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An overview of advances in the standardization of herbal drugs

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ABSTRACT

Herbal formulations have reached extensive acceptability as therapeutic agents for several diseases. The development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a major challenge to scientists. Standardization is an important step for the establishment of a consistent biological activity, a consistent chemical profile, or simply a quality assurance program for production and manufacturing of herbal drugs. WHO specific guidelines for the assessment of the safety, efficacy and quality of herbal medicines as a prerequisite for global harmonization are of utmost importance. An overview covering various techniques employed in extraction and characterization of herbal medicines as well as herbal nanomedicines standardization is reported. In addition, phytosomes increased bioavailability, bhasma as a metal nanocarrier drug delivery system, potential of metabolomics in the development of improved phytotherapeutic agents, DNA based molecular markers in distinguishing adulterants, and SCAR markers for authentication and discrimination of herbs from their adulterants are reported. The extraction of high-valued herbal compounds using microwave–assisted extraction and supercritical phase extraction technology followed by the standardization utilizing various spectroscopic, chromatographic and thermogravimetric techniques individually and/or in combination has been discussed in relation to herbal drugs. Capillary electrophoresis and polarographic techniques contributions towards standardization of herbal drugs is also reported. Nanotechnology based Chinese herbal drugs possess improved solubility and enhanced bioavailability.

Keywords: Herbal drugs, standardization, nanoherbal drugs, phytosomes, DNA marker, chromatographic and spectroscopic techniques.

1. Introduction

In recent years, plant derived products are increasingly being sought out as medicinal products, nutraceuticals and cosmetics and are available in health food shops and pharmacies over the counter as self-medication or also as drugs prescribed in the non-allopathic systems[1,2]. Herbal medicines widely used in health-care in both developed and developing countries are complex chemical mixtures prepared from plants and are limited in their effectiveness because they are poorly absorbed when taken orally[3]. According to an estimate of the World Health Organization (WHO), about 80% of the world population still uses herbs and other traditional medicines for their primary health care needs[4]. Herbal formulations have reached widespread acceptability as therapeutic agents for diabetics, arthritics, liver diseases, cough remedies, memory enhancers and adoptogens[5]. As per WHO definition, there are three kinds of herbal medicines: raw plant material, processed plant material and medicinal herbal products. Herbal drugs are finished labelled products that contain active ingredients such as aerial or underground parts of plant or other plant material or combination thereof, whether in the crude state or as plant preparations. The use of herbal medicines has increased remarkably in line with the global trend of people returning to natural therapies[6]. Herbal medicine products are dietary supplements that people take to improve their health and are sold as tablets, capsules, powders, teas, extracts and fresh or dried plants[7]. Herbals are traditionally considered harmless and increasingly being consumed by people without prescription. However, some can cause health problems, some are not effective and some may interact with other drugs. Standardization of herbal formulations is essential in order to assess the quality of drugs, based on the concentration of their active principles[8]. Quality evaluation of herbal preparation is a fundamental requirement of industry and other organization dealing with ayurvedic and herbal products. The growing use of botanicals (drug and other products derived from plants)

by the public is forcing moves to assess the health claims of these agents and to develop standards of quality and manufacture. It is evident that the herbal industry needs to follow strict guidelines and such regulations are necessary. Herbal drugs regulations in India as well as an overview of regulatory status of herbal medicine in USA, China, Australia, Brazil, Canada and Germany has been reported [9]. According to WHO guidelines, an herbal product needs to be standardized with respect to safety before releasing it into the market.

1.1 Herbal drug technology

Herbal drug technology involves conversion of botanical materials into medicines where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is employed, and various drug delivery technologies used for herbal drugs were reported [10-11]. Conventional pharmaceutical products, herbal medicinal products may vary in composition and properties, and increasing reports of adverse reactions has drawn the attention of many regulatory agencies for the standardization of herbal formulations. In this context, correct identification and quality assurance is an essential prerequisite to ensure reproducible quality of herbal medicine, which contributes to its safety and efficacy[12]. This review article deals with various techniques employed in extraction, characterization and standardization of herbal, polyherbal as well as nanoherbal medicines.

2. Herbal drug standardization

Standardization is a system that ensures a predefined amount of quantity, quality & therapeutic effect of ingredients in each dose[12]. Herbal product cannot be considered scientifically valid if the drug tested has not been authenticated and characterized in order to ensure reproducibility in the manufacturing of the product. Moreover, many dangerous and lethal side effects have recently been reported, including direct toxic effects, allergic reactions, effects from contaminants, and interactions with herbal drugs[6]. Therapeutic activity of an herbal formulation depends on its phytochemical constituents. The development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/ bioactive compounds and other major constituents, is a major challenge to scientists. In view of the above, standardization is an important step for the establishment of a consistent biological activity, a consistent chemical profile, or simply a quality assurance program for production and manufacturing of an herbal drug[13]. The authentication of herbal drugs and identification of adulterants from genuine medicinal herbs are essential for both pharmaceutical companies as well as public health and to ensure reproducible quality of herbal medicine[14].

