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Adulteration methods and current trends in authentic identification of botanical materials used for the pharmaceuticals

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ABSTRACT

Macroscopic and microscopic evaluation and chemical profiling techniques have their own limitations for authentication of plant species. DNA-based markers give the new directions to pharmacognosy. It is important to distinguish accurately the authentic plant sources for herbal drugs because adulteration and substitutions diminish their efficacy and reduce the trust of users as well as it is necessary to convert botanical materials into scientifically valid herbal drugs. Proper integration of molecular techniques as analytical tools with conventional knowledge will lead to the development of a comprehensive system of identification of medicinal plants that can be conveniently applied at the research and industry level for quality control of botanicals. The review derives the advantages of DNA-based method for the purpose of correct identification of herbal material and discrimination of originals from the plant-derived adulterants.

Keywords: Phytopharmaceuticals, medicinal plants, Adulterations, Herbal Preparation

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Introduction

Medicinal plants: integration of primordial tradition to contemporary medicines

Nature has evolved in a very astonishing way to deliver various healing molecules in the form of natural products. These healing Molecules can be derived from microbes to more complex than plants and animals. Use of medicinal plants for treatment of various diseases can be traced back to the origin of humans. Plants were the basis of various established traditional system of medicines in ancient times and encouraged development of various modern medicines. It is the empirical findings of hundreds and thousands of years, which make the plant-based drugs safe and reliable. The antiquity of the plant therapy was evident in the writings on a clay tablet found in the most ancient culture of Mesopotamia dated about 2600 BC (Gurib-Fakim 2006). It is evident that they have been using the Cedar oils, *Cupressus sempervirens* oil, *Glycyrrhiza glabra*, *Commiphora* species and *Papaver somniferum* (Poppy juice) for medicinal purposes (Gurib-Fakim 2006). Bishop's weeds (*Ammi majus*) has been reported for treating vitiligo in Egyptian culture (Gurib-Fakim 2006). Many cultural systems and societies have adopted the phytomedicines as their prime healing method and to protect them from diseases and to stabilize the life on earth. The traditional system of medicine thrived in various countries such as Africa, India, Sri Lanka, China (TCM), Japan (Kampo), Korea, Arab and many European countries especially in Greece (Gurib-Fakim 2006).

Progression in the chemical biology turns the focus towards synthetic drugs, which limited the use of phyto-pharmaceuticals. Chloral hydrate was the first synthetic drug that used as the sedative (Jones 2011). Chemical synthesis allowed the production of the structurally predefined drugs in the desired dosage form. It was not possible with the naturally derived drugs due to their high level of diversity and minimal amount of biological supply. In addition, the process of drug discovery from the natural resources is time consuming and required several steps of extraction, isolation and structure elucidation procedures. On the other hand, some of the natural substances are structurally very complex and hard to mimic by chemical synthesis.

In addition, advances in the scientific methods for isolation and determination of new lead compounds from the natural resources and the side effects of non-biological origin of synthetic drugs retrieved the attention of pharma industry towards traditional medicines (Lahlou 2013).

Thus, the constraints associated with these chemically originated drugs encourage the use of natural compounds as the medicines. Medicinal plants have proved as the great repertoire of the therapeutic molecules and it consist several qualities such as they are easily accessible, inexpensive and show fewer side effects and fit into the immediate personal need to be used as the biological resources of natural products. (Kala et al. 2006). Various bioactive molecules are derived from the medicinal plants. Some of the molecules can be directly used as medicines and some of them provide scaffolds, prototype or precursors to develop drug molecules using combinatorial chemical synthesis. Morphine was the first commercial therapeutic plant product introduced by Merck in 1826 (Veeresham, 2012). Aspirin was the first semi-synthetically developed drug originated from salicin, a natural product isolated from *Salix alba*, introduced by Bayer in 1899 (Veeresham 2012). Success in this direction led to the isolation and commercial production of the plant-derived drugs (Sharma et al. 2014).

At present, nearly 80% of African and Asian population depends on traditional medicines for their primary healthcarepurpose (Gurib-Fakim 2006). and 20% inhabitants of developed countries also use plant products as the prime source of various drugs, nutraceuticals and

dietary supplements (Gurib-Fakim 2006). It is estimated that about 25% of the drugs prescribed worldwide are derived from plants and around 121 such active compounds are used in 252 drugs (Sahoo et al. 2010). Further WHO essential medicine list has shown that 11% of essential medicines are exclusively from plant origin (Sahoo et al. 2010).

Quality is the major concern in translation of old wisdom into current prospects

As stated earlier, interrelation of humans and phyto-pharmaceuticals was established since time immemorial and it is growing continuously across the world in the modern era. In spite of the rapid growth of consumption of herbal remedies, the herbal sector is not devoid of challenges. The issues related to plant-based drugs are dishonoring their reputation and affecting the consumer's belief very negatively.

