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New Tools for Herbal Drug Standardization

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Abstract: *The term "herbal drugs" denotes plants or plant parts that have been converted into phytopharmaceuticals.* The quality control standards of various medicinal plants used in indigenous system of medicine are becoming more relevant today in view of commercialization of formulations based on medicinal plants. For standardization and quality assurance purposes, following three attributes are desirable i) Authenticity ii) Purity and iii) Assay. Authenticity relates to proving that the material is true. Authentication in itself involves many parameters including gross morphology, microscopy, chemical, physical, biological and toxicological analysis. Assay part of standardization is chemical and biological profiling which could assess the chemical effects and curative values. The new era of herbal drug standardization includes chemometrics, Gel Electroporesis, Metabolomic technique, Differenital pluse polarography combination of this all techniques show clear picture and this will help to introduce new molecule or formulation in society which will safe and effective one.

Key words: Herbal drugs, Chemometrics, Gel Electroporesis, Metabolomic technique, Differenital pluse polarography.

Introduction:

The term "herbal drugs" denoted by means of plant or part of plants that have been converted into phytopharmaceuticals by simply means of processes involving collection or harvesting, drying and storage[1]. Herbal medicines have a long history of use for the prevention and treatment of diseases. The use of medicinal plants with therapeutical purposes represents a secular tradition in different cultures[2,3]. Their use was traced back to the first written testimonies of different book of Ayurveda. They have always been part of human culture. About 80% of world populations still rely on medicinal herbs for their primary health care, according to WHO. Not only in India but also in western nations the use of herbal medicine is increasing day by day[4]. Standardization of herbal plant is a critical issue to ensure the quality of the research process for safety and efficacy of the research products.

Herbal drug standardization

"Standardization is a system to ensure that every packet of medicine that is being sold has the correct amount and will induce its therapeutic effect (Chaudhry, 1992)."

The overuse of synthetic drugs with impurities, resulting in higher incidence of adverse drug reactions in more advanced communities, has motivated mankind to go back to Nature for safer remedies. Therefore, quality control standards of various medicinal plants used in indigenous system of medicine are becoming more relevant today in view of commercialization of formulations based on medicinal plants.

Due to varied geographical locations where these plants grow, coupled with the problem of different vernacular names these plants are known by, a great deal of adulteration or substitution is encountered in the commercial markets. Therefore, reproducible standards of each plant are necessary for effective quality control. For this reason WHO recently released guidelines on Good Agricultural and Collection Practices (GACP) for medicinal plants (WHO, 1991). WHO has also issued "Guidelines for quality control methods for medicinal plant materials" in 1992 with a clear objective to provide general test methods for correct botanical evaluation and identification of medicinal plants widely used in traditional and home remedies (WHO, 1998).

"Standardization refers to the body of information and controls necessary to produce material of reasonable consistency. This is achieved through minimizing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing processes (Waldesch *et al*., 2003)."[5]

For standardization and quality assurance purposes, following three attributes are desirable i) Authenticity ii) Purity and iii) Assay. Authenticity relates to proving that the material is true. Authentication in itself involves many parameters including gross morphology, microscopy, chemical, physical, biological and toxicological analysis. Assay part of standardization is chemical and biological profiling which could assess the chemical effects and curative values. The new era of herbal drug standardization includes chemometrics, Gel Electroporesis, Metabolomic technique, Differenital pulse polarography combination of this all techniques show clear picture and this will help to introduce new molecule or formulation in society which will safe and effective one[6].

Parameters for standardization and Quality Control of herbal drugs

1. Organoleptic evaluation/ Morphological evaluation/Sensory evaluation:

Organoleptic (Lit."Impression on the organs") evaluation of crude drugs refers to the evaluation of a drug by colour, odour, taste, size and shape, occasionally the sound or snap of fracture and special fetures including touch, texture, etc. Fractured surfaces in cascara, cinchona, and quillia bark and quassia wood are essential characteristics. Umbelliferous fruits have aromatic odour and liquorice have sweet taste are the example of this type of evaluation. Shape of drug may be conical (aconite), subcylindrical (podophyllum), cylindrical (sarsapilla), fusiform (jalap).Size represents thickness, length, breadth and diameter. Color represents external color which various from white to brownish black are essential diagnostic features. Taste which is a specific type of sensation feel by epithelial layer of tongue. taste may be sweetish (saccharic), sour (acidic), salt like (saline),and bitter or tasteless [7,8,9].

