

**HERBAL DRUG STANDARDIZATION AND APPLICATION OF DNA
FINGERPRINTING – A REVIEW****Dr. Pratiksha R. Gharde^{1*}, Dr. D. S. Chothe² and Dr. Sanjivani Shekokar³**¹PG Scholar Department of Dravyaguna, Government Ayurved College, Nanded. 431601.²Guide & Associate Professor, Department of Dravyaguna, Government Ayurved College, Nanded.³Head of Department, Department of Dravyaguna, Government Ayurved College, Nanded.***Corresponding Author: Dr. Pratiksha R. Gharde**

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ABSTRACT

Ayurvedic herbal medicines played an important role in maintaining health and treating the disease worldwide since ancient times. A commercialization and increased demand safety, quality and assurance of *Ayurvedic* herbal medicines are biggest lacuna. The adulteration of herbal materials usually occurs as a result of materials not having readily distinguishable morphological features, materials sharing similar common names and the substitution of economically valuable materials with inexpensive herbs. Proper authentication and identification process is necessary to prevent the adulteration. Since the standardization of botanical preparations most of the regulatory authorities suggest macroscopic, microscopic and chemical evaluation like TLC, HPTLC and HPLC. However, these methods have limitations because the composition and relative phytochemicals in a particular species of plant varies with growing conditions harvesting period, post harvesting period and storage conditions. The present review mainly focuses on authentication of *Ayurvedic* herbal medicines by DNA based fingerprinting methods to prevent intentional and inadvertent adulteration or substitution of targeted *Ayurvedic* medicinal herbs.

KEYWORDS: Authentication, DNA fingerprinting Herbal Drug Standardization.**INTRODUCTION**

Ayurvedic herbal medicines played a pivotal role in maintaining health & treating the disease worldwide since ancient times. According to WHO guidelines authenticity, purity & safety are important aspects of standardization & in evaluation of traditional medicines, the first step is authentication.^[1,2] Authenticity relates to proving the material is true & corresponds to right identity. As commercialization & increased demand safety, quality & assurance of *Ayurvedic* herbal medicines is biggest lacuna. Herbal medicinal plant materials are often substituted and/or adulterated either accidentally or intentionally with herbs from closely related species which are morphologically indistinguishable or by materials from unrelated plants. The adulteration of herbal materials usually occur as a result of materials not having readily distinguishable morphological features, materials sharing similar common names & the substitution of economically valuable materials with inexpensive herbs.^[3] Proper authentication process is necessary to prevent the adulteration of target plant with other plant materials. For the standardization of botanical preparations most of the regulatory authorities & pharmacopeia's suggest macroscopic, microscopic & chemical evaluation. As macroscopic identity of botanical materials is based on parameters like shape, size, colour, texture, surface

characteristics, fracture characteristics, odour, taste & such organoleptic properties that are compared to a standard references. Microscopy involves comparative microscopic inspection of broken or powdered crude botanical materials. Chemical profiling establishes a characteristics chemical pattern for a plant material. Chromatography tools like Thin Layer Chromatography (TLC), High performance thin layer chromatography (HPTLC) & High-performance liquid chromatography (HPLC) are routinely used for qualitative determination of small amounts of impurities.^[4] Macroscopic & microscopic examinations & chemical analysis can be used as rapid & expensive method for plant identification & detection of contaminants.^[5] However these methods have limitations because the composition & relative amount of chemicals in a particular species of plant varies with growing condition-harvesting period, post harvesting period & storage condition.^[6] Each herb contains large number of compounds & therefore it is also not possible to analyse or trace the presence or absence of all compounds of interest either qualitatively or quantitatively. These serious difficulties in testing for active principle or chemical constituents are well known.^[7]

Authentication of botanicals which are medicinally valuable is an important issue globally because of

unavailability/underutilization of appropriate tools for standardization. Since DNA is more stable & does not vary seasonally & with age of the plant. DNA based fingerprinting techniques have greater role in the authentication of botanicals which are medicinally important. DNA markers are reliable for information on genetic polymorphism as the genetic composition is unique for each species irrespective of plant part used & is not affected by age, physiological condition as well as environmental factors.^[8]

The present review mainly focuses on standardization & authentication of Ayurvedic herbal medicines by DNA based fingerprinting methods to prevent intentional & inadvertent adulteration or substitution of targeted Ayurvedic medicinal herbs.