Consistent quality and efficacy is the biggest challenge for the global acceptance of herbal drugs. Quality checking at each level starting from plant cultivation, collection to processing and marketing is required. Moreover, the challenges related to failure to convince differing regulatory, registration and specific Good Manufacturing Practice (GMP) standards, lack of herbal practitioners in overseas, lack of quality in terms of adulteration, substitution and contamination and insufficiency of regulatory guidelines for different aspects of the complete supply chain can impact on the globalinput (Sahoo and Manchikanti 2013).

The major issues responsible for the low quality of the herbal drugs are described **in the Figure 1.1**

Figure 1.1 Major concerns affecting the quality of herbal preparations.

Lack of universality is one of the main problems, which hinder the global acceptance of herbal drugs. Traditional medicines are the results of therapeutic experiences governed by the indigenous people and the knowledge passes from generation to generation (Sissi & Benzie 2011). These customs and rituals differ

in various civilizations corresponding to their local requirements and availability of the resources. Additionally, the descriptions in the local ancient literatures are very difficult to translate and understand in modern scientific language. Differences in the languages can lead to the misapprehension. It is very difficult

to reconstruct all the knowledge entrenched in the rituals of indigenous people as well as in the old documents and apply directly for proper laboratory investigations.

Lack of proper toxicity analysis is another major concern related to the quality of herbal drugs. Many reports are there which exhibited that abusing of herbal remedies have acute or chronic adverse effects. Most of the marketed products are launched without any efficacy and toxicological evaluation (Sahoo et al. 2010). Lack of good collection as well as lack of controlled and regulatory manufacturing procedures, an uncontrolled chain of supply, and misidentification of the species may lead to the external mixing of any toxic chemical or plant species, heavy metals, and microbes, which would be the reasons for toxic side effects of herbal drugs (Sahoo et al. 2010). Most of the drugs are promoted as food supplements and self-medication without proper prescription and without recognizing their hazardous effects (Ekor 2014). Worldwide surveys revealed the cases of organ failures, paralysis, allergic reactions, nausea, vomiting, bleeding as the consequences of herbal drugs toxicity (Aronson 2009). Some of the toxic herbs like *Ephedra, Aristolochia,* and *Aconitum* have intrinsic toxic constituents, when taken more than recommended doses can create severe reactions (Panda and Debnath 2015). Some herbal drugs required preprocessing (as in Ayurveda known as 'Shodhana') steps to remove the noxious bioactive from the plants. Interactions of herbal drugs with concomitantly administrated chemical based drugs as well as the active constituents of the other herbs in the polyherbal formulations also potentiate the problem of toxicity (Ekor 2014).

Batch to batch variations in the herbal products constrains the high quality of herbal products. The integrity of herbal extracts or solid formulations is essential to maintain the reproducibility of pharmaceutical effects. Lack of uniformity in each step from cultivation or wild plant collection to preparation of drugs may affect the homogeneity in the marketed products. The concentration of bioactive constituents in the medicinal plants can vary with the physical conditions of the plants, harvesting or collection time, varied geographical and climatic conditions as temperature, and exposure to light as well as can change with the genetic diversity of the plant species. Physical and chemical stress to the plants as interaction with any microbial organism, pathogens, use of fertilizers and pesticides are also the governing factors. Irregularity in several steps in the processing of raw material as the method of drying, grinding, storage, transportation and extracting generate the variations in pharmacological activities of the herbal drugs. Irreproducibility in the therapeutic effects and actions of herbal drugs lead to the decline in consumer's belief (Mukherjee et al. 2015).

Adulteration and substitution of genuine raw material with the other plant-derived or synthetic substances is also hampering the production of high quality herbal preparations. Adulteration or debasement is common term include different conditions as deterioration, sophistication, spoilage, admixture, substitution, inferiority. Adulteration may be both unintentional and intentional. Unintentional or accidental adulteration is due to the mixing and substitution with nonprofit motive. It occurs mainly due to the careless collection by unskilled hands having less knowledge about the authentic sources, confusion in the species identity due to same morphology or ambiguous vernacular names or lack of taxonomic key characters during the time of collection. Intentional adulteration is the condition in which mixing and substitution are motivated by some economic benefits. This can be done with the substance having inferior value, with exhausted drugs, toxic materials, synthetic products and other parts of the same species etc. Deliberate adulteration is done with the aim of increasing weight of the material or to gain more profits in lesser investments (Bandaranayake 2006;

Blumenthal 2011).

Contamination of herbal preparations with microbes and various chemical agents as heavy metals, organic pollutants, mycotoxins, endotoxins, pesticides etc. also downgrade the purity and quality of herbal drugs. Several reports are there exhibited a higher percentage of Asian, Chinese and African herbal (Abba et al. 2009; Alwakeel 2008; Justin-Temu et al. 2009) remedies carrying the microbial load including bacteria, viruses and fungi in the herbal products. The bacterial species, which are predominantly carried by herbal drugs, include *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* which are pathogenic in nature. Withering and presence of moisture content due to improper storage leads to the increase in microbial load and simultaneously convert some metabolites into another which can further react with the endotoxins and mycotoxins and produce adverse reactions (Agarwal et al. 2014).