2. Microscopical method :

This method allows more detailed examination of drug, and it can be used to identify the organized drug by their known histological character, it is mostly used for qualitative evaluation of organized crude drug in entire and powder form.

Qualitative evaluation: In this method drugs characteristics structures are studied by microscope from origin of plants. To distinguish the cellar structure of drugs various reagent are used. Constituents study is also include in the microscopic evaluation by application of chemical method to the Drugs. For example a drop of phoroglucinol and concentrate hydrochloric acid give red stain with lignin.

Various characteristics are studied in histological study such as characteristics of cell wall, vessel, trichomes, starch grains etc.

Quantitative evaluation: Quantitative microscope and linear measurements is one of the histological aspect. The various number of parameters studied here are number of stomata and index, palisade ratio, vein islet number, size of fibers etc.

Table 1: Quantitative microscopic parameters with example

Parameter	Examples
Palisade ratio	Datura metel: 0.5-6.5, Nicotinana tabacum: 3.5-4
Vein-islet number	Digitalis purpura: 2.5-3, Ocimum santum: 19-23.
Vein termination	Datura 43-45.
Stomatal index	Atropa belldona: 2.3 to 3.9 to 10.5(Upper surface), 20.2 to 21.7 to 23.0 (lower surface)

3. Chemical Method of Drug Evaluation:

Determination of active constituents in a drug by chemical process is referred to as chemical evaluation. General preliminary phytochemical test is use for identification of secondary and primary metabolites[10,12,13]. Quantitative chemical evaluation gives exact quantity of of substance present in particular substance.
For Ex. Evaluation test of resins: acid value, sulphated ash; Evaluation test of balsams: acid value, saponification value, ester values; Evaluation test of volatile oils : acetyl, iodine value and ester values.

Table 2: Preliminary phytochemical test is use for identification of secondary and primary metabolites.

Chemical constituents	Chemical test
Alkaloids	Dragendroff's, Wageners, Hagers, Mayers test.
Carbohydrate	Molish, Fehling's, Benedict's, iodine test.
Cardiac glycoside	Killer killani, Kedde, Baljet, legal test.
Flavanoids	Shinoda, Bromine water test.

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

Biological evaluation: Some drugs have specific biological and pharmacological activity which is utilized for their evaluation. Actually this activity is due to specific type of constituents present in the plant extract. For evaluation the experiments were carried out on both intact and isolation organs of living animals. With the help of bioassays, strength of drug in its preparation can be evaluated[13-15]. It also include bitterness value, tannins content determination etc.

Toxicological evaluation: It includes the determination of micro-organism, aflatoxins, heavy metels content.

