constituents are separated according to its polarity and gives series of bands[9].

2) High performance liquid chromatography

This method includes recording of chromatograms, retention time of individual peaks and absorption spectra (recorded with a photo diode array detector) with different mobile phases. HPLC are of preparative and analytical type. Traditional LC was based on usage of class for a plastic columns filled with the low efficiency packing materials having large particle size distribution whereas in preparative HPLC larger stainless steel columns packed with the materials having particle size ranging from 10micrometer to 30 micrometer. In HPLC due to presence of efficiencies and faster solvent velocities permits more difficult separation conducted in less time.

The preparative HPLC used in pharmaceutical industry for isolating and standardization of drug. The important parameters like resolution, sensitivity and fast analytical time are the factors which are to be considered in analytical HPLC whereas both the degree of solute purity as well as amount of compound can be produced per unit time i.e. throughout or recovery in preparative HPLC[12] .

3) Gas chromatography

GLC is most selective and versatile form of gas chromatography since there is a wide range of liquid phase used. This method separates volatile substances by percolating a gas stream over a stationary phase. Efficiency of GLC depends on the type of gas used. Most of the time helium or nitrogen is used. It is used for examination of many volatile oils.

5.7.2 Spectroscopic method

1) Ultra-Violet and Visible spectroscopy

The region ranging from 190-380nm in electromagnetic wave spectrum is called as UV region whereas region ranging from 380-900 nm in wave spectrum is called as visible region. There are varieties of instrument available for this type of analysis majorly Single beam type and Dual beam type are used.

2) **FTIR (Fourier Transform Infra-red)**

It is the study of the reflected, absorbed or transmitted light energy in the region of electromagnetic spectrum ranging from 0.8-500 nm. It is divided in to three regions namely- near I.R, mid I.R, far I.R. These spectrophotometers can be single or double beam instruments.

It is an infrared spectroscopy method which involves passing of infrared radiation through a sample and measuring the amount of radiation transmitted or reflected by the sample. FTIR along with the statistical principle component analysis (PCA) was applied to identify and discriminate herbal medicines for quality control in the finger print region 400-2000 cm^{-1} [12]. PCA clusters the herbal medicines in different groups, clearly showing that IR method can adequately discriminate different herbal medicines using FTIR data [14]. This spectrum is unique to the molecular spectrum of the material. Four techniques are used in FTIR method namely transmittance, reflectance, ATR and GAO.

Transmission Mode

In this the radiation which passes through the sample is viewed through the microscope with the clean surface as the background.

Reflectance Mode

In this the amount of radiation that is reflected by the sample is measured by the FTIR when the radiation passes through the sample and here the radiation is viewed through the microscope with the gold surface as the clean background.

ATR mode or Attenuated total Reflectance mode

It uses zinc selenied crystals because it is highly refractive. Germanium crystals are also used in ATR mode. The crystal picks up the amount of radiation reflected by the sample. This mode can be used on a solid object such as soft rubbers without taking a sample from them.

GAO mode or the Grazing Angel Objective mode

It is used to analyze the very thin coatings on metallic substrates. The radiation spectrum is reflected by the metal at an angle and measured[15].

3) Nuclear Magnetic Resonance Spectroscopy

NMR has application in determination of impurities and minor components in mixtures because of ease, speed and specificity of analysis deals with the absorption of radio frequency radiated by substance held in magnetic field. There is an interaction of radiation with the magnetic moment of nuclei in the sample and it occurs at different frequencies for nuclei with chemically different environments within a molecule. It is extremely important tool for elucidation of molecular structure specially the stereochemistry and configuration.

4) Mass Spectroscopy

The important application of mass spectroscopy is in determination of molecular weight of compound. It is concerned with electron ionization, subsequent fragmentation of molecules, determination of mass to charge ratio and relative abundances of ions which are

produced. Knowing the fragmentation pattern, a possible structure of the original molecule can be suggested.

5) Radio Immuno Assay
Use of RIA is increasing because of high sensitivity. The technique uses an antibody specific for the drug being assayed and a labeled form of the sample drug. The label may be a particular radio isotope, an active enzyme or an isotope of carbon or iodine is used[11].