The physicochemical contaminants include heavy metals, organic pollutants, residual solvents agrochemicals, pesticides and radioactive material. The presence of various elements such as zinc, manganese, chromium, copper, iron, lead, nickel, vanadium, cadmium, mercury, lead and arsenic are reported. In a report by Saper et al. (2009), high prevalence of lead and mercury was found in the US and Indian-Manufactured Ayurvedic medicines. Heavy metal contamination was also found in traditional medicines originated from UAE (Dghaim et al. 2015), Malaysia (Uddin et al. 2013), Brazil (Caldas and Machado 2004), Australia (Denholm 2010) etc. Organic pollutants, which are non-biodegradable and persist in soil and environment as DDT, BHC also affect the quality of herbal drugs. Residual agrochemicals and pesticides used during the cultivation can be mixed with the herbal drugs. Detection of these contaminates is mandatory step to provide assured quality and purity of herbal drugs. Various analytical detection techniques as TLC, HPLC, GC combined with MS are recommended for determination of prevalent toxic chemical compounds in herbal products.

AUTHENTICATION/IDENTIFICATION OF BOTANICALS IS THE FIRST STEP IN QUALITY MANAGEMENT

Correct identification of the starting material is the very first step among the various quality parameters. The original plant species should be reliably distinguished from their close relatives, inferior substitutes, adulterants, filler materials and counterfeits (Chen et al. 2014). Case reports are there evidencing the serious ill effects, due to misidentification of the medicinal plant species. For example, In 2004, aristolochic acid nephropathy was diagnosed in three patients. It was reported that they ingested Aristolochiae Mollissimae Herba (Xungufeng) which was derived from the *Aristolochia mollissima* Thunb, in place of non-harmful genuine anti-cancer herb Solani Lyrati Herba (Baiying), which was *Solanum lyratum* Hance. Safety issues regarding the use of Periplocae Cortex (Xiangjiapi) derived from *Periploca sepium* Bge. Have been reported. It was due to the replacement of original *Periplocae Cortex* with *Acanthopanacis Cortex* (Wujiapi) which was derived from Acanthopanax *gracilistylus* W.W. Smith (Chen et al. 2014). In addition, mixing of any inferior or irrelevant filler materials reduces the effective dosage of original drugs. Properly authenticated and scientifically validated plant species are the prime requirement for the preparation of evidenced based drugs. The authenticity of the material should be well established, whether it is required for the research or the clinical evaluation or for the consumption in the market (Smillie and Khan 2010). A survey of 150 herbal drug companies revealed that more than 50% of companies are facing problems in collecting and authenticating raw material and 54 companies (36%) admitted that adulteration of raw materials is very common (Sahoo and Manchikanti 2013). These

facts have raised the alarm to all the personnel's associated with the herbal drug sector to find the scientific solutions.

Reasons for misidentification and plant derived adulteration

Many factors are exemplified below as responsible for the misidentification of the herbs and plant-derived adulteration.

Careless Collection by unskilled collectors

Most of the times, plant collection rely upon the persons who are not expert in the taxonomic skills. Collections from the wild resources enhance the problem intensity. The persons who are employed as collectors, lack proper training for species identification in a scientific way. Mostly the persons are picked from the local communities who do not have appropriate knowledge of botanical names of the species and they only know the plants from their indigenous names. This increases the chances of wrong identification and collection of the species. Cultivation of medicinal plants also depends the farmers who are not literate to the extent that they can grow right species. WHO has recommended some GAP for medicinal plant collection from wild as well as from the fields. All the personnel as growers, collectors, producers should receive adequate knowledge of agriculture, plant science and their conservation and every person involved in the plant collection should be trained and skilled for the procedure of proper sample collection in a scientific way.

Less knowledge about the authentic source

Sometimes the collection place or the sources are hard to explore or inaccessible for searching the authentic species. Easy accessibility to the other areas for collection of the any other similar species can lead to the procurement for analogous species from these areas. As for example authentic source of Nagakesar is *Messua ferrea* which is found abundantly in the Western Ghats of the Himalayan region but the unawareness of the collectors or suppliers and the restriction in collection in the forest areas, the drug is adulterated with the *Callophylum inophyllm* (Shah and Seth 2010).