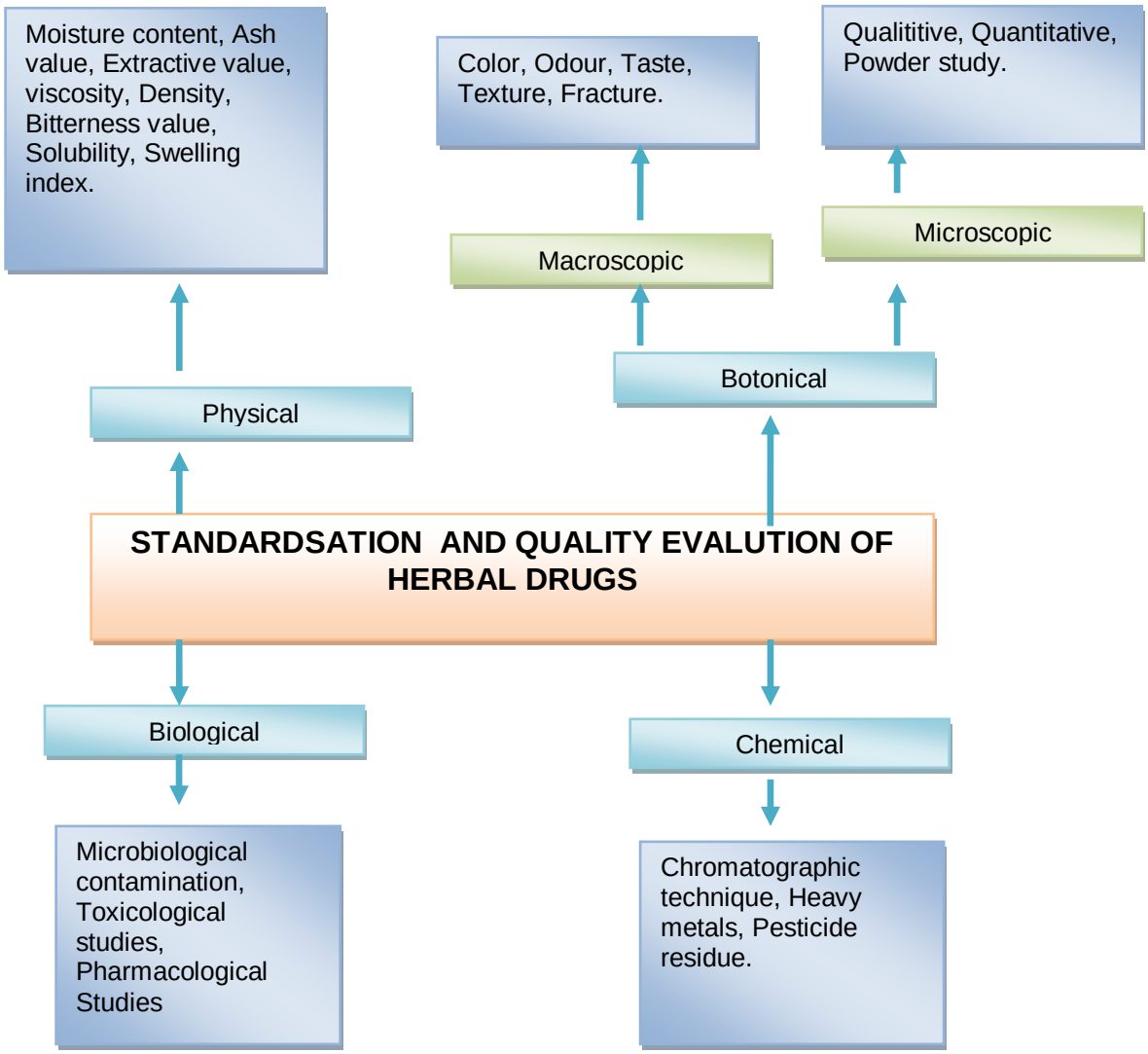


Fig. 1: Methods of Standardisation

Novel techniques of standardization:

CHEMOMETRICS:

Chromatography and spectroscopy techniques are the most commonly used methods in standardization of herbal medicines but the herbal system is not easy to analyze because of their complexity of chemical composition. Many cutting-edge analytical technologies have been introduced to evaluate the quality of medicinal plants and significant amount of measurement data has been produced. Comprehensive methods and hyphenated techniques associated with chemometrics used for extracting useful information and supplying various methods of data processing are now more and more widely used in medicinal plants, among which chemometrics resolution methods and principal component analysis (PCA) are most commonly used techniques.

Common chemometric tools used in chemical analysis of herbal drugs

As is known, HPLC is extensively applied for quality control of herbal drugs due to its sensitivity, superior precision, high resolution and extensive applicability. LC–MS, GC–MS, and LC–NMR have been

increasingly used in complex chemical identification of HDs[16]. This advancement in instrumentation is able to generate enormous amounts of data which record small differences between samples and this enables us to provide large implications for the discrimination of herbal plants. So we discuss here application of chemometrics in the analysis of herbal drugs. But before analyzing data, pretreatment of data is essential because unknown components or unclear interferences cause overlapped peaks and shifted baseline. Thus, we discuss here various commonly used chemometric techniques in herbal drug standardization (HDS) such as similar analysis (SA), principle component analysis (PCA), cluster analysis (CA), discriminate analysis (DA), spectral correlative chromatography (SCC), information theory (IT), local least square (LLS), heuristic evolving latent projections (HELP) and orthogonal projection analysis (OPA) and pattern recognition. These methods play a vital role in the discrimination and classification of medicinal plants.