6) X-Ray Diffraction
It is done to check polymorphism of the drug. The stability of the polymorphic form is determined by this process.

5.7.3 Hyphenated Techniques[1]

1) Liquid Chromatography- Mass Spectroscopy (LC-MS):
Recent LC-MS there electrode spray, thermo spray and iron spray ionization technique which offers a unique advantage of high detection sensitivity and specificity, Liquid secondary ions mass spectroscopy, later laser mass spectroscopy with 600MHz offers accurate determination of substances like protein, peptides etc.

2) Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR):
This combination technique is one of the powerful and time saving methods for separation and structural elucidation of desired compound. It is specially done for light and oxygen sensitive substances.

3) 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.

4) Gas chromatography-Flame Ionization Detector (GC-FID):
A number of detectors are used in gas chromatography; the most commonly used are flame ionization detector (FID) and thermal conductivity detector (TCD). Both are sensitive to a wide range of components and both work over a wide range of concentrations. FIDs are primarily sensitive to hydrocarbons than TCDs. Both detectors are also quite robust. TCD is non-destructive and hence it can be operated in series before an FID, thus providing complementary detection of the same analysis[9,]

5) High performance thin layer chromatography:
HPTLC is used for process development, identification, detection of adult, substituent in the herbal products this method is used for substance having low or moderate polarities. HPTLC has following advantages over HPLC method:

- HPTLC requires less amount of mobile phase than HPLC.
- The mobile phase having pH 8 or more than 8 can be used in HPTLC.
- HPTLC efficient method because it involves repeated detection (scanning)[9].

5.7.4 Other Techniques

1) DNA Finger printing
DNA analysis has been proved as an important tool in herbal drug standardization. This technique is useful for identification of phytochemical indistinguishable genuine drug from substituted or adulterated drug. DNA finger printing is based on the principle that DNA forms the basic genotype that is genetic identity of an organism which in turn determines the phenotype that is physical feature of organism. A particular DNA profile can be assigned to a particular organism. This profile is unique as a finger print; it is specific to that individual. The availability of intact DNA provides analysis even after processing of the sample[13].The other application of DNA Finger printing is the availability of intact genomic DNA specificity in commercial available herbal drugs which helps in distinguishing adulterants even in processed samples[12.]

Method to obtain a DNA Fingerprint
There is specific method through which DNA fingerprints are obtained.

- Cells of some part of the plant like roots, leaves, stems, etc. are taken.
- DNA molecules are digested with special kind of enzymes which are called as restriction enzymes. These enzymes cut the DNA at specific places and make small fragments. By using the technique of electrophoresis, cut DNA pieces are sorted and then are passed from seaweed agarose gel. This technique of screening to determine the size of the particles.
- The DNA fragments which are separated are dispersed on the nylon sheet which is soaked in the agarose gel.
- DNA probes are usually colored to identify the specific sequences in the DNA molecules. These colored or radioactive probes are then also dispersed on the nylon sheet. Each probe will find its complementary strand and stick to it.
- Through these steps, the DNA fingerprints are obtained which can be used for identifying indistinguishable genome of same or different species[13].

Techniques used in DNA Fingerprinting

a) SSR

Simple sequence repeats (SSR) are microsatellites.1-6 nucleotides in length which show a high degree of polymorphism like any DNA fragment, SSRs can be detected by specific dyes or by radio labeling using gel electrophoresis. The main advantage of using SSRs for finger printing is that small amounts of DNA are required compared to restriction fragment length polymorphism (RFLP) method. This is due to large amount of SSRs present in any genome. Further, assays involving SSRs are more robust than random amplified polymorphic DNA (RAPDs), making up them seven times more efficient.