Confusion in species identity

The method of description of medicinal plants is different in the modern era and in the ancient medical literature. In ayurvedic literature, plants were classified on the basis of their therapeutic properties. Lack of apposite synchronizations between Sanskrit and botanical names, lead to the incorrect identification of plant species. Wrong interpretation of the plant description may cause the confusion which is sometimes influenced by the local languages and indigenous culture. The similarity in vernacular names based on the belief of indigenous people also one of the reasons of erroneous identity of the plants. These drugs were considered as 'Sandigdha dravyas' or 'Controversial drugs' (Dixit 2011). Jivanti is one of such example. Three different species *Lepatdenia reticulata, Desmotrichm fabricatum, Cimifuga foetida* all are known as Jivanti (Dixit 2011). Some other example include Ashoka and Brahmi. The three species *Shorea robusta, Polyalthia longifolia* and *Saraca asoca* all are confused as 'Ashoka drug' (Khatoon and Mehrotra 2009), while *Bacopa monnieri* and *Centella asiatica* both are traded under the same vernacular name Brahmi (Kumar 2007). Sometimes the herbs which are available abundantly in different regions can be used as adulterant or substituent. As for example the two species, one is *Fumeria parviflora* which is known as Parapatta and the other is *Mollugo pentaphylla* which is used in Siddha medicines as Parpadagam. In the north Indian region the former plant species is traded both as Parapatta and Parapadgam and in the south India the later species is sold (Shah and Seth 2010). Similarly, Ginseng a very important drug used in traditional system of medicine. Different species are traded in different countries. *Panax ginseng* known as

Chinese/Asian ginseng, *Panax quinquefolius* is American ginseng, Siberian ginseng is *Eleutherococcus senticosus*, *Withania somnifera* and *Acanthopanax senticoccus* known as Indian and Russian ginseng respectively (Kumar 2007).

The morphological similarity is also one of the reasons for the use of misidentified herbs. Lots of confusion associated with the genus Phyllanthus. The three morphologically near species *P. fraternus, P. amarus, P. urinaria* are considered as Tamalaki or Bhuiamalaki. Moreover, *P. amarus* and *P. niruri* sometimes considered as synonyms or the previous species is considered as a subspecies of the other. *P. fraternus* also considered as synonyms of *P. niruri* (Khabiya 2012; Theerakulpisut et al. 2008). Similarly, the source of drug Puunernava that is *Boerhavia diffusa* also has conflicts of identification with other *Boerhavia* species (Selvaraj et al. 2012). *Tribulus terrestris* also share similar morphology with *T. lanuginosus* and *T. subramanyamii*. Fruits of all the three are known as goksura (Balasubramani et al. 2010). Some of the medicinal plant species have more than one name. The more numbers of synonyms poses difficulty and can create misunderstanding. As for the example *Cassia acutifolia* also vernacularly known as Alexandiran senna and have different synonyms as *Senna Alexandria*, *C. senna*, *C. obtusata, C. sophera* (Kumar 2007).

Absence of taxonomic key characters during collection

In certain plants identification is possible only at a particular stage of life or may be relied upon particular organs which may or may not be present during the time of plant collection. As for example leaves of *Saraca asoca* and *Polyalthia longifolia* have close resemblance to each other (Begum et al. 2014). It would be difficult to differentiate them on the basis of leaves for untrained collectors. Flowers can help somehow as a character for identification.

The problem becomes more prevalent in lower taxa as in algae, fungi, bryophytes, pteridophytes and conifers in which reduce taxonomic characters are present in much reduced form. As for instances, *Equisetum arvense* known as horse tail is the member of pteridophytes and can be admixed with the toxic *E. palustre* (Mills and Bone 2005).

Deficiency of the authentic species

Increasing demands of herbal drugs leads to their overexploitation. This condition is responsible for the collection of species other than authentic one. Some authentic species are endemic to a particular area; in such circumstances the species found abundantly in other areas are preferred for gathering. As for example *Hypericum perforatum* is the species of European region but in India *H. patulum* is sold as *H. perforatum,* which can be found copiously in the Indo-Nepal region (Shah and Seth 2010).

Intentional mixing of other vegetative parts of the same plant

Many secondary metabolites synthesized in the plants in tissue-specific manner. As in *Withania sommnifera* withaferin A is accumulate in leaves whereas withanolide A is root specific metabolite, which are used as the anticancer compounds and the neuroprotective compounds respectively (Gupta et al. 2013). In some medicinal plant species, specific plant part or organ is consumed as the drug. As for example bark of *Cinchona, Saraca asoca,* fruits of *Terminalia chebula,* and roots of *Clerodendrum multiflorum* (Burm F.*)* etc. Different plant parts show different pharmacological activities. Mixing or substitution of organs and plant parts with the therapeutically important plant tissue of the same plant can produce negative impact.

Methods for traceability of botanicals

Various methods are available to inhibit the use of incorrect starting material and to ascertain the identity of any herbal material.

Conventionally, this is done by examining morphological characters, anatomical characters and chemical analysis using chemical fingerprinting or by evaluating specific

markers. These methods have some limitations which can be overwhelmed by the adoption of DNA-based technologies.
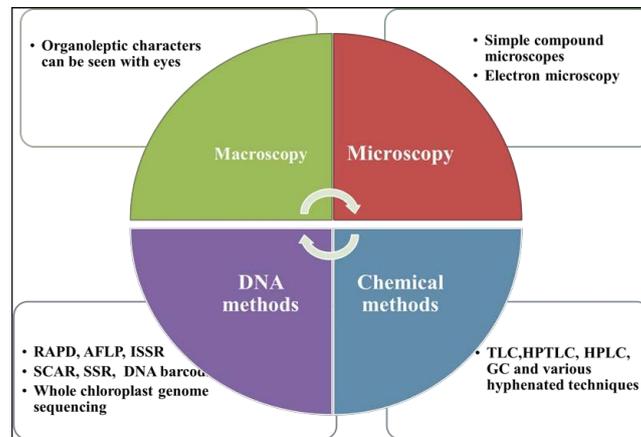

Figure 1.2 illustrates the scientific methods used for authentication of herbal material.