Tools for preprocessing data: To eliminate or reduce unwanted sources of variations due to instrumental responses from hyphenated techniques and to obtain more efficient results, data preprocessing techniques are applied. Commonly applied preprocessing techniques include LLS and normalization.

Normalization: Variation in sample concentration might affect the multivariate analysis of the entire chromatographic profile. So normalization of data is examined before carrying out multivariate analysis[17].

Local least square (LLS) method: Major problem in analyzing HDs is occurrence of signal shift. It causes a significant influence in chromatographic profile. To eliminate the chromatographic shift, the LLS method is used to correct retention time shift[18].

Tools for extracting chemical information: The aim of these methods is to detect similarities or extract useful information from the data obtained from analytical instrument. These methods are important for finding out the interested analyte in complex mixture of plant fingerprint and similarity analysis (SA), SCC and IT are commonly used tools.

SCC:- It is a technique used to identify the chemical component present in different chromatograms as acquired from hyphenated instrument. It is based on the fact that the same chemical components should have the same spectra no matter what or how they are eluted through diverse chromatographic columns. The spectral information is utilized to pick up the targeted component from the other two-way chromatograms[19].

Information theory (IT):- The value of information content depends on the separation degree and concentration distribution of each chemical component in a chromatogram. The more they separate with uniform concentrations, the higher the value of information contents, i.e., more chemical information can be obtained from this chromatogram[20].

Tools for displaying data

PCA:- The central idea of PCA is to reduce the dimensionality of a data set consisting of a large number of interrelated variables, while keeping maximum variation in the data set. PCA a multivariate tool, is used to find out the main source of variability present in the data sets. It is used to detect cluster formatting and to establish relationship between object and variable[21,22,23].

Application of chemometrics in HDS

The application of chemometrics in HDS by giving detailed examples regarding authenticity, efficacy and chemical analysis of HDs.

Authenticity: Ascertaining authenticity is the important step to assess the quality of plant medicine. Each medicinal plant contains certain characteristic constituents. Therefore, constituents including their respective chemical ratios can be analyzed to identify medicinal plants and distinguish the fakes. The analysis of fingerprint of 46 Cassia seeds samples was carried out by applying chemometric methods at two wavelengths of HPLC-UV. Samples were clustered into four groups according to the plant sources and preparation procedures. Chemometric tools were effectively applied to predict the category of the four different samples in the test set[24]. Several studies concerning the quality control of Camellia sinensis have been undertaken. Different regression models such as PCA and OPA were applied for the prediction of total antioxidant capacity using chromatographic fingerprints[25,26]. Ultra-high performance liquid chromatography- Electrospray ionization-mass spectrometry (UHPLC–ESI-MS) was utilized for metabolite profiling of saponins[27,28].

Efficacy and consistency The efficacy of plant medicines is closely related to their chemical constituents and their concentrations. Consistency may slightly vary according to differences in climate, cultivating and harvest time, possessing procedure and storage. Evaluating the recorded Fingerprints, minor differences in concentrations might in influence the quality of the herbal plant while small differences between the fingerprints can discriminate among species. PCA, SSC and LDA can classify and discriminate medicinal plants fingerprints effectively. Fingerprint analysis of Chrysanthemum morifolium was developed by combining chemometric methods such as SA, HCA, and PCA with ultra-performance liquid chromatography[29].

GEL ELECTROPHORESIS

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. It is used in clinical chemistry to separate proteins by charge and/or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments[30] .A genetic marker may be defined as gene or a nucleotide sequence on a chromosome that has the potential to differentiate cells, individuals or species. As the DNA sequences are 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.

