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Application of Molecular Techniques in Quality Control of Drugs of Natural Origin; A Review

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Authors' contributions

This work was carried out in collaboration between all authors. Author JB designed the study, wrote the protocol and wrote the first draft of the manuscript. Author SA managed the analyses of the study. Author ZAM wrote the bioinformatics portion. Authors SA, JI and SUH managed the literature searches. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

Pharmacognosy is a long established pharmaceutical science, which played a diverse role in the discovery, characterization, production and standardization of drugs. Molecular pharmacognosy using molecular biological tools has extended the scope of pharmacognostical science and plays an important role in the safe and efficient usage of crude drugs. So, these crude drugs are required to be authenticated. DNA Molecular profiling is an additional tool for quality control of herbal drugs as DNA is more basic component of living organisms, whereas chemical and phenotypic expression is controlled by arrangement and expression of genes in the DNA". This novel approach has several significant advantages over morphological and chemical methods. It is possible to use DNA based identification methods to identify species and different constituents of an herbal medicines and transcriptomics,

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proteomics, metabolomics based methods for the functional analysis of key genes involved in synthesis of desired medicinal materials. It serves as a complementary tool to standardization of drugs.

Keywords: Molecular pharmacognosy; DNA molecular profiling; RNA transcripts; natural products; quality control.

1. INTRODUCTION

Traditional medicine is an ancient and culture-bound health care practice, a realistic medical system based on customary beliefs. The foundation of traditional medicine is chiefly created on prescription of medicinal herbs assumed to work in concert mutually for the cure purpose. It is the timeless human quest to prepare extracts from natural products, as the natural products do have innate superiority over synthetic chemicals and are used to alleviate his sufferings of illness and injuries. However, in the past knowledge of medicinal properties of plants was entirely based on, guess work, trial & error, during searching food, by superficial resemblance between the plant's parts and the affected organs i.e. "signature of nature" and by observing other animal's instinctive discrimination between toxic & palatable plants. Based on these observations ancient people acquired a volume of knowledge about drugs. Disease-preventive effect of natural herbal materials inspired mankind to turn back to "Nature". Use of herbs has reduced many illnesses like Alzheimer's disease by using turmeric in daily diet has long been in practice. Herbal materials are obtained either from their wild natural habitats or cultivated in a suitable environment.

In herbal medicine, transformation of plant constituents into drugs is carried out and the herbs may be in the raw form or as an extract either taken internally or applied externally. It is a standardized system connecting quality control procedures, modern scientific techniques and traditional knowledge. Medicinal supplies originated from natural sources include microbes, fresh or treated plants, animals and minerals.

Assurance of safety and quality of herbal drug is necessary for the reproducible efficacy. So, the standardization and pharmaceutical quality of all the phyto-pharmaceuticals must be determined. The herbal material safety evaluation is very crucial according to reports of World Health Organization [1] and is complicated by different processing techniques because of inadequate knowledge and lack of documentation by herbal drug manufacturers. The quality of herbal drugs must be as high as that of other medicinal preparations. Natural heterogeneity, phytochemically or morphologically indistinguishable varieties, varied geographical locations coupled with different vernacular names are the major causes of fluctuations [2].

In the finished herbal medicinal products quality is difficult to achieve and is more complex than for other pharmaceuticals so WHO recommends quality specifications for herbal materials that consist of practices of GMP, proper labeling and licensing schemes to manufacture, import and market a product [1,3]. Natural products from medicinal plants due to unique chemical range have infinite prospects for novel drug leads. Commercialization of formulations based on natural herbal materials requires reproducible standards for the effective quality control.

2. ROLE OF EFFECTIVE PHARMACO-VIGILANCE SYSTEM

Due to the immense global consumption of herbal materials, they are required to be added in pharmacovigilance systems. Population exposure and increased cases of poisoning are demanding to identify the risks and set standards for the safety of drugs of natural origin. There is need to establish an effective pharmacovigilance system for the welfare of the community. However, implementation of regulations is facing difficulties that include:

1) Regulatory status, as medicinal plants are differently categorized in different countries either as a food or medicine which raises serious concerns in defining herbal medicine for the purpose of state drug regulations besides this also baffling patients [1] e.g. in the US natural products are regulated by DSHEA, U.S. Food & Drug Administration, (2012) that consider a dietary supplement as a product meant to

cure deficiency and contain a “dietary ingredient” e.g. vitamin, mineral, herbetic.
2) Another challenge is restrictions on sharing regulatory information with regulatory authorities and pharmacovigilance centers.
3) Standardization and quality assurance, which requires three desirable attributes i.e. authenticity, purity and assay that are to be considered strictly.