Morphological Identification

Morphological identification methods comprise an examination of the taxonomic key characters which can be easily visualized with eyes. Macroscopic characters depend on the analyses of organoleptic characters of whole plants or the plant part. For example shape, size, color, texture and odor of leaves, flowers and fruits, leaf type, leaf margin, leaf tips, flowers and their characteristics, inflorescence etc. Identification of the unknown sample can be possible by comparing it with the plant reference material in the form of herbarium or the voucher specimens. Morphological identification is the easiest way to validate the identity of any plant material in its intact form. But, it becomes impossible when the sample for identification is not complete and when the taxonomic key characters are reduced. The plant samples devoid of key taxonomic characters (as, the absence of flowers) also present challenge in the identification with the classical botany. These methods are not sensitive enough for intraspecific gradations as ecotypes and chemotypes (Techen et al. 2004).

Microscopic Identification

Microscopic identification methods go a step further and can be applied to establish authenticity and identity of original botanicals and adulterants in the broken or grounded state, in which morphological characters are indistinguishable. Microscopy allows examining the sample identity using distinguished characters as histology, cell morphology, cell type and cell contents. Advanced microscopy techniques as the application of phase contrast microscopes, fluorescence and confocal microscopes, scanning electron microscope has increased the accuracy and precision of identification (Smillie and Khan 2010). However, cost factor limits the use of high- end microscopes over light microscopes. Microscopic identification methods for various medicinal plant species were illustrated in various reports (Zhang et al. 2015). However, microscopic analysis for identification also requires specialized trained persons. Moreover, it is difficult to found distinguishable characteristics in two closely related or co-generic species because most of the time they share similar anatomical features (Smillie and Khan 2010).

Analytical chemical methods

Analysis of phytochemistry and detection of chemical phytoconstituents of medicinal plants is the other most preferred approach in order to ascertain the identity of particular species. This can be done in two ways by generating chemical fingerprints (pattern oriented) or through identification of specific chemical /biological marker compounds (compound oriented) (Govindaraghavan et al. 2012). Many analytical methodologies as TLC, HPTLC, HPLC and GC along with various hyphenated detection modes can be used for authentication of botanicals using the either approaches (Smillie and Khan 2010; Steinmann and Ganzera 2011).

Chemical fingerprint analysis and more complex complete metabolic profiling have demonstrated their potential in various researches (Gao et al. 2012; Li et al. 2011). However, to generate the established and properly validated chemical fingerprints for a particular plant species, a number of authenticated reference plant materials from the multiple populations are required. As exposure to differing environmental conditions and genome structure can bring the spatial and temporal variations in the chemical profile, even in the same species, so statistically significant number of representative samples are must (Dhami and Mishra 2015; Smillie and Khan 2010). Amalgamation of chemical fingerprinting data with the statistical techniques as hierarchal cluster analysis and principle component analysis to generate chemometrics can provide automation and improve the quality of analysis through chemical profiling (Bansal et al. 2014). This multicomponent approach including chemical fingerprinting followed by chemometric analysis, was illustrated for the authentication purpose of Cassia seeds (Lai et al. 2010), licorice roots (*Glycyrrhiza glabra*, *G. uralensis*, and *G. inflate*) (Simmler et al. 2015), analysis of formulas containing *Taraxacum officinalis*, *Cynara scolimus*, *Silybum marianum*,

Hypericum perforatum, *Chelidonium majus* and *Lycopodium clavatum* (Pop et al. 2013).

Chemical fingerprinting based on one or more known characteristic chemical or biological markers enhance the specificity over uncharacterized fingerprinting. Chemical markers can be defined as the fully characterized chemical constituent/s of the plants which can be used for qualitative as well as quantitative quality control of herbal drugs including species authentication. The chemical markers having any therapeutic value are considered as biological markers (Li et al. 2008). Some other categories for chemical makers were proposed by various scientists. They also proposed their selection criteria to be optimally used in the standardization of herbal drugs (Li et al. 2008). The major issues related to widespread adoption of chemical markers for authentication is that, very few markers are well defined for very less number of herbal drugs and sometimes two different plants share the same markers. Commercial availability of the markers in lower costs is also a limiting factor because chemical synthesis or the extraction and isolation of the each identified marker compound is not possible. Environmental variations can cause phytochemical variability even in the same species. Universally applicable optimizations are required for methodological approaches for isolation and preparation of reference compounds and should be regulated properly to minimize the experimental variations (Govindaraghavan et al. 2012; Li et al. 2008; Smillie and Khan 2010).