Types of molecular markers: A vast numbers of molecular markers are available of which the more common are Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSR), Sequence Characterized Amplified Regions (SCAR), Loop Mediated Isothermal Amplification (LAMP), Single Nucleotide Polymorphisms (SNPs) and others. DNA barcoding, microarrays based markers and Next Generation Sequencing (NGS) based markers are of relatively recent development. Each of the techniques are targeted towards a particular component of the genome or is completely arbitrary, face unique methodological, technical and material challenges; thus no DNA marker can be considered ideal.[31].

Table no. 3: Comparison of different DNA markers used in biological sciences

	RFLP	RAPD	ISSR	SCAR
Genomic Abundance	High	Very high	Medium	High
Genomic DNA required	2-5µg	15-30ng	15-30ng	30-50ng
Inheritance	Co-dominent	Dominent	Dominent	Co-dominent
Primers/probes used	Specific	Random	Specific	Specific
Reproducibility	Very high	Very low	Medium	Very high
Applicability in plant authentication	Yes	Yes, but not to be used (it is genetic diversity marker)	Yes, but not to be used (it is genetic diversity marker)	Yes
Cloning/sequencing	Yes	No	No	Yes
Use of radioactivity	Yes	No	No	No
Cost	High	Low	Medium	Medium
Use of automation	Not easy	Very easy	Easy	Easy

Restriction fragment length polymorphisms (RFLP)

RFLPs are considered as one of the first developments in the field of genetic markers, responsible for initiating the field of molecular genetics. The technique is based on the principle of variation that is present due to occurrence of mutations in restriction enzyme binding and cleavage sites; additionally, any re-organization in the genomic region flanked by restriction sites that also disrupts their distribution and thus causes

polymorphism also contributes to RFLP. The digested fragments vary in size, have to be separated using Southern blot analysis and accordingly visualized by hybridization to specific probes which could be homologous or heterologous in nature. When the flanking regions of nucleotide sequence are known, the region meant for RFLPs could be amplified through polymerase chain reaction. However, it must be kept in mind that If the length polymorphism is caused by a relatively large (> approx. 100 bp depending on the size of the undigested PCR product) deletion or insertion, gel electrophoresis of the PCR products should reveal the size difference and when the length polymorphism is caused by base substitution at a restriction site, PCR products must be digested with a restriction enzyme to reveal the RFLP. The positive point of this marker is its co-dominant nature.

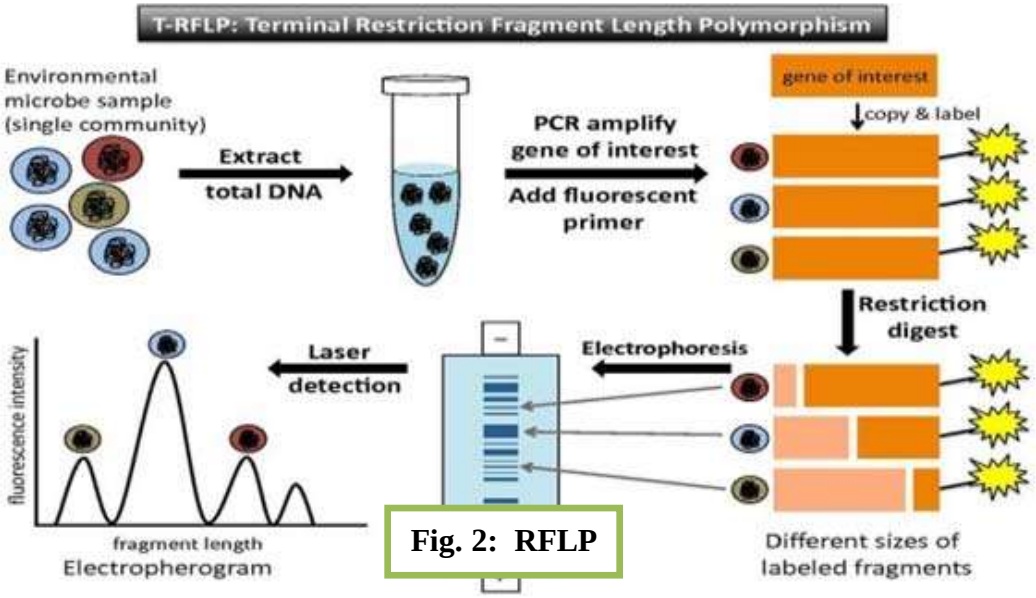


Fig. 2: RFLP

Limitation of RFLPs

• The limited sensitivity of detection associated with RFLPs is the serious problem; because it is very difficult to get valid profiles from trace biological evidence or from samples that are too aged or have significantly compromised environmental insults.