Authentication means to prove that material is true in itself, it involves many parameters like morphology, microscopy, chemical analysis and DNA fingerprinting. Previously, regulatory principles most of the times emphasized upon just quantifiable analysis of formulations [4]. Currently there is marked increase in natural products investigation that leads to,

- Search for new leads for drug development
- Biotechnological applications for pharmaceuticals
- Nutraceuticals
- Authentication of old-fashioned prescriptions
- Significance in phytotherapy

In fact Pharmacognosy is a science developed over the years and adapted itself with continuous changing environment and the challenges of the future [5].

3. QUESTIONABLE QUALITY OF NATURAL PRODUCTS

Many people consider products stamped "natural" are always safe which is not essentially true. Phytochemical constituents of herbal formulation are responsible for the pharmacological properties and are complex compared to traditional pharmaceuticals whether it is a particular herb formula or a polyherbal production of a uniform herbal product of reliable quality and distinct ingredients for consistent therapeutic effects are not so easy. Authentic analytical techniques consistently figure out the phytochemical structure with measurable analysis of marketed combinations is a major concern [1]. Passionate work of Pharmacognosist played a vital role in introduction of new technologies to check the quality of pharmaceutical preparations, initially microscopy was introduced in 19th century and Pharmacognosy remained with these methods for too many years. Then new directions were exposed due to the great efforts of some visionary Pharmacognosist Ergon Stahl, use of one such example is TLC (1967) besides this pharmacognosists were amongst the innovators in the plant material analysis in the field of GC and HPLC. These are still considered important tools for the study of active compounds then spectroscopic methods such as MS and NMR helped Pharmacognosist in search for new biologically active natural compounds [6].

4. PERILS OF NATURAL SOURCES

Herbal materials are facing enumerable risks i.e. inter or intra species variations due to ecological factors, harvesting time and post harvesting conditions. They influence the quality of herbal material directly or indirectly. Absence of questioned or presence of pharmacologically inert ingredient in herbal remedy can be harmful and against law & regulations.

4.1 Plant Material Impurities

Herbal drugs containing plant materials are either substituted, contaminated or adulterated [7,8].

4.1.1 Debasement

Adulteration is a practice of replacing actual crude drug partly or totally with identical ones. It includes diverse conditions like substitution with low-standard marketable varieties, low-grade drugs or synthetic industrial products e.g. *Levisticum officinale* roots are often detected instead of *Angelicae pubescentis radix* and *Angelicae sinensis radix* [9].

4.1.2 Misidentification of raw materials

The major contributors to misidentification include absence of certified herbal reference materials and herbal nomenclature. Presently, the common name, the translated or pinyin name, the pharmaceutical name and the binomial botanical name are in use. As there is no definite standard for the determination of rank in classification of plants so finding a authenticated herb can leads to ambiguity [10]. Pinyin and common names for herbal materials occasionally conceal many botanical species for other commercial reasons i.e. unavailability or rate which mainly results either in poor quality or even unsafe product [11,12], e.g. substitution in a famous weight loss medicine *Stephania tetranda* with nephrotoxic *Aristolochia fangchi* (nephrotoxin aristolochic acid) [7]. Besides this historical naming pattern also makes the botanical identity controversial as homonyms and

a synonym creates confusion [13], e.g., *Aralia continentalis* and *Angelica pubescens* (a synonym of *A. biserrata)*used in Korean and Chinese customary medicine for the same purpose [14,15]. Similarly, root *Angelica pubescens* labeled as the similar herbal medicine, specifically Du-Huo in the Chinese Pharmacopeia "Defining Dictionary for Medicinal Herbs in Korea Institute of Oriental Medicine," 10 January (2016). However, confusion by the common name can be removed by using binomial names (binomial synonyms) for herbal material e.g. *Artemisia absinthium* with different common names so it is required to provide the exact scientific name of the plant and description related to part used and manufacturer's specs, but this can be achieved only with effective union of those involved in isolation of drugs of natural origin [16].

4.2 Indefinite Concentration of Active Ingredient

The therapeutic activity of the herbal material is dependent upon the presence of the certain amount of active ingredient. If the intensity of biologically active ingredient is unknown or concentration of putative active ingredients either low or too high in a formulation will lead to inappropriate dose e.g. a patient went into coma by taking herbal medicine with phenytoin to increase usefulness [17]. Herbal materials cause intrinsic reactions (predictable toxicity or idiosyncratic reaction i.e. type A or type B) due to active medicine and extrinsic reactions (contaminants e.g. microbes, pesticides or heavy metals) due to inappropriate handling or failure of GMP [18].