Standardization and Quality Control of Herbal Medicines – Concept and Scope

As per WHO [1996 a and b, 1992]: Standardization and Quality Control of herbals is the process involved in the physicochemical evaluation of crude drug covering aspects, such as selection and handling of crude material, safety, efficacy and stability assessment of finished product, documentation of safety and risk based on experience, provision of product information to consumer and product promotion.

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. The quality assurance is also required during cultivation, harvesting, primary processing, handling, storage, packaging and distribution. Therefore, there has been introduced a set of criteria and guidelines by WHO to be followed at each step as an intergral part of quality control standards:

1. Good Agricultural and Cultivation Practices
2. Good storage practices
3. Good Manufacturing practices
4. Good laboratory Practices

The main Objective of these guidelines is to contribute to the quality assurance of medicinal plant materials used as the source for herbal medicines, which aims to improve the quality, safety and efficacy of finished herbal products and minimize the risks involved in any pharmaceutical production that cannot be eliminated through testing the final product.^[9]

Need for Standardization

In the global perspective, there is a shift towards the use of medicine of herbal origin, as the dangers and the shortcoming of modern medicine are getting more apparent. It is the cardinal responsibility of the regulatory authorities to ensure that consumers get the medication, which guarantees identity, purity, quality, potency, efficacy and stability.

WHO Guidelines for Quality Standardized Herbal Formulations

- 1) Quality control of crude drugs material, plant preparations and finished products.
- 2) Stability assessment and shelf life.
- 3) Safety assessment; documentation of safety based on experience or toxicological studies.
- 4) Assessment of efficacy by ethno medical information and biological activity evaluations.

Caraka clearly described the parameters to be assessed for a drug to be standardized in *Vimanasthana* 8th chapter where the concept of quality, safety, and efficacy was explained elaborately.^[10]

Etham Avam Prakriti Avam Gunam avam Prabhavam asmin Deshe Jatam Asmin Rutav Avam Gruhitam Avam Nihitam Avam Upaskrutam Anayach Matraya Yuktam Asmin Vyadhav Avam Vidhasya purushasya area tavantam Dosham Apakarshati Upashamayati va Yat Anyat Api Cha avam Vidham Bhesajam Bhavet tat cha anen Visheshen Yuktam iti.

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- *Etham avam prakrutim* [Name & natural order of plant] – Correct and well identified species should be selected.
- *Avam gunam* –[Rasa, Veerya, Vipak,]- Qualities & active principles of the drug.
- *Avam Prabhavam* – Pharmacokinetics of drug.
- *Desha jatam* – Habitat, Source, Soil character. Eg. *Guggulu* should be taken from Rajasthan.
- *Rutam avam* – Seasonal characteristics
- *Rutam gruhitam* – Proper time of collection & mode of preservation eg. *Chandana* after 20 years *guggulu* after 8 years.
- *Avam nihitam* – Mode of storage & packaging.
- *Avam upskrutam* – Types of processing
- *Anaya ch matraya* Yuktam – Dose & time schedule.
- *Vyadhi prayuktam* – Clinical drug research indicating the use of medicines in specified diseases.
- *Avam Vidhasya purushasya tavantam dosham apakarshayati* – Assessment of the drug for its eliminating activity on body elements.
- *Avam vidhasya purushasya atavantam dosham upshamayati* – Prediction of palliative action of the drug.
- *Yat anyad api bhesajam bhavet tat ch anen visheshen yuktam* – Controlled & comparative study of effects of trials drugs with unknown & proven therapeutic agent.

When Standardization

1. Raw material standardization – Before starting Research.
2. In process – Stepwise & timely observations
3. Quality control / finished product standardization – Before Animal experimentation.