2.1 Conventional methods for standardization of herbal formulation

Standardization of herbal raw drugs include passport data of raw plant drugs, botanical authentification, microscopic & molecular examination, identification of chemical composition by various chromatographic techniques and biological activity of the whole plant[5]. Macroscopic and microscopic evaluation and chemical profiling of the herbal materials for quality control and standardization have been reported by various workers[15-17]. Macroscopic identity of medicinal plant materials is based on sensory evaluation parameters like shape, size, colour, texture, odour and taste while microscopy involves comparative microscopic inspection of powdered herbal drug. Further, advances in microscope technology have increased the accuracy and capabilities of microscopy as a mean of herbal crude material identification due to the implication of light and scanning electron microscopes (SEM) in herbal drug standardization[18]. Furthermore, various advanced methods such as chromatographic, spectrophotometric and combination of these methods, electrophoresis, polarography, and the use of molecular biomarkers in fingerprints are currently employed in standardization of herbal drugs[5,17,18-22]. The history of some important events such as government policies, quality control and standardization of herbal drugs is given in Table 1. A schematic representation of herbal drug standardization is shown in Figure 1.

2.2 Standardization of herbal formulation

Standardization of herbal formulation requires implementation of Good Manufacturing Practices (GMP) [15-17]. In addition, study of various parameters such as pharmacodynamics, pharmacokinetics, dosage, stability, self-life, toxicity evaluation, chemical profiling of the herbal formulations is considered essential[19]. Other factors such as pesticides residue, aflatoxine content, heavy metals contamination, Good Agricultural Practices (GAP) in herbal drug standardization are equally important[40].

Table 1 History of important events in herbal drug standardization.

Year	Important events	Reference
1983	The first National Health Policy 1983 claims that India's is the richest source of herbs and the drugs should be standardized.	23
1995	A separate Department for Indian Systems of Medicine and Homeopathy (ISM&H) now known as AYUSH (Ayurveda, Yoga, Unani, Siddha, Homoeopathy) was established in March 1995 to promote indigenous systems.	24
1996	World Health Organization has recommended the drug control agency to regulate the quality and safety profile of herbal products.	17
1999	World Health Organization (WHO) had given a detail protocol for the standardization of herbal drugs comprising of a single content.	25
2002	The Indian Herbal Pharmacopoeia. Mumbai, Indian Drug Manufacturer's Association, 2002.	26
2002	Analytical approaches like Herboprint use three-dimensional HPLC and attempt to develop tools for activity-based standardization of botanicals.	27
2003	Department of Indian Systems of Medicines & Homoeopathy (ISM&H) established in 1995 renamed into Department of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy (AYUSH)	28
2003	WHO. Guidelines on good agricultural and collection practices (GACP) for medicinal plants. Geneva, Switzerland: World Health Organization; 2003.	29
2004	WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems.	30
2004	In Canada, the Natural Health Products Regulations (NHPR) [13] under the Food and Drugs Act came into force on 01 January 2004.	31
2005	National Policy on Traditional Medicine and Regulation of Herbal Medicines - Report of a WHO Global Survey	32
2006	The manufacture of herbal medicines	33
2007	WHO. Guidelines for assessing quality of herbal medicines with reference to contaminants and residues. Geneva, Switzerland: World Health Organization; 2007.	34
2007	WHO Guidelines on good manufacturing practices (GMP) for herbal medicines. Geneva, Switzerland: World Health Organization; 2007.	35
2009	AYUSH department with collaboration with Quality Council of India (introduced certification scheme for AYUSH drug products	36
2009	USP. United States Pharmacopeia 32/National Formulary 27. Rockville,MD:The United States Pharmacopeial Convention; 2009.	37
2011	An EU directive passed in 2004 erects "disproportionate" barriers against herbal remedies by requiring them to be "licensed" before they can be sold. It's called the Traditional Herbal Medicinal Products Directive (THMPD), Directive 2004/24/EC.	38
2011	Draft Guidance for Industry: Dietary Supplements: New Dietary Ingredient Notifications and Related Issues." The document was published in the Federal Register on Tuesday, July 5, 2011.	39