4.3 Absence of Chemical Standardization and Characterization

Appropriately designed QC and QA procedures are needed to minimize the risks. A substantial portion of QC procedures in herbal formulation manufacturing must be conducted. Previously, the amount of herbal extract in preparation conforms to claim on label but the suppliers these days provide standardized extracts, to achieve target strength. If the selective marker is present in high concentration or a non-standardized pharmacologically active component is present it will not contribute appreciably to the herb activity. The plants are variable raw materials as many factors contribute to this variation [19]. Such variants contribute considerably in many batches of a product so modify its efficacy. Defining the authenticity and quality of drugs of natural origin remain as much a frontier as it is a vital science in guaranteeing clinical use of herbal drugs [11].

Herbal authentication is a quality assurance practice to confirm exact plant species and part used in herbal medicine therefore it should be critically monitored. Besides this herbal drug standardization is the need of the hour to guaranty that every pack of drug sold must possess quantity responsible for its therapeutic effect.

It is a fundamental science, as drugs from natural origin are used widely in the world. It is essential to investigate current market of drugs of natural origin and find reasons for various confusions not solved by conventional methods [21]. Real and consistent identification of herbal materials can only be attained by exercising advance molecular genetic tools [19,20,21,22,23]. Along with such advanced applications it is proposed that an authority on authentication of herbal materials should be founded as a physical institute or as an electronic database [11].

5. TRADITIONAL METHODS OF AUTHENTICATION

Herbal medicine is identified as a "black box" due to overabundance of unidentified compounds it hold [24] and many components work synergistically or antagonistically with each other [25]. Following are the three traditional methods currently in practice for the authentication of herbal materials and drugs of natural origin.

5.1 Morphological Authentication

This mean of authentication depends on traditional morphological inspections e.g. *Radix codonopsis*, identified as little curved elongated cylinder-shaped root [26,27] and the odor is characteristic, aromatic and tastes sweet ("Herbasin Chinese herb database,"). It is simple but entirely based on examiner's view and needs a passport data of herb.

5.2 Histological Authentication

It means a microscopic examination that reveals the characteristics of cell components or content of a manufactured products and used as marker for the identification of the plant source e.g. *Herba dendrobii* [28]. Although these are very useful but cannot be applied to extracts,

injections and even interrelated species do share same histological characteristics that make this method not so perfect.

5.3 Chemical Authentication

Phytochemicals are structurally diverse range of chemicals synthesized by plants and are classified as primary and secondary metabolites. Plant's natural growth and maturity is completely based on primary metabolites while signaling and plant defense are thought to be accomplished by secondary metabolites [29]. Secondary metabolites are widely examined as part of herbal regulations because of distinctive characteristics of plant species and specific plant genus as an indicative of herb individuality [30], however, they represent significant risk to humans [31]. Potentially, hundreds of analytes are present in herbal medicines although most of them in low concentration. Application of TLC to identify *Tribulus terrestris* [32] and *Fructus xanthii* [33], HPLC to cassia bark analysis *Cortex cinnamomi* [34] and Ephedra [35,36], including UV [37], IR [38],GC/MS [39], LC/MS [40] and LC/MS/MS [41] are the regular methods in practice for the chemical authentication.

However, this method of authentication is restricted by the analytical method development practices that are too time consuming and compositional differences which hinder authentication process and even misleading as closely related species share similar compounds and can be ambiguous if intentionally adulterated besides this many key steps rely heavily on worker skill, providing modest reproducibility and it's semi computable nature [42].

6. GENOMIC AUTHENTICATION

This advanced method of authentication has extended the scope of pharmacognostical science [21] to molecular level which involves molecular biotechnological tools. Along with the morphological, pharmacognostical and chemical examination, molecular marker analysis should also be added as a parameter for authentication to control the quality of natural products [43].

6.1 Major Concerns and Tasks of Molecular Pharmacognosy

Molecular Pharmacognosy has been given the following main tasks:

- Systematic assortment of varieties of herbs and study of quality standardization.
- Conservation of medicinal plant and animal biodiversity and research of sustainable utilization of crude drugs resources.
- Medicinal plant marker breeding and new variety cultivation.
- Gene regulation of metabolic pathway and directional control of the quality of herbal medicines.
- Genetic engineering and tissue culture technique to achieve high level expression and production of natural active ingredients or genetically modified ingredients.
- Genetic engineering and green pollution free medicinal plant.