Conventional Methods for Standardization of Herbal Formulation

Phytochemical standardization encompasses all the possible information generated with regard to the chemical constituents present in an herbal drug. Hence, the phytochemical evaluation for standardization purpose includes the following:

- Preliminary testing for the presence of different chemical groups. [e.g. total alkaloids, total phenolics, total triterpenic acids, total tannins etc.]
- Quantification of chemical groups of interest.
- Establishment of fingerprint profiles based upon single or multiple markers.^[11]

Standardization of herbal raw drugs include passport data of raw plant drugs, botanical authentication which include microscopic & molecular examination, physical parameters like moisture content, ash value, extractive value etc, identification of chemical composition by various chromatographic techniques and determination of biological activity of the whole plant.

Standardization of herbal formulation requires implementation of Good Manufacturing Practices [GMP]. 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. Good Agricultural Practices[GAP] in herbal drug standardization are equally important.

Advanced Techniques for Herbal Drug Standardization

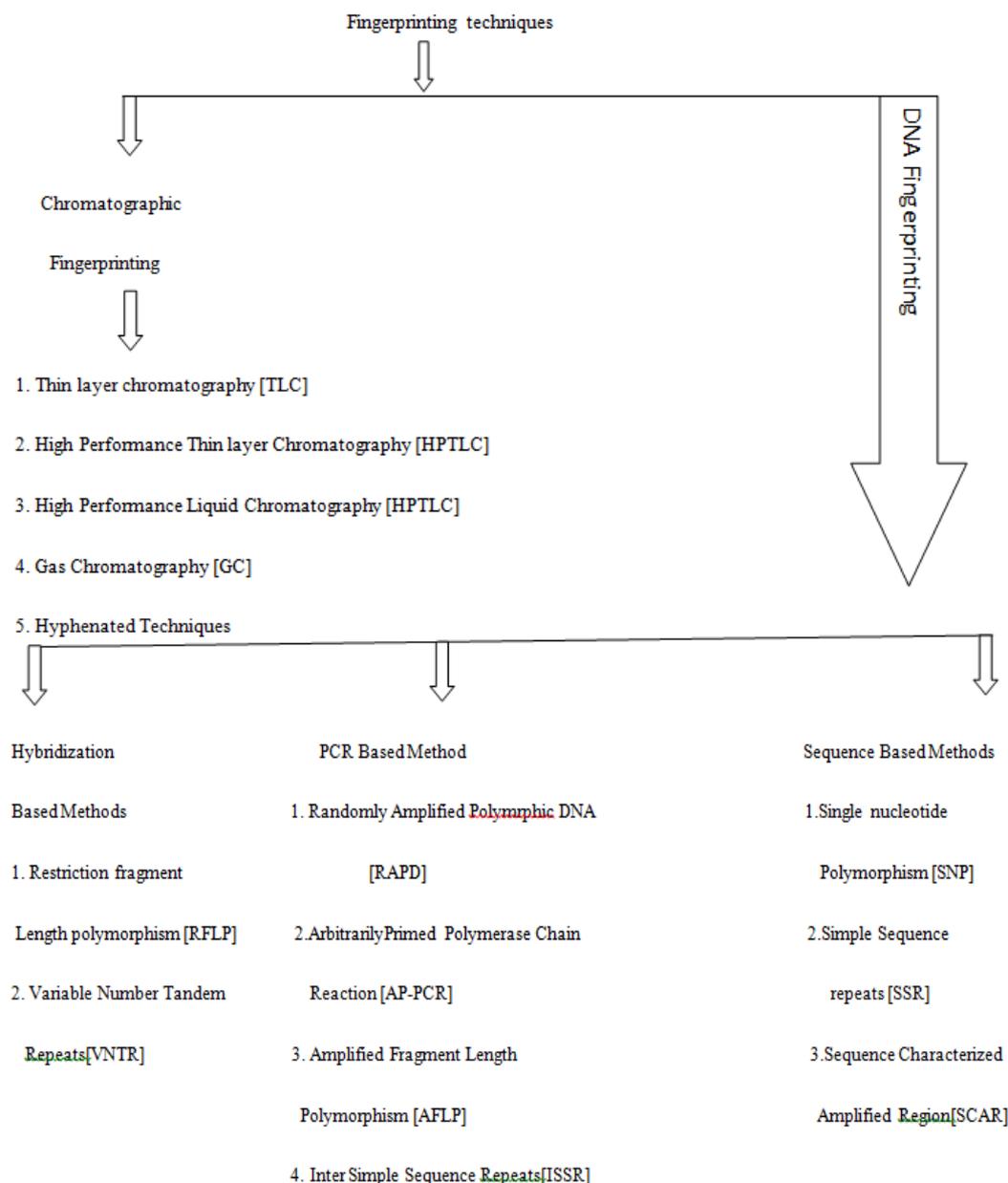
Quality control of herbal medicines is a tedious and difficult task. Herbal medicines differ from that of the conventional drugs and so some innovative methods are necessary for quality assessment of herbal drugs. Fingerprint analysis approach is the most potent tool for quality control of herbal medicines because of its accuracy and reliability. Fingerprinting is a process that determines the concentration of a set of characteristic chemical substances in an herb. Knowing the relative concentration is a means of assuring the quality of herbal preparations. It can serve as a tool for identification, authentication and quality control of herbal drugs. Based on the conception of phytoequivalence, the chromatographic fingerprinting and DNA fingerprints of herbal medicines could be utilized for addressing the problem of quality control of herbal medicines.^[12]