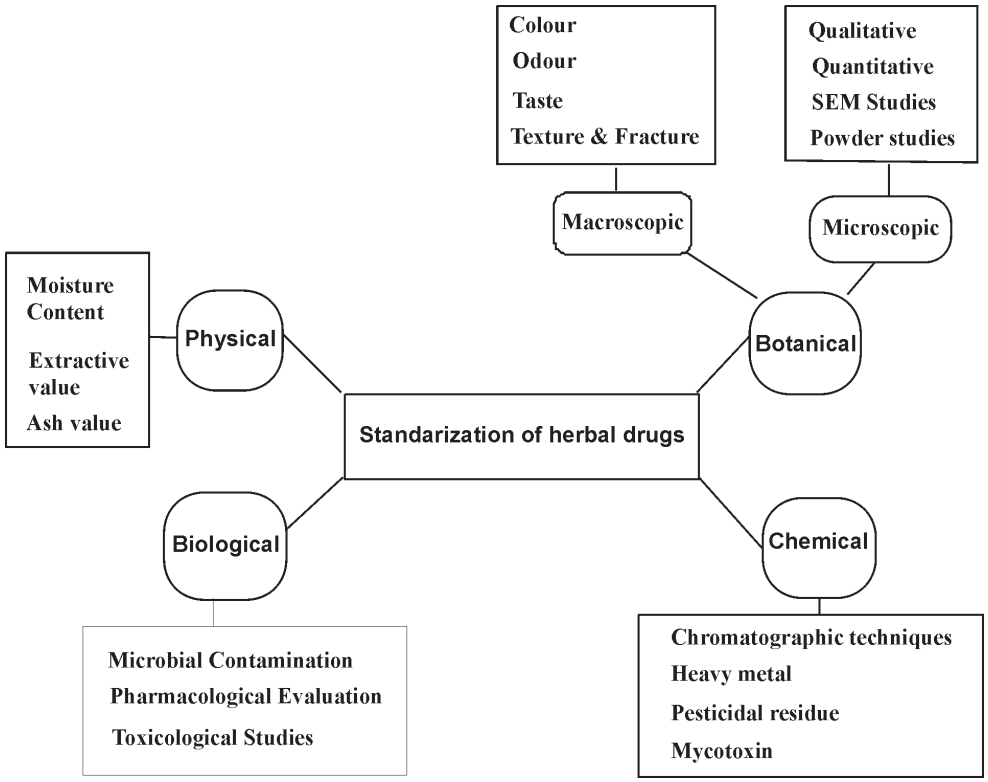

Figure 1: A schematic representation of herbal drug standardization

2.3 Standardization of polyherbal formulations

Standardization is an important aspect for maintaining and assessing the quality and safety of the polyherbal formulation as these are combinations of more than one herb to attain the desire therapeutic effect[41]. The polyherbal formulation of hyperlipdemia has been standardized on the basis of organoleptic properties, physical characteristics, and physico-chemical properties[42]. The formulation and standardized of a polyherbal formulation (Artrex®) designed for the treatment of arthritis containing four botanicals was carried out using modern scientific tools and with known markers, has been granted a US patent [43].

The standardization of various marketed herbal and polyherbal formulation [Madhumehari Churna (Baidynath) containing the mixture of eight herbal antidiabetic drugs *Momordica charantia* (seeds), *Syzigium cumini* (seeds), *Trigonella foenum* (seeds), *Azadirachta indica* (leaves), *Emblica officinalis* (fruits), *Curcuma longa* (rhizomes), *Gymnema sylvestre* (leaves), *Pterocarpus marsupium* (heart-wood)][44], Pancasama Churna known to be effective in gastrointestinal disorder [45], Dashamularishta, a traditional formulation, used in the normalization of physiological processes after child birth[46], Gokshuradi Churna, Megni, Jawarish-e-Darchini[47-49] have been reported. But still there are many polyherbal formulations which require standardization as these are frequently used based only on their ethanobotanical use[50]. Standardization minimizes batch to batch variation; assure safety, efficacy, quality and acceptability of the polyherbal formulations[51]. Methiorep Premix (a combination of herbs viz. *Cicer arientinum, Phaseolus mungo, Mucuna pruriens, Triticum sativum, allium cepa* & richer source of protein with highly bioavailable methionine) has been recommended as a safe product to replace synthetic methionine in poultry ration and for supplementation in basal diet for regular usage[52]. TLC and HPTLC fingerprint profiles were used for deciding the identity, purity and strength of the polyherbal formulation and also for fixing standards for this Ayurvedic formulation[53].

2.4 DNA fingerprinting technique

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[54]. 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[55]. Several studies have been done in past few years to distinguish relation between DNA markers with phytochemical composition among closely related species[56]. Interspecies variation has been reported using random amplified polymormphic and random fragment length polymorphism DNA marker in different genera such as *Glycerrhiza*, *Echinacea*, *Curcuma* and Arabidopsis[57]. Proper integration of molecular techniques and analytical tools generated a comprehensive system of botanical characterization that can be applied in the industry level to ensure quality control of botanicals. DNA markers are helpful to identify cells, individuals or species as they can be used to produce normal, functioning proteins to replace defective ones. Moreover, these markers help in treatment of various diseases and helps in distinguishing the genuine herb from adulterated drug[57].