So based on above data it can be rightly stated,

> "DNA Molecular profiling is an additional tool for quality control of natural drugs as DNA is more basic component of living organisms, whereas chemical and phenotypic expression is controlled by arrangement and expression of genes in the DNA" [44].

These techniques have advantages as,

- Genotype rather than phenotype is assayed
- Particular sequence can be selected
- New approach,

  a) To identify adulterants /substitution
  b) To identify genetic diversity (closely related spp.)
  c) Decipher the manner by which genes and corresponding proteins help in management and control of basic cellular process

- Constituent properties of medicinal plants by genetic information [45].

7. MOLECULAR MARKERS

Markers are chemically defined constituents or a group of constituents analyzed for the qualitative and the quantitative purpose. A marker can be a therapeutically active compound, a portion of DNA, a bio-molecule like protein or enzyme or analytically significant constituent [46,47].

7.1 Plant Based Markers

Various types of plant based markers (chemical, biochemical, genetic) are in practice. Chemical

markers are the biochemical constituents like primary and secondary metabolites, proteins, glycoprotein, steroids, glycosides etc. particularly present in root, stem or leaves e.g. *Nardostachys jatamansi* contain major constituent jatamansone [48]. Biochemical markers are either proteins or isoenzymes that are alloenzymes having functional similarity but different physical characteristics e.g. isoenzyme pattern of glucose-6-phosphate dehydrogenase used for the identification of *Eclipta prostrate* [49]. Besides above mentioned marker genetic marker is unique indicating genotype of the individual carrying it and genotype of one or several loci linked to the marker. A genetic marker is a measurable character that can detect variation. Molecular markers are based on biochemical macromolecule deoxyribonucleic acid (DNA). These have revolutionized research activities in biological sciences. A macromolecule used as marker should be ideally neutral to environmental effects and management practices. DNA markers are identifiable DNA pieces mark the location of a particular gene, which can be a particular gene, a complete portion of genome and depends on the study that which molecular marker is to be selected. DNA based molecular markers act as efficient and versatile tool and complement classical strategies for the genetic analysis. Since, the development of DNA based molecular markers they are constantly modified to increase their use and the automation of the process of genome analysis. The genetic markers present in plants are playing their role in genome based procedures e.g. SNP [50,51,52,53] and micro-satellites (SSR) [54,55,56,57,58,59]. The application of sequence independent array technology to determine genomic polymorphism [60,61], PCR for the amplification of a minute locus of genomic DNA [62] and RT-PCR to quantify amplified markers is exclusive progress [63].

7.2 Molecular Based Markers

However, molecular markers are categorized as:

7.2.1 DNA-based molecular markers

DNA-based molecular markers are the popular means of identification and authentication. It includes various techniques such as RAPD, AP-PCR, PCR-RFLP, AFLP, DALP, SCAR and SSR. They were applied e.g. RAPD to differentiate *C. pilosula* species for locality authentication [64,65,66], DNA finger prints to distinguish Dangshen (herb), *C. pilosula (root),* wild *Dendrobium officinale* and phylogenetic analysis of *C. lanceolata* and when used with Eastern blotting analysis helped to authenticate Panax specie. Besides, this PCR, PCR-RFLP were used to differentiate *Fritillaria* species and AFLP DNA patterns to distinguish *Plectranthus* species [67,68,69,70,71,72]. Owing to simplicity and convenience, RAPD analysis has been used extensively in the identification of other herbal materials, for example, to analyze the genetic similarity among four *Glycyrrhiza* species and their relationship with commercial licorice roots [73].

Types of DNA-Based Molecular Markers:

On the basis of method of analysis these are of two types:

1) Hybridization based markers
2) Polymerase chain reaction based markers

1. Hybridization based markers

In these profiles are visualized by hybridizing the restriction enzyme digested DNA fragments to a labeled probe which is a DNA fragment of known origin/unknown sequence e.g., RFLP, RLGS.

2. PCR based markers

It involves in vitro amplification of a particular DNA sequence, using specific or arbitrary oligo nucleotide sequences known as primers.

Deploying Arbitrary Primer: RAPD, AP-PCR, DAF.

Using Specific Primers:

1) Semi-specific primer e.g., AFLP
2) Sequence specific primers e.g., SNP
3) Microsatellite /mini-satellite based markers e.g., SSR, ISSR
4) Transposon/Retrotransposan based markers e.g., TD
5) Other markers e.g., Intron-exon splice junction markers, SCAR.