• RFLP is time consuming, involves radioactivity and is laborious and difficult to automate. However, this can be overcome by PCR-based DNA typing systems.

Random amplification of polymorphic DNA (RAPD):

In RAPD technology, random short synthetic oligonucleotide primers (10–12 base pairs) are used to amplify the genomic DNA through polymerase chain reaction under low annealing temperature. The amplicons generated are separated on agarose gels based on sizes. As stated that primer size is short, therefore annealing temperature range is 28–38 °C. At this temperature range, primers anneal wherever they find complementary sequences from the genome and the profile of amplified DNA vary in size that depends on nucleotide sequence homology and the primer at the end of each amplified product. As it is obligatory that primers for RAPD are arbitrarily chosen, therefore, a minimum of 40% GC and the absence of palindrome sequence is a prerequisite for RAPDs[31].

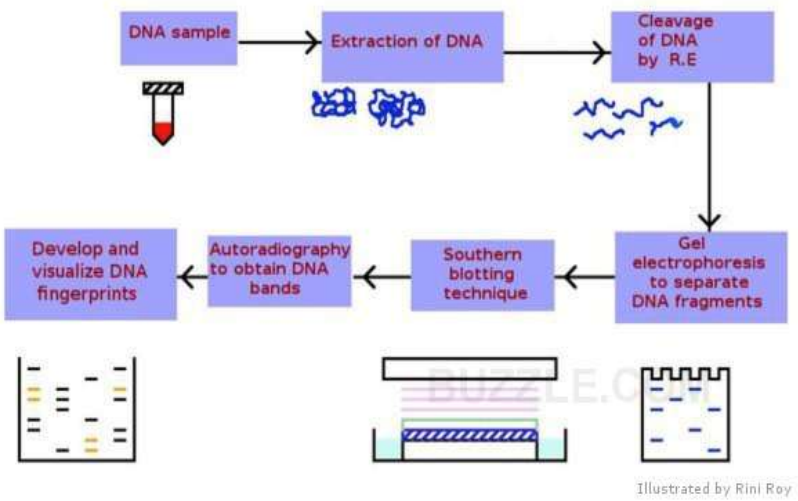


Fig. 3: RAPD

The bands obtained may vary in intensity which could be due to the differences in copy number or relative sequence abundance; therefore, may work for distinguishing homozygote dominant from heterozygote because more bright bands are expected for the former. However, some workers have found no correlation between copy number and band intensity. The possible occurrence of fainter bands could be due to the varying degree of primer mismatch. The other drawback associated with RAPD markers is the low reproducibility.

Limitations of RAPDs

•RAPD is a less reproducible marker. Because the annealing temperature for such marker is low (28–38°C) therefore, there are chances of wrong annealing.

•It is a dominant marker

Inter-simple sequence repeats (ISSR)

ISSR is one of dominant markers involved in amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. This technique relies on the principle of using microsatellite as primers which target multiple genomic loci to amplify inter simple sequence repeats of the genomic DNA of different sizes. Microsatellite used as primers for ISSRs are di-penta nucleotide. The primers for the ISSRs could either be unanchored or anchored at 3`-5` end having 1–4 degenerate bases extended in to the flanking sequences. Compared with RAPDs the size of the primers in ISSRs are much longer (18–25 mers), which permit the annealing of the primers at higher temperatures leading to higher stringency. The DNA fragments obtained are generally 0.5–2.0 kb long and could be detected both by agarose and polyacrylamide gel electrophoresis. Survey of literature reveals that ISSR markers show high reproducibility than RAPDs although it varies with the detection method used, reported greater than 99% reproducibility level after performing repeatedly tests for ISSR markers with the DNA samples of the same cultivar grown in different locations, DNA extracted from different aged leaves of the same individual, and by performing separate PCR runs. However, in other studies, the reproducibility of ISSRs amplification products are in the range of 86–94%, with the maximum when the priority is given to polyacrylamide gel electrophoresis and AgNO3 staining over agarose gel electrophoresis and faint bands are totally excluded while scoring the bands.