DNA based methods- from genome profiling to DNA barcoding

Traditional methods from morphological assessment to chemical analysis for authentication and identification of medicinal plants have contributed immensely since the advent of pharmacognosy by Austrian physicist Schmidt. These techniques are the primarily mentioned in the pharmacopoeias for

the quality control of the herbal drugs including their authentication (Chen et al. 2014). Limitations of these approaches provoked the need for additional methods. DNA-based methods are prominently drawing the attention of scientific communities due to their various benefits over conventional methods. Genetic composition of a particular species is generally not affected by age, type of tissue, diverse physiological and environment conditions. DNA is very rigid molecule so chances of affecting the detection due to physical state of the samples are less. These approaches are not completely dependent upon the personal expertise as macro and microscopy. Small amount of experimenting material is required for detection. DNA- based methods can be used as stand-alone or as strengthening tool to the conventional methods (Joshi et al. 2004). Patents were granted in this field proves the need and progress of these techniques for authentication (Shaw et al. 2009). In a recent report by Palhares et al. (2015), it was recommended that authenticity of the plant samples must be evaluated with the DNA based methods. They observed that the samples which were identified as the substituents by the DNA methods were passed in the quality checking by chemical marker analysis. They raised concerns about the deliberate mixing of synthetic chemical markers in the drugs which can enable them to be passed during chemical authentication. Genome-based approaches for herbs traceability and molecular identification of the medicinal material are progressing (Galimberti et al. 2013; Sucher and Carles 2008). Various DNA techniques have been using for medicinal plant species identification which can be broadly classified as DNA fingerprinting and Sequence-based methods

(A) Non-targeted DNA fingerprinting/genome profiling

In general, DNA fingerprinting here stands for the techniques which are arbitrarily/specifically primed to generate DNA banding patterns through *in vitro* amplification/ PCR. These techniques are very helpful when small genetic information of a particular species is available. These are the multilocus practices which cover the variations spreading in the whole genome and not dependent upon the prior information of genome sequences. These methods are relatively cheap and generate multiple markers in one run (Poczai et al. 2013). Randomly amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Inter simple sequence repeats (ISSR) are some of the examples of such basic techniques applied for molecular identification of medicinal plant species and their discrimination with the plant-derived adulteration.

RAPD is based on the amplification of genome with arbitrary oligonucleotides of 10-15 bases in length. The amplification is carried out at low annealing temperatures. The primers bind several priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products if these priming sites are within an amplifiable distance of each other. Banding patterns thus generated are analyzed on agarose gels. RAPD was reported for molecular authentication of various medicinal plants (Guo et al. 2010; Khan et al. 2011; Lam et al. 2015). However, robustness and reproducibility is the major problem in RAPD. The technique is prone to changes in experimental conditions, for instances, poor template quality, presence of too much RNA, inconsistent interpretation of mixed-intensity banding patterns, competition between primer target sites, generation of hetero duplex molecules and generation of primer-derived, nonspecific amplification products, change in the thermo cyclers units (Weising et al. 2005).

ISSR or Inter simple sequence repeats is the technique based on the variations detection between the microsatellite regions. Primers for amplification are designed based on the sequence repeats which resulted into the finger prints; belong to the genomic regions between

two repeat sites, oriented in the opposite direction (Godwin et al. 1997). ISSR are considered as more reproducible than RAPD due to longer primer length which allowed amplification on the higher annealing temperatures (Reddy et al. 2002). However, reproducibility may be negatively affected by sensitivity of the detection methods and can lead to the wrong interpretations (Ganie et al. 2015). ISSR markers have been used for genetic diversity measurement and to authenticate plants as *Sida* spp.(Thul et al. 2011), *Tribulus terrestris* (Sarwat et al. 2008) etc.

AFLP or amplified length polymorphism is the more accurate and reproducible fingerprinting technique. AFLP is based on the amplification of specifically restricted genomic DNA. It combines the reliability of the RFLP technique with the flexibility and rapidity of PCR technique. The technique involves three steps: (i) Restriction of the DNA with two enzymes rare and frequent cutter (ii) Ligation of oligonucleotide adapters (iii) selective amplification of restriction fragments having one to three random oligonucleotides at the 3'ends. AFLP have been used to ascertain the identity of *Panax ginseng* and *Panax quinquefolius* (Ha et al. 2002), *Chlorophytum* and *Asparagus* (Misra et al. 2007), *Embelia ribes* burm.F and *Embelia tsjeriam*- Cottam A. DC.(Gowda et al. 2010), *Swertia* species (Misra et al. 2010), *Crocus sativus* (Busconi et al. 2015) etc.