Advantages of DNA-Based Markers:

- Small amount of DNA is enough.
- No need of radioisotopes for polymorphism detection.
- No prior sequence information is required.
- High level of polymorphism generates many genetic markers.
- Screening of many genes simultaneously.

7.2.2 DNA sequencing-based markers

DNA sequencing based markers were used to find DNA polymorphism by determining the nucleotide sequence in a defined region and aligning the sequence with homologous region of related organisms [74] and offers a reproducible analysis at various levels, distinguish species, analysis of phylogenetic relationship , population genetics, systematics and evolution [75] e.g. ITS, (rDNA) for investigation of angiosperms [76], *trn*K genes [77], cytochrome b [78] and cpDNA to determine pollen morphology [79]. These were applied to species identification of *Codonopsis* species, *Dendrobium* species [80,81,82,83]. Similarly, ITS region was used to authenticate Feng [84] dou shihu and distinguish *D. chrysanthum* [85]. Substitution of *Radix Adenophorae* originated from the roots of *A. stricta* & *A. tetraphylla* is determined by amplification of 5S rDNA spacer domain, diversity was observed and served as marker [86]. For centuries *Rhizoma curcumae* was used to remove blood stasis and to relieve the pain. Diversity in its sequence was used for quality control of *Curcuma* species by investigating 18S, trnK, 12S, cytochrome b genes, ARMS analysis is also suitable for authentication of Curcuma drugs [77], phylogenetic relationship of Panax from Sino-Japanese floristic regions was established using cp *trnK* gene and nuclear 18S rDNA sequences [87], Sailonggu a Chinese crude drug authentication using allele specific diagnostic primers [88], Byakujutsu & Sojutsu discriminated by two regions inside cp *trnK* [89], Ephedra by chlB gene of light-independent proto-chlorophyllide reductase [90,91].

7.2.3 DNA Microarray-based markers

DNA Microarray-based markers utilizes biochip surfaced as favorable tools for the high throughput investigation of genomic data e.g. *Dendrobium* specie. Biochips serve as a good tool for genomic high throughput data analysis and revolutionized traditional way of genome studies i.e. one gene in one experiment [92]. Microarray using ITS sequences determined the identity of *Dendrobium* species and authentication from its adulterant orchids. Even in complex medicinal formulations of *Herba dendrobii* microarray yield positive results and *D. lohohense* was detected which was not listed in the Chinese Pharmacopoeia. Toxic herbal materials and *Ginseng* has also been authenticated using microarray technologies [93,94]. However, results are greatly influenced by experimental conditions and these techniques in combination increases the accuracy of quality [95].

An ideal marker selection:

DNA markers are most easily recognizable and play an important role in authentication of crude natural herbal materials and presence of adulterants as part of standardization and quality control. Following factors should be considered when selecting a marker:

- Abundance
- Level of polymorphism
- Low specificity
- Co-dominance
- Reproducibility
- Labor intensity and safety
- Technical demand
- Operational costs
- Development cost
- Quantity of DNA required for analysis

Applications of molecular marker:

DNA fingerprinting is one of the remarkable tool for identification of species and determination of genetic diversity [96].

1) For genomic profiling or genotyping of medicinal plants
2) In pharmacodynamics for the discovery of new diagnostic and prognostic indicators
3) In pharmacogenomics to show side effects of herbal drug
4) In pharmacognosy for botanical identification and crude plant material authentication to ensure quality control and standardization
5) In toxicogenomics
6) Detection of adulterants in medicinal plants

8. IDENTIFICATION, QUANTIFICATION AND AUTHENTICATION USING PCR TECHNIQUE

Genomic DNA isolation is the crucial and most important step. It is very difficult especially from the plants producing large amount of secondary metabolites. Many times if genomic DNA is isolated even then either it's concentration is very low or further downstream process does not work due to impurities (either in the form of complex reagents used during isolation or the alkaloids, proteins, carbohydrates and other secondary

metabolites) which have not been removed during isolation. So a simple or a rapid method is needed. Various methods like, Doyle and Doyle (1987), Doyle and Dickson, (1987), Cullings, (1992), Dellaporta (1983), CTAB, Murray and Thompson, (1980), HiPurA plant genomic DNA isolation and purification miniprep spin kit and Khanuja (1999) are available.

