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AUTHENTICATION METHODS FOR DRUGS USED IN AYURVEDA, SIDDHA AND UNANI SYSTEMS OF MEDICINE: AN OVERVIEW

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ABSTRACT

Traditional systems of medicine in India include Ayurveda, Yoga, Naturopathy, Unani and Siddha. Among them Ayurveda, Siddha and Unani systems (ASU) use plants, minerals and animal products as main drugs to cure various ailments. There has been a boom in the usage of ASU drugs and export is appreciably high in the last two decades. ASU drugs may vary in composition and properties, unlike conventional pharmaceutical products, which are usually prepared from synthetic, chemically pure materials by means of reproducible manufacturing techniques and procedures. Counterfeits and drugs of poor quality degrade the clinical effects of ASU drugs. Thus authentication is a critical step for successful and reliable clinical applications and for further experimental studies on ASU drugs. The authentication methods for herbal, mineral and marine products are discussed in detail which broadly include microscopy, spectroscopy, chromatography, chemometry, immunoassays, DNA fingerprinting etc. A simple method like organoleptic characteristics may hold good to authenticate certain drugs but some may require highly sophisticated techniques too, based on the adulterants and similarity in the chemical constituents. So it is in the hands of the researchers to choose the right method suitable for the drug of interest which would match the reference standard after confirmation from the traditional vaidhyas/practitioners. This article will address about the various common and sophisticated techniques used to authenticate drugs of plant, mineral and animal origin.

INTRODUCTION: The traditional systems of medicine have become significantly more popular all over the globe because of the curative property, less toxic and minimal side effects. It is more widely used for the human ailments from time immemorial. It has been estimated that 70-80% of world's population relies on traditional healthcare. The mode of preparation and plant used in traditional medicine varies from place to place. In addition acceptance of traditional medicines, especially herbal medicines in the developed world is sharply increasing [1, 2, 3].

In ASU systems plants, minerals, and animal products are used as main drugs to cure various ailments [4]. There is a global resurgence in the use of these medicines along with a growing scientific interest in them as a source of new drugs [5]. There has been a boom in the usage of ASU drugs and export is appreciably high in the last two decades [6].

There has been an increase in science based research in ASU drugs for the purpose of globalization. One of the most critical issues involved in any research study is the quality of the test material.

A study cannot be considered scientifically valid if the material tested is not authenticated and characterized such that the material can be reproduced [7].

ASU drugs may vary in composition and properties, unlike conventional pharmaceutical products, which are usually prepared from synthetic, chemically pure materials by means of reproducible manufacturing techniques and procedures. Correct identification and quality assurance of the starting material is therefore an essential prerequisite to ensure reproducible quality of these medicines which contribute to its safety and efficacy [8, 9]. Counterfeits and drugs of poor quality degrade the clinical effects of ASU drugs. Thus authentication is a critical step for successful and reliable clinical applications and for further experimental studies on ASU drugs.

Authentication is especially useful in cases of drugs that are frequently substituted or adulterated with other varieties which are morphologically and chemically indistinguishable. Several herbal drugs in the market still cannot be identified or authenticated based on their morphological or histological characteristics. Use of wrong drugs may be ineffective or it may worsen the condition [10].

The method of evaluation of drugs by veteran collectors experience should be confirmed by scientific methods before starting the research work. Evaluation has become even more difficult when several different individual species were powdered and mixed together in a proprietary medicine [11].

A. **Authentication of Herbal Drugs:** Authenticated raw material is the basic starting point in developing a botanical product. In addition, each step of harvest, storage, processing and formulation may dramatically alter the quality and consistency of final product. Therefore methods to ensure quality control in manufacturing and storage are requisite tools to ensure optimal efficacy and safety of these products. Furthermore, such controls are critical for the evaluation of pharmacological, toxicological or clinical studies involving botanical products.

1. **Taxonomic method:** The initial step in the identification and authentication of botanical materials entails classical botanical methodologies for collection and documentation of the plant at its source. The botanical origin of the drug is identified and its scientific Latin binomial (i.e. genus species) name is determined based on this method. It is the first step for authentication [12].

   Information such as botanical name, vernacular names, site of collection of plant material, details of collector, habitat, season of collection, altitude and part collected etc. are the essential prerequisites even before authentication [12].

2. **Herbarium voucher sample:** The sample of collected material should be kept as a voucher sample in a herbarium or a research institute for future references [12].

3. **Macroscopic method:** Macroscopic identity of botanical materials is based on parameters like shape, size, color, texture, surface characteristics, fracture characteristics, odor, taste and such organoleptic properties that are compared to a standard reference material [13].