2.4.1 ISSR (Inter-Simple Sequence Repeat)

ISSR, a PCR-based application is unique and inexpensive popular technique of DNA finger printing which include the characterization of genetic fingerprinting, gene tagging, detection of clonal variation, phylogenetic analysis, detection of genomic instability, and assessment of hybridization[58]. *Cannabis sativa* and *Arabidopsis thaliana* L. Heyne have been differentiated from their adulterated species by using ISSR markers[57]. Molecular characterization by Sequence-characterized amplified region (SCAR) markers allows effective and reliable authentication and discrimination of herbs from their adulterants. In addition, morphologically similar plant species can be differentiated using SCAR markers[58]. DNA based molecular markers have been found to be useful in differentiating different accessions of *Taxus wallichiana*, *Azarchdichta indica*, *Juniperus communis* L., *Codonopsis pilosula* ,*Allium schoenoprasum* L., *Andrographis paniculata* collected from different geographical regions[59].

2.5 Guidelines for the standardization of herbal drugs

The guidelines set by WHO: a) botanical characters, sensory evaluation, foreign organic matter, microscopic, histological, histochemical assessment, quantitative measurements, b) physical and chemical identity, fingerprints chromatography, ash values, extractive values, moisture content, volatile oil and alkaloids tests, quantitative estimation protocols, c) estimation of biological activity, the values of bitterness, astringency hemolytic index, a factor swelling, foaming index, d) detail-toxicity pesticides residues, heavy metals, microbial contamination as viable count total, pathogens such as *E. coli, Salmonalla, P. aeroginosa*, *S. aureus*, *Enterobacteriaceae*, e) microbial contamination and radioactive contamination are followed[60] .

3. Phytosomes/ pharmacosomes: A novel drug delivery system for herbal drugs

Pharmacosomes commonly known as phytosome are drug-phospholipid complexes having active ingredients of the herb and can be formulated in the form of solution, suspension, emulsion, syrup, lotion, gel, cream, aqueous microdispersion, pill, capsule, powder, granules and chewable tablet[61,62]. Plants namely *Silybum Marianum*, *Ginkgo Biloba*, and ginseng showed better efficacy than conventional herbal formulations[63]. In addition, the clinical trials of phytosomes have shown increased bioavailability in comparison to conventional herbal formulations generally containing polyphenols and flavonoids in humans[63,64]. Several phytosomal herbal drug delivery systems have been reported[65]. Researchers demonstrated increased bioavaliblity of four polyphenol phytosome preparations (curcumin, silybin, flavan-3-ol catechins and proanthocyanidin) and this effect was due to the intermolecular bonding between individual polyphenol molecules and one or more molecules of the phospholipid, phosphatidylcholine[66]. Phytosomal herbal drug delivery systems are mainly used i) to deliver systemic antioxidant (mainly polyphenols, flavonoid and terpenoid components), ii) useful in treatment of the disease like blood pressure, liver disease, cancer, skin disease and iii) helps in protecting the brain lining[66].

4. Standardization of herbal nanomedicines

Herbal nanotechnology helps in incorporation of the active phytoconstituents to obtain desired therapeutic effect. The increased solubility, stability, bioavailability,

pharmacological activity of many popular herbal extracts including Milk thistle, *Ginkgo biloba*, grape seed, green tea, hawthorn, ginseng using nano dosage forms such as polymeric nanoparticles nanospheres & nanocapsules, liposomes, proliposomes, solid lipid nanoparticles, and nanoemulsion has been reported[67,68]. Other advantage of herbal nanomedicine include protection from toxicity, improving tissue macrophages distribution, sustained delivery, protection from physical and chemical degradation[68]. Silver nanoparticles of *Ocimum sanctum* extract exihibited maximum antibacterial activity at a dose of 150µg in wistar rats[69].The herbal drug incorporated antibacterial nanofibrous mat fabricated by electrospin provided a potential application for use of wound dressing[70]. Nanotechnology patents issue in Chinese herbal medicine have been reported and proliferation of nano-based Chinese herbal medicine patents in China was due to the illusions of biomedical technology progress extensively[71].