(B) Sequence-based methods

The above mentioned descriptions are the examples of DNA fingerprinting techniques used for the molecular identification of the medicinal plants. The advantage of these techniques comprises no requirement of prior knowledge of genome. However, their universal application may be hampered because their reproducibility and robustness changes in some extent with reaction conditions, template concentration, ratio of primer and template DNA, methods of DNA isolation, tissue source of DNA and quality as well as integrity of DNA. Results may be varying based upon the local lab expertise and difference in analysis can cause the errors (Boiteux 1999; Crawford et al. 2012; Jones et al. 1997). Genetic diversity of a particular species, hybridization and polyploidization history can also create a negative impact on the analysis especially when the purpose of the study is species authentication (Galimberti et al. 2013). In a report by Peng et al. (2016), ISSR markers were found insufficient to discriminate *Tetrastigma hemsleyanum* from the species of the same genus.

The advancement and cost reduction of sequencing procedures increase their popularity and fondness over gel based DNA fingerprinting, for various genome-based analysis integrating authentication (Mishra et al. 2015). These techniques need access to a sequence of the particular genomic region and can give more precision, robustness and improve cost/effectiveness ratio in comparison to discontinue fingerprinting techniques mentioned above (Galimberti et al. 2013). Sequence-based approaches have added smartness to the studies and can be used to obtain information directly from a defined locus with more transferability among labs than random fingerprinting techniques. Access to the sequence information in various databases eases the identification processes and makes them approachable and fast. Regarding DNA sequence-based approaches different techniques like SSR, SNP and SCAR are used widely for the authentication and several other genomic applications. These approaches are described below.

SCAR or sequentially characterized amplified region is the advance single locus targeted version of random markers. Dependent upon PCR they give rapidity, stability, reliability with specificity. These markers originated from the fingerprints, so give the advantage to be utilized where no any prior knowledge of genome

sequence is available. To generate SCAR markers distinct bands were selected, eluted, cloned and then sequenced. The resulted sequence can be utilized to develop primers for the development of both single plex as well as multiplex species-specific PCR-based identification assays. Most of the SCAR markers were derived from the RAPD due to its easy setup. Derivation of SCAR from the more robust techniques like AFLP can give the benefit of more reproducibility and fineness. SCAR markers were developed to authenticate three species of *Phyllanthus* (Theerakulpisut et al. 2008), *Angelica decursiva* (*Peucedanum decursivum*), *Peucedanum praeruptorum* and *Anthricus sylvestris* (Choo et al. 2009), *Panax notoginseng* (Kwon et al. 2009), *Crocus sativus* (Marieschi et al. 2010), *Ipomoea mauritiana* (Venkatasubramanian et al. 2011), Liriope and Ophiopogon (Li and Park 2012), *Cuscuta reflexa* and its adulterant *Cuscuta chinensis* (Abdin et al. 2012), *Knema andanamica* (Sheeja et al. 2013), *Bulbus fritillariae* (Xin et al. 2014).

Simple sequence repeats (SSR) or microsatellites are the markers having repeated units of nucleotides which are known as motifs. Each repeating unit may contain mono, di to six nucleotides and number of repetition of each unit can be polymorphic, hence it is a type of length polymorphism. These repeating units may be spread both in the coding or noncoding part of the nuclear as well as in the organelles genome (chloroplast and mitochondria). They are advantageous in terms of co-dominance, high allelic polymorphism and reproducibility. Many microsatellites markers were identified using different methods and for different purposes as genetic characterization, diversity analysis at the genomic level, cultivar identification, marker assisted selection breeding and mapping of medicinally important plants. Microsatellites markers were isolated from medicinally important plant, *Dendrobium officinale* in 2007 by

Gu et al., for authentication purpose along with an analysis of genetic diversity, population structure. SSR markers were also used to authenticate *Bupleurum chinense* (Sui et al. 2009)*, Picrorhiza kurrooa* and to detect its adulterant *Lagotis cashmiriana* (Hussain and Bedi 2012).

DNA barcoding is the most advance concept developed for molecular identification of the animal or plant species which have been potentially extended to molecular standardization of herbal material. The above-mentioned techniques (SCAR, SSR) showed great potential to ascertain the identity of botanicals. However, lack of universality and standardized experimental procedures limit their use for authentication and simultaneously, transferability of these procedures is confound to a particular taxon and their close relatives (Galimberti et al. 2013). They are not sufficient for exact taxonomic annotation of a species, therefore, needed a more broadly accepted commercial tool which should be universally applicable to each species on the earth (Mishra et al. 2015). DNA barcoding has potential to define species boundaries very precisely. Cryptic species can also be identified. The concept of DNA barcoding was given by Hebert (Hebert and Gregory 2005) at first in animals which meant for utilization of variations present in the sequence of the single gene or intronic region as a unique identifier of a particular species; such that one sequence represents one single species. The standardized experimental procedure for DNA barcoding includes amplification of barcode region with universal primers, sequencing and then sequence comparison with databases. This is universally applied procedure and not limited to particular species and any particular stage of the life cycle for species identification. The sequence recommended for barcoding should contain some attributes as they should universally amplifiable with a single set of primers, should not suffer during sequencing and should diverge enough

between species than within species. The genes endorsed for barcoding in animals (gene for cytochrome oxidase) cannot be immediately used for plants due to its comparatively slow evolution pace (Kress et al. 2005). The inherent properties of plants make them harder to discriminate and species delimitation is somewhat difficult for this kingdom (Rubinoff et al. 2006).