Polymerase chain reaction (PCR) technology has been applied for amplification of a minute locus of genomic DNA [62] and then identification is done on the basis of comparison of DNA sequences that are deposited in public repositories like GenBank and it is proved to be a significant tool to check the quality of natural products. It is very practical for dried samples too, using any part of organs and is prepared from small amount of tissues. PCR is both thermodynamic and an enzymatic process, its invention by K. Mullis and co-workers in 1985 revolutionized molecular biology and molecular medicine. To identify particular specie and even can be applied at various levels in many assays by direct sequencing of PCR products obtained from herbal compositions. PCR is a powerful molecular tool of scientist/researchers used to amplify trace quantities of DNA. Single locus has proved sufficient and demanding for range of land plants than in animals. A standardized short sequence of DNA are often used, a global joint work of researchers directed to the identification of four areas utilized in land plants with practical success i.e. *rbcL, matK* and intergenic spacer *trnH-psbA* the three plastid markers [97] and one nuclear ribosomal marker and *ITS* proved to be quiet appropriate for specie discrimination in plants [23].

9. THE LIMITATIONS OF GENOMIC AUTHENTICATION

However, these methods have their own limitation and draw backs such as:

- Lack of complete genome sequence of many herbal plants.
- Presence of target DNA does not give quantitative report.
- Presence of target DNA does not disapprove adulterant presence.
- Phenotypically similar plants have much of their DNA similar.

The list of completely sequenced herbal plants is very short. Many herbal plants do not have any DNA sequence at all in any genome database, only *m*RNA sequences that can be common across many species. Any herbal concoction is a mixture of many plants and their parts, on the average 8 to 9 different plants. As current DNA based techniques only show the presence of a target DNA, Simply verifying the presence of a species does not give any indication of its quantity in the mixture. It's a limitation of PCR based DNA identification technique.

DNA based identification techniques are also very restricted in their application for purification. While we can use them in herbal medicine to identify the presence of a required ingredient, we cannot immediately use them to disapprove the presence of adulterant materials. As in order to check the adulterant via DNA we must first have the DNA sequences of all possible adulterant species. The large quantity of such possible adulterants makes this a daunting task. Still even with these current draw backs it is expected that the DNA databanks across the world will increase with the introduction of NGS (Next Generation Sequencing) as more effort is made to document and experiment upon different herbal medicine. DNA identification techniques will grow to meet the challenges.

It is necessary to discern the false herb from the genuine herb in terms of their origins, distribution areas [98]. Only in this way quality can be guaranteed [99].

10. TRANSCRIPTOMIC AUTHENTICATION

We are entering the technologically advanced era where synthetic drugs are in practice but still we can not deny the importance of health benefits we get from nature. The need of the hour is to promote the standardization of these life saving drugs and potential candidates of novel compounds. This can only be achieved by knowing the real facts of variations in the natural products and ultimately functional analysis of medicinal plants [100].

Unlike the genomic authentication which excludes mutations transcriptome analysis is playing a vital role the qualitative and quantitative analysis of medicinal plants [101]. Transcriptome involves the production of RNA molecules in response to physiological stimuli (geographic locations,cultivation conditions, natural growth environment). Transcriptomic authentication is based on global analysis of gene expression profile at the messenger RNA level.

10.1 Transcriptomic Technologies

10.1.1 Hybridization-based approaches

It involves incubation of fluorescently-labelled c DNA with microarrays (custom/high density oligo microarrays). Thus, we can characterize it as microarray technology for gene expression profiling. Other techniques include, probes spanning exon junction,high-resolution tiling arrays (quantify large genome).

10.1.2 Sequence-based approaches

It is used to find c DNA sequences e.g Sanger sequencing but this method is costly and show inefficient quantification. So, to make it economical other tag based techniques are utilized e.g., SAGE (serial analysis of gene expression), CAGE (cap analysis of gene expression), MPSS (massively parallel signature sequencing [102].

10.1.3 RNA sequencing

Recent technique of RNA sequencing is independent of reference genome.

It is a deep sequencing technology involving preparation of cDNA library from population of RNA (total or fractionated) which later sequenced (high throughput) [103].

Hybridization-based approaches need previous genome sequence with narrow dynamic range of detection. Unlike this sequence based approaches are more reliable with few limitations as portion of transcript to be analysed in tag based methods is short. However, RNA sequencing allows whole transcriptome to be surveyed [104].