DNA Fingerprinting

DNA fingerprints are a bar code like patterns generated by amplification of chromosomal DNA of an individual which can distinguish the uniqueness of this individual from another.^[13] Also called DNA typing, genetic fingerprinting, DNA profiling. The basic technique of DNA fingerprinting was discovered by Great Britain geneticist ALEC J. Jeffrey in 1984. DNA analysis has been proved as an important tool in herbal drug standardization which is useful for the identification of

phytochemically indistinguishable genuine drug from substituted or adulterated drug. DNA fingerprinting genome remain the same irrespective of the plant part used while the phytochemical constituents will vary with the part of plant used, physiology and environment.^[14]

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.



Types of DNA fingerprinting techniques used in plant genome analysis

Various types of DNA based molecular techniques are utilized to evaluate DNA polymorphism which include :-

1. Hybridization based methods
2. Polymerase chain reaction [PCR] based methods
3. Sequencing based methods

Sub types of above three are explained in above flow chart.

Methodology of DNA fingerprinting

The basic methodology of DNA profiling in plant involve first the isolation of DNA from plant cells, quantification and quality assessment of isolation. The important steps involved in DNA fingerprinting are isolation of DNA from plant part like leaves, root etc than check quality and quantity of isolated DNA afterward running the polymerase chain reaction for

amplify the specific genotyping and interpretation of result and lastly matching has to be done with sample recovered and control sample of suspected herb.

DNA Isolation

DNA from plant tissue is isolated by removal of cell wall and nuclear membrane around the DNA and the separation of DNA from other cell components such as cell debris, proteins, lipids or RNA without affecting the integrity of the DNA. The DNA is isolated from tissues of plants, fresh leaves are preferred.^[15]

DNA Quantification and Quality assessment

DNA quantification and quality assessment is done by using UV-VIS spectrophotometry. Normally quality check is performed through the A260/A280 ratio that is 1.8 value shows the highest purity, if more than 1.8

shows the presence of RNA contamination and less than that indicates protein contamination.^[16]

Polymerase chain Reaction[PCR]

The DNA amplification by thermal cycling called polymerase chain reaction is in vitro method that can be used to amplify a specific DNA segment from small amounts of DNA template or duplex into millions of copies.

Application of different genotyping methods like RAPD, AFLP, RFLP, ISSR are done. Some of them are discussed here:

Simple sequence repeats [SSRs]:- Microsatellites are simple sequence repeats 1 to 6 nucleotides in length, which show a high degree of polymorphism. Specific microsatellites can be isolated using hybridized probes followed by their sequencing. Like any DNA fragment, SSRs can be detected by specific dyes or by radio labeling using gel electrophoresis.^[17]

Advantage:- 1. Fast and highly polymorphic
2. Can be automated
3. Only for very small DNA

Disadvantages:-1. High developmental and start up costs
2. Sometimes difficult interpretation because of stuttering.