Limitations of ISSRs

• ISSR markers do have more value compared to RAPDs; however, the marker has the reproducibility issues.

• This marker is also dominant

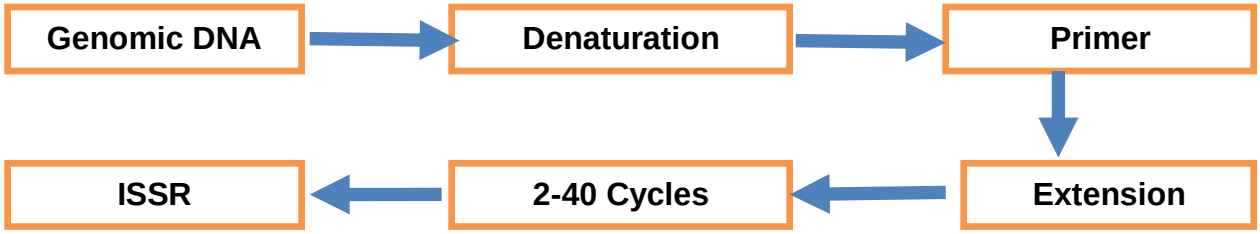


Fig. 4: ISSR

Sequence characterization of amplified regions (SCAR)

The most important marker for authentication of medicinal plants is SCAR. It is sequence-based mono-locus and a co-dominant marker in which forward and reverse primers are designed from the particular region of a cloned AFLP, RAPD and ISSR DNA fragment linked to a trait of interest. SCAR may be a specific gene or random DNA fragment in the genome of an organism and the primers for amplification are located at any suitable position within or flanking the unique AFLP, RAPD, ISSR amplicon. SCAR is fast, reliable and highly reproducible marker used in molecular biology. The designed primers are used to identify the target species from the pool of related species by the presence of a single, distinct and bright band in the desired sample. The length and GC content is also concern for SCAR markers and generally 20–25 oligonucleotide bases are sequence specific. SCAR markers developed from AFLP and SSR though is more reproducible but simultaneously such markers are costly, time consuming and more difficult (particularly in AFLP where silver staining is required for the elution of DNA fragment from polyacrylamide gels). To convert a selected unique RAPD, AFLP, ISSR or SSR band to a SCAR marker, each unique band is eluted, cloned and sequence verified. The nucleotide sequence of the unique DNA band is analyzed for uniqueness by comparing with the known DNA sequences available at various databases for synthesizing specific SCAR primers. These markers have the advantage of being co-dominant and are highly reproducible. **Limitations of SCAR markers**

•The need for sequence data to design the PCR primers is prerequisite for SCAR markers[31].