Trancriptomic studies of medicinal plants is a practical approach in estimation of levels of metabolites and identification of genes that regulate production of metabolites of high medicinal value e.g., Salvia miltiorrhiza Bunge [105]. Oligo microarray is widely used however DNA microarray also help in systemic authentication of closely related species. Besides this current next generation sequencing technologies (illumine,454, solid approaches) revolutionized the generation of Expression sequence tags (ESTs) as in case of *Colinus virginianus L*. and red earthworm [106].

11. PROTEOMIC AUTHENTICATION

Proteome is the complete complement of proteins it even includes modified proteins due to some physiological change like post-translational modifications [107]. Still a lot of work is required in this study although tandem mass spectrometry enable de novo sequencing of proteins which will improve in near future.

12. METABOLOMICS AUTHENTICATION

The science of profiling all the metabolites produced in an organism is termed as “Metabolomics”. As thousands of metabolites are produced in organisms this technique helps us to find quantitative changes of metabolites in medicinal materials. We will be able to generate metabolic fingerprinting of medicinal plants for both authentication and quality control of phytopharmaceuticals.

MS and NMR methods are most commonly used for metabolic fingerprinting [108]. Gas chromatography-time-of-flight-mass spectrometry quantify number of metabolites in tissue specific manner [109]. Not only this comparative metabolomics strategy coupled with cell and gene based assays classify species i.e., Echinacea species [110].

Lipidomic technology identify novel biomarkers and lipid quantification [111].

13. EPIGENOMICS AUTHENTICATION OF MEDICINAL MATERIALS

In this epigenetic elements (Regulatory elements involving chromosomal modifications especially histone modifications) are studied considering whole genome. Mechanisms of gene expression changes in medicinal plants are evaluated in epigenomics.

14. BIOINFORMATICS ROLE IN MOLECULAR AUTHENTICATION

Informatics help in storing information we get through molecular authentications which we later use for analysis. It is a computer based program help in identification of unknown sample by comparing with reference sequences. We can develop test procedure and tools to identify medicinal materials.

Public databases:

Various public databases are available for nucleotide information are as follows,

- Flora of China
- NCBI Nucleotide database
- Barcode of Life Data Systems (BOLD)
- Medicinal Materials DNA Barcode Database (MMDBD).
- Kyoto Encyclopedia of Genes and Genomes (KEGG)
- KNApSAcK
- The International Ethnobotany Database (ebDB)
- NAPALERT for natural product analysis
- The United States Department of Agriculture (USDA) for medicinal plants
- Herb Ingredient's Target (HIT)
- Therapeutic Target Database (TTD)
- Protein Data Bank (PDB)
- Uniprot
- Pfam
- TCM-ID
- CMKb (Australian aboriginal medicinal knowledge base)
- EGENES database (Transcriptome-based information related to plants)
- Medicinal Plants Genomics Resource (MPGR) for transcriptome and metabolome analysis.

Bioinformatics acts as a suite for the analysis and interpretation of huge volume of information. This computational methodology will help to store the scattered information and will solve herbal mixtures optimization related issues.

QCAR (Quantitative composition-activity relationship) *i.e* chemical composition and biological activity is similar to QSAR (Quantitative structure-activity relationship) [112].

Besides this many statistical tools are also required for analysis e.g., Clustering algorithms (cluster genes having like characteristics), Classification algorithm (authentication and quality control). J48, random forest (RF), simple logistic (SL) etc.

15. HERBAL PHARMACEUTICAL PRODUCT DEVELOPMENT IN PAKISTAN

Pakistan is the world's sixth largest country with an area of 796095 Km^2 and population approx. 185 millions. However, 71.07 million live in urban areas and around 113 million in rural areas [113].

Due to this large number of Population in rural areas with low daily income complementary medicine is taken as first line of treatment. As a direct result of this, the Unani and homeopathic system are practiced quite heavily, so there is need to develop a relationship between conventional allopathic physicians and complementary alternative medicine [114]. Orthodox medicine restricts the CAM practice labeling them as antiscientific due to multiple valid reasons among them the most important is lack of regulations to check the quality of the herbal materials and herbal products. Current evidence suggests that physicians should accept as adjuncts to conventional and the need is to develop balanced health policy keeping in mind the health seeking behavior of the population as there is 80% increase in its use globally [115,116,117]. It is required to establish a pharmaceutical laboratory at federal level. Presently, research and development, standardization and quality control of traditional medicine is conducted by,

I. National Institute of Health, Islamabad
II. Hamdard University, Karachi
III. Pharmacognosy Department, University of Karachi, Karachi
IV. HEJ Research Institute, University of Karachi

In Pakistan for herbal industry to follow pharmacopoeial standard is Hamdard Pharmacopoeia of Eastern Medicine [118] and official standards for regulatory purposes is Drug Regulatory Authority of Pakistan Ministry of National Health Services Regulations and Coordination Islamabad. Drug Regulatory Authority of Pakistan Act, 2012 enlist the rules for the Alternative Medicines and Health Products. Complementary system of medicine in Pakistan is known under the Act of Parliament known as the Unani, Ayurvedic and Homeopathic Act, 1965, which regulates it's education as well as practice [119].