4. **Microscopic method:** Microscopy is used to determine the structural, cellular and internal tissue features of botanicals. It is usually used to identify and differentiate two herbals that are similar [14, 15]. This is the commonly used technique, convenient, quick and can be applied to proprietary medicines too [16]. An example of a botanical that can utilize microscopic techniques to aid in its identification is star anise (*Illicium verum Hook.f*). As the name suggests, star anise is star shaped fruits that taste like anise; originally a native of southern China, it has now been introduced throughout the tropics and subtropical Eastern Asia.

   The fruit is used principally as an aromatic spice in China and India to flavor food and confectionary items [17]. It is known for its therapeutic value in traditional Chinese medicine for treating rheumatism, back pain and hernias. Unfortunately, an increasing number of cases of infants, suffering from acute neurological effects- such as seizure, vomiting and rapid eyeball movement have been reported in western countries and United States after the consumption of star anise herbal tea [18].

These diverse events were suspected to be due to adulteration of Chinese star anise with Japanese or "Bastard" anise [19]. Japanese star anise *(Illicium anisatum)* is well known to contain the toxic sesquisterpenes [20-22].

a. **Fluorescence Microscope:** Using the microscope to determine the identity of herbal medicines, namely, microscopic authentication refers to observing cell structure and internal features using a microscope and its derivatives. Besides the ordinary light microscope, polarized and fluorescence microscopes can also be used to enhance the accuracy of authentication. Use of these microscopes expands the number of features available for use in identification. For example, it has been found that starch grains, crystals of calcium oxalate, stone cells, vessels and fibers have stable and special polariscopic characteristics [23, 24].

The fluorescence microscope reveals the fluorescence emitted from herbal tissues under illumination. Many herbal tissues, by virtue of their chemical structures or secondary metabolites, have the ability to emit light of a specific wavelength following the absorption of light with a shorter wavelength and higher energy [25]. For example, in recent years, the fluorescence microscope has been applied to distinguish the medicinal herb *Oldenlandia diffusa* from other species of the same genus which are confused with it, in herbal markets [26]. The fluorescence microscope and micro spectrometer can be used to authenticate powdered ASU drugs and measure the distribution of chemicals in the cross section of these drugs.

5. **Physicochemical methods:** Physicochemical parameters include total ash, water soluble ash, acid insoluble ash and sulphated ash. These values of the individual drugs or the proprietary medicines can be compared with the standard values of Indian pharmacopoeia and thus the identity can be ascertained [27].

6. **Chemometric and Spectral methods:** Initially the use of infrared (IR) spectroscopic method is restricted only for structural elucidation of isolated compounds from the herbal matrices. It is also found useful in phytochemical studies as a "fingerprinting" device, for comparing a natural with synthetic sample [28]. With the advance of computer technology, chemometric method has become a leading tool among the scientific communities towards faster analysis and shorter product development time [29]. Among others, an unsupervised pattern recognition technique such as Principal Component Analysis (PCA) is the most often used method for handling multivariate data without prior knowledge about the study samples [30].

While the supervised classification procedure using Soft Independent Modeling of Class Analogy (SIMCA) based on making a PCA model to assign unknown samples into the predefine class model has also been applied to the analysis of infrared spectra [31]. A study using FTIR transmission spectroscopy, associated with the appropriate chemometric methods (PCA and SIMCA) was done to classify *Orthosiphon staminens* Benth (well known as Java tea for treating infection of the urinary tract, kidney and bladder stone disease) based on its geographical origin and varieties from the obtained characteristics infrared spectrum. Chemometric analysis of spectra is rapid and simple since no chemical treatment of samples is required [32].

7. **Chromatographic methods:** High Performance Liquid Chromatography (HPLC), Capillary Electrophoresis (CE) and Thin Layer Chromatography (TLC) are the most commonly used analytical methods for herbal products.[33]The analysis of volatile compounds by gas chromatography is very important in chemical analysis of herbal medicines [34].

a) **Thin Layer Chromatography (TLC):** TLC was the common choice for the analysis of herbs before instrumental chromatography methods like GC and HPLC were established. Even nowadays, TLC is still frequently used for the analysis of herbal medicines since various pharmacopoeias still use TLC to provide first characteristic fingerprints of herbs [35, 36]. TLC has the advantages of many fold possibilities of detection in analyzing herbal medicines. In addition, TLC is rather simple and can be employed for multiple sample analysis.