4.1. Bhasma as a nanoherbal medicine technology

Bhasmas are the Ayurvedic metallic preparations in which metal act as a nanocarrier for drug delivery and are widely recommended for treatment of a variety of chronic ailments and are taken along with milk, butter, honey, or ghee to eliminate the harmful effects of metals and enhancing their biocompatibility in the body[72]. Neutron activation analysis of twenty metallic based bhasmas such as calcium, iron, zinc, mercury, silver, potassium, arsenic, copper, tin, and gemstones confirmed the purity of these bhasma as the other elements such as Na, K, Ca, Mg, V, Mn, Fe, Cu, and Zn were found in microg/g amounts and Au and Co in ultratrace (ng/g) amounts[73]. Various techniques like atomic forced microscope, transmission electron microscope, scanning electron microscope and energy dispersive spectroscopy) have been employed for the estimation and characterization of bhasma. The Swarna bhasma (nanoparticles of Au) analysis qualitatively through X-ray diffraction, Fourier transform infrared spectroscopy) showed that the particle size are about 56 nm containing pure Au (gold) which act as a nanocarrier for drug delivery[72]. Mineral arsenicals mainly in the form orpiment (As_2S_3), realgar (As_4S_4), and arsenolite (contains arsenic trioxide, As_2O_3) have been used in traditional medicines for treatment of various diseases, however, arsenic can be highly toxic and carcinogenic[73,74]. Some commercially bhasma acting as a metal nanocarrier are given in Table 2.

Table 2. Marketed bhasma containing metal as a nanocarrier for drug delivery.

Marketed Bhasama	Basic metal which act as a nanocarrier
Shanka Bhasama	Calcium present in sea products
Swarna	Gold
Mukta	Pearl
Abrak	Manganese
Godanti	Gypsum stones
Loha	Iron
Trivang	Aluminium and Zinc
Naga	Lead
Parad	Mercury

5. Regulation of herbal medicines

WHO has evolved guidelines to support the member states in their efforts to formulate national policies on traditional medicine and to study their potential usefulness including evaluation, safety, and efficacy[17,75]. In India, traditional medicine is governed by the Drugs and Cosmetics Act, 1940 and the provisions of the Act are implemented by the state governments. The first Indian National Health Policy 1983 claims that India's is the richest source of herbs and the drugs should be standarized[60]. The department of AYUSH, Government of India, launched a central scheme to develop standard operating procedures for the manufacturing process to develop pharmacopeial standards for Ayurvedic preparations[76]. The Regulation for herbal drug products in Europe and United states are more stringent than in India[77].

5.1 Pharmacovigilance of Herbal Medicines

Pharmacovigilance means the science and activities relating to detect, assess, understand, and to prevent the adverse effects or any other possible drug-related problems, which is not only confined to chemical drugs, but extended to herbal, traditional and complementary medicines, biological, vaccines, blood products and medical devices[78]. There is an increasing recognition of the need to develop safety monitoring systems for herbal medicines[79,80]. The herbal products of ginseng are in great demand as it is considered as a safe herbal drug for human health in spite of few reports on adverse drug reactions. But this is not applicable to every herbal product. Therefore, pharmacovigilance is essential for herbal drug

before being considered as a safe for human health[81]. WHO has set specific guidelines for the assessment of the safety, efficacy and quality of herbal medicines as a prerequisite for global harmonization. The Medicines and Healthcare Products Regulatory Agency's, UK had launched 'yellow card' scheme for monitoring the safety of herbal medicines. Medicinal herbs as potential source of therapeutics aids have attained a significant role in health care system all over the world for human beings not only in the diseased condition but also as potential material for maintaining proper health [82]. Canadian Health Care department has analyzed various unapproved Ayurvedic medicinal products that contain high levels of lead, mercury, and arsenic in various Indian formulations [Karela capsules (Himalaya Drug, India), Maha Sudarshan Churna (Zandu Pharmaceuticals, India), Safi liquid (Hamdard, India & Pakistan), Shilajit capsules (Dabur, India)] and some herbal products were found to contain 0.1 to 0.3 mg of betamethasone which produced corticosteroid-like side effects. Reports have been received by drug safety monitoring agencies of prolonged prothrombin times, increased coagulation time, subcutaneous hematomas, and intracranial hemorrhage associated with the use of Ginkgo biloba[83].

6. Techniques in extraction of herbals

6.1. Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is the most preferable process for the extraction of the bioactive chemical from the medicinal and aromatic plants[84] SFE has emerged as a highly promising technology for production of herbal medicines and nutraceuticals with high potency of active ingredients[85]. SFE techniques have been found useful in isolating the desired phytoconstituents from the herbal extracts[86].

6.2. Microwave-assisted extraction (MAE)

MAE technology includes the extraction of high-value compounds from natural sources including phytonutrients, nutraceutical and functional food ingredients and pharmaceutical actives from biomass[87]. MAE finds utility in production of cost effective herbal extracts and helpful in extraction of carotenoids from single cells, taxanes from taxus biomass, essential fatty acids from microalgae and oilseeds, phytosterols from medicinal plants, polyphenols from green tea, and essential oils from various sources. Compared to conventional solvent extraction methods, advantages of this technology include: a) improved product, -purity of crude extracts, -stability of marker compounds and use of minimal toxic solvents. b) reduced processing costs, increased recovery and purity of marker compounds, very fast extraction rates, reduced energy and solvent usage[88,89].