The practioners of these systems have to be registered with National Council for Tibb (NCT) and National Council for Homoeopathy (NCH) respectively.

There is a long list of national and multinational herbal pharmaceutical companies in Pakistan. The most famous are Hamdard Laboratories

producing six hundred herbal products in three different dosage forms for all age groups, Herbion (Pvt.)Ltd. globally operating in more than 200 countries providing standardized raw materials and 44 formulations, Hashmi, the company Mohammad Hashim Tajir Surma, Qarshi Industries (Pvt.) Ltd. having humble dawakhana to Qarshi Industries (Pvt.) Ltd. [120] and manufacture more than 200 products, Tayyebi Dawakana manufacturing 350 different medicinal and health care products and own 25 patented products, Marhaba Laboratories leaders in herbal medicine and Medics Laboratories pioneer in ethical marketing of phytopharmaceuticals in Pakistan through healthcare professionals with a team of qualified pharmacists, chemists and herbalists [121].

As we all know herbs are precious natural source and economic crops due to their enormous health benefits. Unceasing developments of more effective synthetic drugs have not faded therapeutic effects of medicinal plants around the world.

Although Pakistan is an agricultural country and produce crude drugs still is unable to meet its total requirements import major part of medicinal plants from Nepal, Sri Lanka, India, China, Kenya and Uganda. The herbs are sold either dried or fresh to the local traders who sell them to wholesalers and ultimately to Pharmaceutical concerns or exporters. Pakistan's major exporters of medicinal herbs and herbal medicines are Hamdard Laboratories (Pvt.) Ltd; Herbasian (Pvt.) Ltd; Hashmi Surma; Qarshi Industries (Pvt.) Ltd; Tayyebi Dawakana; Marhaba and Medics Laboratories out of them Hamdard Laboratories is one of the leading stake holders.

Efforts should be made to explore the herbal product development. As safety is imperative. Safety of natural origin medicines has become a major concern to both national health authorities and the general public [15]. Therefore, regulatory policies on herbal medicines need to be standardized and strengthened on a global scale and with this adequate training regarding use of herbal products with prescription or nonprescription medicine to conform to standards of safety, quality, and efficacy are to be considered for public health. Regulations and WHO support is helping in the preparation of model guidelines in this field and the herbal medicines and phytonutrients or Nutraceuticals continues to grow worldwide.

DNA based molecular markers however are important tool in quality assurance of medicinal plant species in the plant kingdom. This serves as qualitative /quantitative diagnostic tool for identification of medicinal herbs from harvest to finished product. Recent era is the use of molecular biological techniques that helps in identification of species [122]. Although, species specific constituents are helpful in the identification of species e.g., three kinds glycoumarin, glabridin and licochalcone A (*G. uralensis, G. glabra, G. inflata*) but with the help of this technology we can distinguish their kinds.

Along with traditional this new innovative automated assays and specific tools DNA analysis are emerging and contribute to the next generation of technologies e.g. mini-sequencing [123,124] nano-scale DNA sequence, Next Generation sequencing NGS [125,126]. Further, extremely promising development is nanopore technology for identification of DNA bases and APEX (enzymatic genotyping to analyse variations of genome in single multiplexed reaction) [127,128], OLA to detect highly polymorphic gene. These will provide complete genome analysis, high multiplexing capacity and future taxon identification [129].

Recently, massive parallel sequencing technology produces millions of DNA sequence reads i.e. Giga base pairs in single run. These revolutionized research in medicine. However, there is no perfect DNA-typing method and the choice of a particular technique is often a compromise that depends on a number of factors, including: resources of the laboratory, financial constraints, available expertise, time limitations and more importantly, the research question pursued. All factors should be scrutinized to avoid an inappropriate choice.

16. CONCLUSION

Application of above mentioned molecular techniques can be utilized to authenticate the commercialized drugs obtained from natural sources and maintain the quality,safety as well as legality of economically important medicinal materials which are basic ingredient of many important drugs.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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