Being existent in the nuclear genome ITS region can disentangle these problems as it is bi-parentally inherited, have low functional constraints, flanked by highly conserved genic sequences, high evolutionary rates give more discriminatory power and can be amplified from degraded DNA also (Hollingsworth 2011). Concerted evolution is the reason for the existence of identical copies of this array, but sometimes incomplete concerted evolution leaves the non-homogenized copies within the genome. The presence of multiple copies can mislead the analysis. Orthology is needed for correct assessment of species resolution and presence of paralogous copies can hinder the investigation (Alverez & Wendel 2003). These factor disgraces the value of ITS. Despite, many scientists proposed ITS as a solid candidate for barcoding in different groups of plants with more emphasis for flowering plants (Chen et al. 2010; Group et al. 2011, Techen et al. 2014; Tripathi et al. 2013; Yao et al. 2010). In the view of the failures of single loci barcoding, many scientists recommended inclusion of two or three barcode candidates (multilocus approach) (Li et al. 2014). The plant working group of the consortium of the barcode of life stamped the two-locus system rbcL + mat K as suitable DNA barcode for plants (Group et al. 2009). Other barcode combinations as rbcL with and/or the internal transcribed spacers and ITS+ trnH– psbA, have also been found equally potential (Kress et al. 2005; Kress and Erickson 2008; Pang et al. 2012; Tripathi et al. 2013). In addition to the multi loci barcoding, two-tiered approach was proposed by Newmaster et al. (2006) in which two barcode

loci should be used sequentially. Particularly, for medicinal plants rbcL can be used as first barcoding loci followed by ITS/ITS2 (Mishra et al. 2015).

Various international and national institutes (CBOL, iBoL) are working together to catalog each species on the earth with DNA barcoding and also provide a platform for sequence deposition which is freely accessible. Particularly for medicinal plants, MMDBD or Medicinal Materials DNA Barcode Database **(http://137.189.42.34/mherbsdb/index.php)** is the database provides sequence information related to medicinal plants materials recorded in the Pharmacopoeia of China and USA. This database, updated in May 2014 with 1661 species and 51,375 sequences available related to plants, fungi and animal. Furthermore, an online DNA barcoding database specific to herbal materials has been created **(http://www.tcmbarcode.cn)** based on the ITS2 and psbA–trnH as the core and supplementary DNA barcodes, respectively. This database also contains barcoding data related to adulterants, substitutes and closely related species (Chen et al. 2014).

Future developments towards NGS

The advent of high throughput sequencing technologies termed as next generation sequencing or NGS expand the boundaries of DNA barcoding beyond the single or two locus barcodes. Sequencing of whole chloroplast genome is represented as the potent alternative of single or multilocus barcodes (Li et al. 2014). Consideration of whole chloroplast genome for plant species identification can increase the discrimination power and resolve the closely related species. In comparison to nuclear genome chloroplast genome is smaller in size so easy to be applied. However, unavailability of high-quality DNA, finished assembled sequence, lack of reference sequences or still high cost of sequencing restrict the use of whole chloroplast genome as barcode in routine practice (Li et al. 2014; Ivanova et al.

2016).

CONCLUSION

Conclusively, the significance of medicinal plants has already evident in the ancient writings and continues at present. Inputs of advanced technologies have revealed the structural diversity of plant-derived therapeutic molecules which has infinite prospects to provide ingredients for novel drugs. Synergism of technological advances with traditional medicinal systems has established medicinal plants as resources of more novel therapeutic agents. This regeneration of medicinal plants have brought more opportunities but also fetched various challenges in standardization and scientific validation of the herbal products.

Medicinal plants have their glorious past and still used as the medicinal resources. But, admiring the magnificent therapeutic potential of these plants is not sufficient to make them a reliable source of medicines. Scientific validation of phytopharmaceuticals is essential to make them consistently safer and efficacious. Quality in the herbal products is the major concern in their global acceptance. This has raised the need for scientific evaluation of herbal drugs from the initial step from plant collection or cultivation to the preparation of formulation. Correct identification of the species or the raw material is the first step for the production of standard herbal drugs. This is possible only through imposing strict legislations assisting with various scientific methods from morphological analysis to DNA-based methods. DNA-based methods are the most advanced approach and can be used stand alone.

Furthermore, these can be combined with the other conventional practices to develop comprehensive approaches for authentication of botanicals. The review derives the advantages of DNA-based method for the purpose of correct identification of herbal material and discrimination of originals from the plant-derived adulterants.

Consent for publication

We certify this manuscript has not been published elsewhere and is not submitted to another Journal.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

LS conceived the study design, interpreted and manuscript preparation. AD and MS drafting of the article details with edit of the manuscripts. LS contributed with critical revision of the manuscript. All authors read and approved the final manuscript.

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