6.3. Solid phase extraction (SPE)

SPE technique is applied for isolation of analytes from a liquid matrix and purified herbal extracts. This technique has many advantages such as: high recoveries of the analyte, concentartion of analyte, highly purified extracts, ability to simultaneously extract analytes of wide polarity range, ease of automation, compatibility with instrumental analysis and reduction in organic solvent in comparison with more traditional sample preparation techniques[90].

The solid-phase extraction was introduced for determining thirteen organochlorine pesticide residues including alpha-benzene hexachloride (BHC), betaBHC, gamma-BHC, delta-BHC, p,p'-dichloro-diphenyl-dichloroethylene (pp'-DDE), p,p'-dichloro-di-phenyl-dichloroethane (pp'-DDD), o,p'-dichloro-diphenyl-trichloroethane (op'-DDT), pp'-DDT, heptachlor (HEPT), aldrin, heptachlor epoxide (HCE), dieldrin and endrin in *Scutellaria baicalensis, Salvia miltiorrhiza, Belamcanda chinensis, Paeoniae lactiflora, Angelica dahurica, Arisaema erubescens, Fructus arctii, Anemarrhena asphodeloides and Platycodon grandiflorum*. The organochlorine pesticides were extracted from herbs with mixed solvents of acetone and n-hexane by ultrasonic and cleaned up by Florisil solid-phase extraction column [91]. Solid phase extraction was used to prepare the test solution for the analysis of aristolochic acid I and II in herbal medicines[92].

7. Techniques in herbal drug identification and characterization

7.1 HPLC

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) [93,94]. 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 [95]. Vasicine, the major bioactive alkaloid of *Adhatoda vusica*, was estimated by HPLC in two polyherbal drug formulations - Shereeshadi Kashaya and Yastyadivati, and its content was found to be 18.1 mg/100 g in Shereeshadi Kashaya and 0.7 mg/100g in Yastyadivati[96]. HPLC analysis of Senna leaves provided informations about sennoside content, kaempferol 3-O-D-gentiobioside , aloe-emodine 8-O-D-glucopyranoside , rhein 8-O-D-glucopyranoside , torachrysone 8-O-D-glucopyranoside and isorhamnetine 3-O-D-gentiobioside L [97]. Standardization of the Triphala (an antioxidant-rich herbal formulation) mixture of *Emblica officinalis, Terminalia chebula* and *T. belerica* in equal proportions has been reported by the HPLC method by using the RP18 column with an acidic mobile phase[98]**.** The combination of HPLC and LC/MS is currently the most powerful technique for the quality control of Chinese herbal medicine Gan-Cao (licorice) [99].

7.2 High performance thin layer chromatography (HPTLC)

TLC is the common fingerprint method for herbal analysis. Four species of herbal medicines were identified easily by TLC of the resins[100].With this technique, authentication of various species of *Ginseng* and *Radix Puerariae* is possible, as well as the evaluation of stability and consistency of their preparations from different manufactures[101]. HPTLC fingerprint is mainly used to study the compounds with low or moderate polarities, but Di et al. established a fingerprint of fungal polysaccharide acid hydrolyzates by using automated multiple development[102].

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 [103]. HPTLC technique was reported for simultaneous determination of Withaferin A and beta-sitosterol-d-glucoside in four *Ashwagandha* formulations[104].

Syzygium Jambolanum was quantitatively evaluated in terms of stability, repeatability, accuracy and phytocconstituents such as glycoside (jamboline), tannin, ellagic acid and gallic acid by HPTLC[105]. HPTLC was used for detection, monitoring and quantification of bacoside A & B in *Bacopa monnieria* and its formulations[106].The standardization of *Cannabis stavia* was done by estimating the content of cannabinoidses in urine sample using HPTLC[107]. HPTLC was used to estimate Withaferine A, a constituent of *Withania somnifera* in herbal extract and polyherbal formulations[108]. HPTLC method has been reported for quantitative estimation of swetiamarin in different marketed polyherbal formulations and small fruits, big fruits and fresh fruits variety of *E. littorale*[109]. Chandanasava known to be effective in karsya (malnutrition) was standardised by organoleptic study, physico-chemical analysis, TLC and HPTLC[110].

Ultra-performance liquid chromatography (UPLC) was used to evaluate decocting-induced chemical transformations and chemical consistency between traditional and dispensing granule decoctions[111,112].