Restriction fragment length polymorphisms [RFLP]:- RFLPs are unequal length 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 do the number and location of restriction enzyme recognition sites. On an agarose gel, RFLPs can be visualized using radiolabeled complementary DNA sequences. There is no need for PCR amplification of DNA in this method.^[18]

Advantages:- 1. Unlimited number of loci
2. Detects in related genome
3. No sequence information required

Disadvantage:- 1. Fairly expensive
2. Large quantity of DNA needed
3. Needs considerable degree of skill

Amplified fragment length polymorphism [AFLP]:- AFLP is a PCR based derivative method of RFLP in which sequences are selectively amplified using primers. AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments are then amplified using primers complementary to the adaptor and part of the restriction site fragments. The amplified fragments are visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies.^[19]

Advantages:- 1. Small DNA quantities required
2. No sequence information required

Disadvantage:- 1. Marker clustering
2. Can be technically challenging

Random amplified polymorphic DNA [RAPD]:- RAPD is one of the most commonly used primary assays for screening the differences in DNA sequences of two species / individuals of plants. RAPD consist of fishing/searching for the sequence using random amplification. Here, plant genomic DNA is cut and amplified using short single primers at low annealing temp resulting in amplification at multiple loci, by running a 2 dimensional electrophoresis gel, it is possible to determine the change in sequence pattern by superimposing 2 gels. Once the band of interest is identified, the gel is cut, and the DNA is isolated and sequenced.^[20]

Advantages:- 1. Results obtained quickly & fairly cheap
2. No sequence information required
3. Relatively small DNA quantities required & high genomic abundance

Disadvantage:-1. Highly sensitive to laboratory changes
2. Low reproducibility
3. Cannot be used across

Inter Simple Sequence Repeat [ISSR]:- ISSR is a general term for a genome region between microsatellite loci. The complementary sequences to two neighboring microsatellites are used as PCR primers. The variable region between them gets amplified. The limited length of amplification cycles during PCR prevents excessive replication of overly long contiguous DNA sequences, so the result will be a mix of a variety of amplified DNA strands which are generally short but vary much in length.^[21]

Advantages:- Highly polymorphic
Disadvantage:- Species specific

DNA fingerprinting is an efficient, precise and sensitive method for identifying components for churnas that has been established and will contribute significantly in quality control.^[22] As the DNA sequences are very highly specific, they can be identified with the help of the known molecular markers, which can find out a particular sequence of DNA from a group of unknown.

Various DNA based methods are used for standardization, authentication and adulteration detection in medicinal plants that have been explained in *Ayurveda* are published some of them are as below:

1. For Identification & Authentication

- *Swertia* species known as *Kiratatikta* in *Ayurveda* are authenticated using AFLP & SCAR markers & identified 483 bp amplicon primers which are specific to *Swertia chirayita*.^[23,24]

- ISSR markers for authentication of *Withaniasomnifera* (*Ashwagandha*) are used.^[25] AFLP markers for identification of *Zingiberofficinale* (*Ardraka*) from its various species are done.^[26]

2. For Adulteration Detection

- RAPD markers used for authentication & identification of genuine & adulterant samples of *Emblia officinalis* (*Amlaki*)^[27], *Dioscorea bulbifera* (*Varahikanda*).^[28]
- Identification & differentiation of the *Taxus baccata* aerial parts from its species using technology of single Nucleotide Polymorphism (SNP).^[29]

3. For Identification in Compound Formulations

- RAPD Fingerprinting was also employed for determination of the components in precedes *Ayurvedic* herbal prescription, *Rasayana Churna* contains dried stem of *Tinosporacordifolia* (*Guduchi*), dried fruit of *Embliaofficinalis* (*Amalaki*) & dried fruit of *Tribulusterestris* (*Gokshura*), identification & quantification of *T. cordifolia*, *E.officinalis* & *T.teresrtis* in *Rasayanachurna*. Primer OPC-6clearly differentiates all componants of *Rasayanachurna*.³⁰

CONCLUSION

DNA Fingerprinting has a wide range of utility & used to authenticate medicinal plants. DNA fingerprinting can differentiate between individuals, species & populations & has been proved as method for the authentication & identification of different adulterants. DNA-based tools for authentication of medicinal plants is an evolving new pharmacognostic measure aimed at quality control & quality assurance in medicinal plant research as well as in clinical usage. These markers have most effective utility in quality control of commercially important medicinal herbs which are adulterated & these tools are also utilised in any form of the drug i.e. processed or unprocessed.

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