ISSR (Inter Simple Sequence Repeats) is a PCR based application which is unique and inexpensive popular technique of DNA Finger printing. It includes the characterization of gene tagging, DNA Finger printing, Clonal variation detection, phyllogenetic analysis, genomic instability and assessment of hybridization[12].

b) RFLP

Restriction Fragment Length Polymorphisms (RFLP) are unequal lengths of DNA fragments obtained by cutting variable number of tandem repeat (VNTRs) sequences up to 30 sequences long with restriction enzymes at specific sites. VNTRs vary between plant species as the number of and location of restriction enzyme- sites vary. RFLPs are visualized using radiolabelled complementary DNA sequences. RFLP are used to identify the origins of a particular plant species.

c) AFLP

Amplified Fragment Length Polymorphism (AFLP) is a PCR based derivative method of RFLP in which the sequences are selectively amplified using primers. It is reliable and efficient method of detecting molecular markers. DNA is cut with the help of two restriction enzymes to generate specific sequences which are then amplified. It is capable of determining a large number of polymorphisms.

d) RAPD

Random Amplified Polymorphic DNA (RAPD) is one of the most commonly used primary assays for screening the differences in DNA sequences of two species of plants. RAPDs lack specificity, however due to low annealing temperatures and easier reaction conditions[13].

2) SCAR Marker:

Sequential Characterized Amplified Region (SCAR) allows effective authentication and discrimination of herbs from their adulterants. In addition morphologically similar species can be differentiated using scar marker[12.]

3) SFC (Supercritical Fluid Chromatography)

The supercritical fluid chromatography offers potential applications for drug analysis[7]. SFC permits the separation and determinations of group of compounds that are not are not conveniently handled by gas or liquid chromatography[12]. SFC has been applied to a wide variety of materials including natural products, drugs, food and pesticides [16]. In this method the mobile phase is a gas (CO_2) maintained at its super critical state, i.e. above its critical temperature and pressure. The SFC mobile phase has a low viscosity, approximating that of a gas, and high diffusivity between those of capillary gas chromatography and liquid chromatography [7]. SFC enables the resolution of unknown components and known markers such as azadirachtin A and B, nimbin in neem seed extracts [17].

4) CE (Capillary Electrophoresis)

Several CE studies dealing with herbal drugs have been carried out and majorly on medicinal compounds-alkaloids[18] and flavanoids[19] have been studied extensively. The methodology of CE was introduced to evaluate one drug in terms of specificity, sensitivity and precision, and the results were in agreement with those obtained by the HPLC method[7]. Moreover in CE, the analysis time was two times shorter than that in HPLC and the solvent consumption was more than 100 fold less[20.] Comparison of CE and HPLC finger prints of Radix scutellariae showed a decrease in analysis time from 4o to 12 minutes for CE, but also a decrease in detected peaks from 14 to 11 [21].

5) 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[22].TGA may be operated under sub ambient conditions to analyze ethanol in herbal formulations such as asavas and arista[23].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 350^0 C which corresponded to melting temperature of mercury sulphide[24]. The optimized extraction obtained by distillation showed the presence of volatile oil in dry ginger as a component of volatile oil-betacyclo-dextrin inclusion compound using DTA[25]. DSC thermo gram data confirmed the formation of phospholipids complex with emodin (an anthraquinone) [26] and naringen [27.]

6) Differential Pulse Polarography (DPP)

DPP 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 calendulae

flowers by DPP [28]. Accumulation of heavy metals, namely Pb, Cd, Cu and Zn was estimated in market as well as genuine samples of important herbal drugs of India viz., Alpinia galanga, Artemisia parviflora, Butea monosperrma, Coleus forskohlii, Curcuma amada, Euphorbia prostrate. The concentration of Pb and Cd was found beyond the WHO permissible limits in most samples [29]. 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[30].

6. CONCLUSION

Now those days are gone when a vaidya used to collect, select, prepare and dispense medicines by himself. This has necessitated the establishment of standards for ayurvedic drugs and formulations so as to ensure proper use of the medicines so prepared for the benefit of the end user without any unwarranted complications. Standardization of herbal drugs comprises total information and controls to essentially guarantee consistent composition of all herbals including analytical operations for identification, markers and assay of active principles. Thus Standardization is needed to establish quality control parameters for each traditional drug before it is released for use without the fear of toxicity and contamination. India can emerge as the major country and play the lead role in production of standardized therapeutically effective ayurvedic formulation. India needs to explore its medicinally important plants .This can be achieved only if the herbal products are evaluated and analyzed using sophisticated modern techniques of standardization such as chromatographic, hyphenated, spectrometric and modern methods which are mentioned above. These guidelines for the assessment of herbal medicines are intended to facilitate the work of regulatory authorities, scientific bodies and industry in the development, assessment and registration of such product.

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