Combined chromatographic fingerprinting with metabolomics enables the working mechanism of traditional Chinese medicine (TCMs) and to further control their intrinsic quality. In addition, the intensive study of chromatographic fingerprinting coupled with multivariate analysis tools developed in bioinformatics and chemometrics strengthened the working mechanisms of TCMs and to further control and strengthen TCMs' intrinsic quality in a comprehensive manner[113].

7.3. Liquid chromatography- mass spectroscopy (LC-MS)

LC-MS has become method of choice in many stages of drug development[114]. Chemical standardization of an aqueous extract of the mixture of the 20 herbs provided 20 chemical compounds serving as reference markers using LC-MS[115] Further, LC-MS analysis of aminoglycosides showed that these drugs are highly soluble in water, exhibited low plasma protein binding, and were more than 90% excreted through the kidney. Further this technique helps in analysis of aminoglycosides in plasma samples with ion pairing chromatography[116].

Two HPLC methods, one combined with a photodiode array detector (LC/UV) and another with mass spectrometry (LC/MS), were reported for the analysis of aristolochic acid I and II in herbal medicines. The LC/UV method was carried out using a Cosmosil 5C18-MS column with a gradient solvent system composed of

phosphate buffer-acetonitrile and a UV detector (390 nm) while the LC/MS method was performed using an acetate buffer-acetonitrile solvent system and positive-ion electrospray ionization MS. The characteristic fragment ions for aristolochic acid I were selected at *m/z* 359, *m/z* 324, *m/z* 298, and *m/z* 296, and for aristolochic acid II at *m/z* 329, *m/z* 294, and *m/z* 268[92].

7.4. Liquid chromatography- Nuclear magnetic resonance (LC-NMR)

LC-NMR improves speed and sensitivity of detection and found useful in the areas of pharmacokinetics, toxicity studies, drug metabolism and drug discovery process[117-119]. The identification of adulterants in a Chinese herbal medicine was done by LC-NMR technique[120].

7.5. Gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS)

GC-MS instruments have been used for identification of large number of components present in natural and biological systems[121]. The identification and quantification of chemical constituents present in polyherbal oil formulation (Megni) consisting of nine ingredients, mainly *Myristica fragrans*, *Eucalyptus globulus*, *Gaultheria procumbens* and *Mentha piperita* was analyzed by GC-MS method[122]. A headspace solid-phase microextraction method was reported for analysis of the volatile compounds in a traditional Chinese medicine (TCM), *Rhioxma Curcumae Aeruginosae.* Thirty-five volatile compounds were separated and identified[123]. An effective, fast and accurate capillary gas chromatography method was employed for determining organochlorine pesticide residues in *Scutellaria baicalensis, Salvia miltiorrhiza, Belamcanda chinensis, Paeoniae lactiflora, Angelica dahurica, Arisaema erubescens, Fructus arctii, Anemarrhena asphodeloides and Platycodon grandiflorum*. The SPE extract was separated by capillary column (30 m x 0.25 mm i.d. x 0.25 microm) using electrochemical detector. The split ratio obtained was 1:2.2 using the carrier gas N_2 (99.999%) with the flow rate of 1.4 mL/min. The injector temperature was 220 degrees C and the detector temperature was 330 degrees C. The column temperature was increased by the rate of 20 degrees C/min from 100 degrees C to 190 degrees C (hold for 1. 0 min), then to 235 degrees C by the rate of 4 degrees C/min and hold for 7 min at 235 degrees C. The good linearities were obtained for thirteen organochlorine pesticides. The detection limits between 0.064-0.61 microg/L, average recoveries between 87.3%-102.3% and relative standard deviations of 1.3%-6.8% were obtained[91].

7.6 Supercritical fluid chromatography (SFC)

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[124]. SFC enables the resolution of unknown components and known markers such as azadirachtin A and B, salannin, and nimbin in neem seed extracts[125].

7.7 Capillary electrophoresis (CE)

Researchers evaluated the importance of CE for quality control of herbal medicinal products [126]. Several CE studies dealing with herbal medicines have been reported and two kinds of medicinal compounds i.e. alkaloids[127] and flavonoids[128] have been studied extensively.