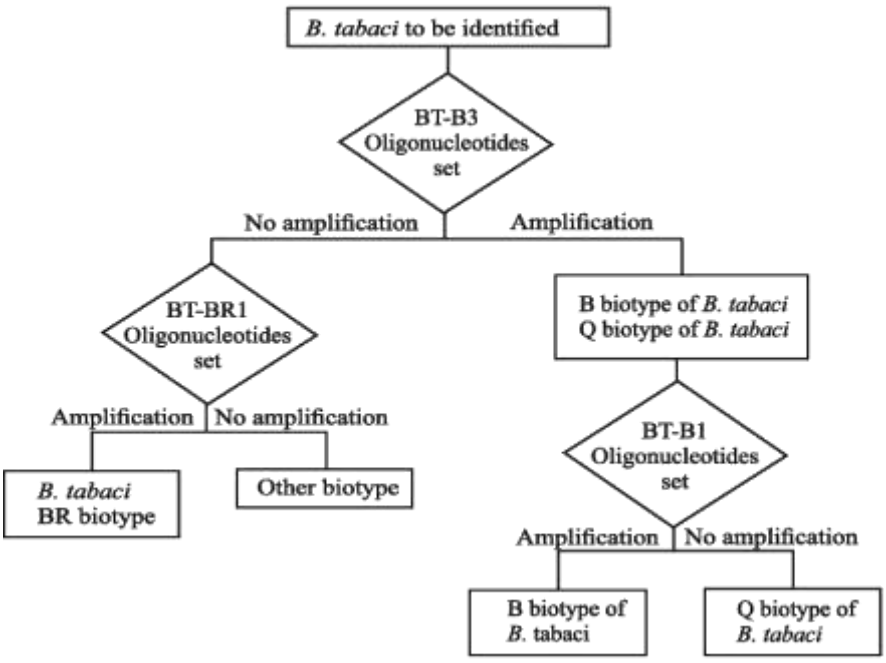
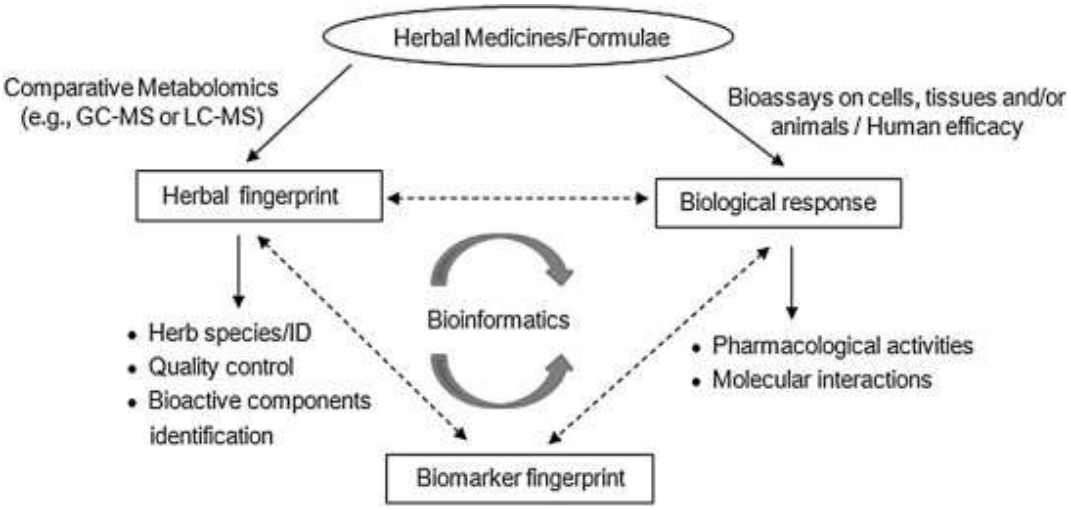


Fig. 5 : SCAR

METABOLOMIC TECHNIQUES

Metabolomics is a advanced emerging field of 'omics' research that is concerned with characterizing large numbers of metabolites using NMR, chromatography and mass spectrometry. It is commonly used in biomarker identification and the metabolic profiling of cells, tissues or organisms. The data processing challenges in this technique are quite unique and often require specialized (or expensive) data analysis software. Metabolomics has been used for identification of active phytoconstituents from herbal medicine[32].

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. The greater potential of metabolomics has been reported in the developmentof active secondary metabolites from medicinal plants as novel or improved phytotherapeutic agents[33-34]. 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[35].



DIFFERINTIAL PULSE POLAROGRAPHY (DPP)

DPP can be used to study trace amounts of chemicalswith very small detection limits on the order of 10-8 M. Some heavy metals, including lead,cadmium, zinc, copper and iron were successfully identified and determined in chamomile and calendulea flowers by DPP [36-37].

Accumulation of heavy metals, namely Pb, Cd, Cu and Zn was estimated in marketed as well as genuine samples of important herbal drugs of India viz., Alpinia galanga, Artemesia parviflora, Butea monosperrma, Curcuma amada, Euphorbia prostrate, Malaxis accuminata etc.

The concentration of Pb and Cd was found beyond the WHO permissible limits in most samples[38]. Trace amounts of selenium in Chinese herbal medicines[39] and flavonoids in small amount of medicinal herb samples were determined by DPP. A DPP method has been for the determination of total hypericin in phytotherapeutic preparations in various buffer systems over the pH range 3.5 – 10.0[39].

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