The methodology of CE was established to evaluate one herb drug in terms of specificity, sensitivity and precision, and the results were in agreement with those obtained by the HPLC method. Furthermore, the analysis time of the CE method was two times shorter than that in HPLC and solvent consumption was more than 100-fold less[129]. A characteristic fingerprint of *Flos carthami* established using CE, simultaneously contributed to several objects in a study: identifying the raw herb, helping distinguish the substitute or adulterant and further assessing the differences of *Flos carthami* grown in various areas of China[130]. Comparison of the CE and HPLC fingerprints of *Radix scutellariae* showed a decrease in analysis time from 40 to 12min for CE, but also a decrease in detected peaks from14 to 11[131]. The hyphenated CE instruments, such as CE-diode array detection, CE-MS and CE-NMR, have been utilized, however, some limitations of CE hyphenations with respect to reproducibility were reported.[132]

7.8 Metabolomics technique

This technique has been used for identification of active phytoconstituents from herbal medicine[133,134]. Metabolomic approach was employed to identify the chemical constituents in *Sophora flavescens*, which were further analyzed for their effect on Pregane X receptor activation and Cytochrome P3A regulation[135]. The greater

potential of metabolomics has been reported in the development of active secondary metabolites from medicinal plants as novel or improved phytotherapeutic agents[134,135]. The recent studies showed that NMR-based metabolomics approach combined with orthogonal projections to latent structure-discriminant analysis identified the purity of an herbal medicine[136].

7.9 Thermal analysis of herbal drugs

Thermogravimetric analysis (TGA), differential thermal analysis (DTA) and differential scanning calorimetry (DSC) have been employed to study any physical or chemical changes in various products including herbal drugs and also used to study preformulation or drug excipient compatibility[137]. TGA may be operated under subambient conditions to analyse ethanol in herbal formulations such as asavas and arista[138]. 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 354° C which corresponded to melting temperature of mercury sulphide[139]. The optimized extraction obtained by distillation showed the presence of volatile oil in dry ginger as a component of volatile oil-beta-cyclodextrin inclusion compound using DTA[140]. DSC thermograms data confirmed the formation of phospholipid complex with emodin (an anthraquinone)[141] and naringen[142].

7.10 X-ray powder diffractometry (X-RPD)

This technique is used to identify minerals, crystalline materials and metallic based herbal formulations. The tin based herbal drug Vanga Parpam was estimated by XRD and the intense sharp diffraction peaks clearly confirmed the presence of high crystallinity in Vanga Parpam [143]. XRD analysis of metallic based Indian traditionally medicine Ras-sindoor indicated the presence of mercury sulphide which is represented by sharp peak[139]. X-ray powder diffractometry data confirmed the formation of phospholipid complex with emodin[141], naringenin [142], quercetin [144], gallic acid [145].

7.11 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 calendulea flowers by DPP [146,147]. 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, Artemesia parviflora, Butea monosperrma, Coleus forskohlii, Curcuma amada, Euphorbia prostrate, Leucas aspera, Malaxis accuminata and Pueraria tuberose.* The concentration of Pb and Cd was found beyond the WHO permissible limits in most samples[148]. Trace amounts of selenium in Chinese herbal medicines [149] and flavonoids in small amount of medicinal herb samples were determined by DPP[150]. 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 [151].

7.12 Infrared spectroscopy

FTIR along with the statistical method principal component analysis (PCA) was applied to identify and discriminate herbal medicines for quality control in the fingerprint region 400-2000 cm^{-1}. The ratio of the areas of any two marked characteristic peaks was found to be nearly consistent for the same plant from different regions, thereby, an additional discrimination method for herbal medicines. PCA clusters herbal medicines into different groups, clearly showing that IR method can adequately discriminate different herbal medicines using FTIR data [152]. Near-infrared spectroscopy technique has been used for rapid determination of active components, species, geographic origin, special medicinal formula, on-line quality control, identification of counterfeit and discrimination of geographical origins of Chinese herbal medicines [153-155]. Two-dimensional near-infrared (NIR) correlation spectroscopy was applied to the discrimination of *Fructus lycii* (a traditional Chinese medicinal herb) of four different geographic regions [156].

8. Conclusions and perspectives

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. There is no legal control model over medicinal plants. Different countries define medicinal plants or products derived from them in different ways and have adopted different approaches to licensing, dispensing, manufacturing and trading to ensure their safety, quality and efficacy. Fingerprinting of herbal medicines is utilized for the authenticity and quality control of herbal medicines

and herbal preparations. Chemical fingerprints obtained by chromatographic, spectroscopic, thermogravimetric analysis, capillary electrophoresis and polarography techniques have become the most potent tools for quality control of traditional herbal medicines. Moreover, all herbal products manufacturers must follow WHO guidelines for quality control. Further, the combination of qualitative fingerprinting and quantitive multicomponent analysis is a novel and rational method to address the key issues of quality control of herbal medicines. The advancement of analytical techniques will serve as a rapid and specific tool in the herbal research, thereby, allowing the manufacturers to set quality standards and specifications so as to seek marketing approval from regulatory authorities for therapeutic efficacy, safety and shelf life of herbal drugs. The applications of high-technology oriented advanced hyphenated techniques will serve as a rapid and unambiguous tool in the herbal research, thereby, benefiting the entire pharmaceutical industry.

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