For each plate, more than 30 spots of samples can be studied simultaneously in one time [37]. In summary, the advantages of using TLC to construct the fingerprint of herbal medicines are its simplicity, versatility, high velocity, specific sensitivity, simple sample preparation and its economy. Thus TLC is a convenient method to determine the quality and possible adulteration of herbal products [38].

b) **High Performance Liquid Chromatography (HPLC):** HPLC is a popular method for the analysis of herbal medicines, because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. In general HPLC can be used to analyze almost all the compounds in herbal medicines [39].

c) **Gas Chromatography (GC):** The GC of the volatile oil gives a reasonable fingerprint and can be used to identify the plant. The extraction of the volatile oil is relatively straight forward and can be standardized and the components can be readily identified using GC-MS analysis. The advantages of GC clearly lie in its high sensitivity of detection for almost all the volatile chemical compounds [34].

d) **Capillary Electrophoresis (CE):** Capillary electrophoresis (CE) allows an efficient way to document the purity/complexity of a sample and can handle virtually every link of charged sample components ranging from simple inorganic ions to DNA. CE is promising for the separation and analysis of active ingredients in herbal medicines, since it needs only small amounts of standards and can analyze samples rapidly with a very good separation activity [34].

8. **Chemical Fingerprinting:** A chemical fingerprinting is a unique pattern that indicates the multiple chemical markers within a sample [40]. The European Medicines Agency (EMEA) defines chemical markers as chemically defined constituents, or group of constituents of herbal medicinal product which are of interest, regardless whether they posses any therapeutic activity [41]. The quantity of a chemical marker can be an indicator of the quality of herbal medicine.

The study of chemical markers is applicable to many research areas, including authentication of genuine species, search for new resources or substitutes of raw materials, optimization of extraction and purification methods, structure elucidation and purity determination.

9. **Molecular markers:** Molecular markers generally refer to biochemical constituents, including primary and secondary metabolites and other macromolecules such as nucleic acids. DNA markers are reliable for informative polymorphisms as the genetic composition is unique for each species and is not affected by age, physiological conditions as well as environmental factors [42]. DNA can be extracted from fresh or dried organic tissue of the botanical material and hence the physiological form of the sample for assessment does not restrict detection [43, 44]. Various types of DNA based molecular techniques are utilized to evaluate DNA polymorphisms. These are hybridization based methods, polymerase chain reaction (PCR) based methods and sequencing based methods [45, 46, 47].

a) **Hybridization based methods:** Hybridization based methods include Restricted Fragment Length Polymorphism (RFLP) and variable number tandem repeats.[48] Labeled probes such as random genomic clones, cDNA clones, probes for micro satellite[49] and mini satellite[50] sequences are hybridized to filters containing DNA, which has been digested with restriction enzymes. Polymorphisms are detected by presence or absence of bands upon hybridization.

b) **PCR based methods:** PCR based methods involve *in vitro* amplification of particular DNA sequences or loci, with the help of specific or arbitrary oligonucleotide primers and the thermo stable DNA polymerase enzyme. PCR based techniques where random primers are used include Randomly Amplified Polymorphic DNA (RAPD) [51, 52], Arbitrarily Primed PCR (AP-PCR) [53] and DNA amplification fingerprinting [54, 55]. A recent approach known as Amplified Fragment Length Polymorphism (AFLP) [56, 57] is a technique that is based on the detection of genomic restriction fragments by PCR amplification.

c) **Microchip method:** A DNA micro array is a multiplex technology used in molecular biology and in medicine. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing Pico moles (10-12 moles) of a specific DNA sequence, known as probes. This can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (called target) under high stringency conditions. Probe-target hybridization is usually detected and quantified by detection of flurophore, silver, or chemiluminescence's labeled targets to determine relative abundance of nucleic acid sequences in the target.

Since an array can contain tens to thousands of probes a micro array experiment can accomplish many genetic tests in parallel. [58]. DNA based techniques have widely used for authentication of plant species of medicinal importance. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically or phytochemically indistinguishable [59].

Dried fruit samples of *Lycium barbarum* were differentiated from its related species using RAPD markers [60]. The RAPD technique has also been used for determining the components of a Chinese herbal prescription Yu-feng-san. In this study, the presence of three herbs (*Astragalus membranaceous, Ledebouriella seseloides and Atractylodis macrocephala*) in the formulation has been detected using a single RAPD primer [61].

B. **Authentication of Minerals:** The use of minerals as source of drugs is largely used in Indian systems of medicine next to herbals, especially in Siddha medicine. They are processed with herbal juices and made into their calcified or oxide forms and administered as drugs. Hence their identification is equally important to herbal drugs.

1. **Physical properties:** The physical properties of individual minerals will be a powerful tool for identification. These properties include nature, colour, streak, tenacity, transparency, luster, hardness, fracture, cleavage or parting, magnetism and specific gravity. These unique physical characteristics of minerals can be used for the authentication.

2. **Optical properties:** Optical properties include application of optical crystallography in identification of minerals that are crystalline and transparent irrespective of their chemical composition. This can be determined within limits by means of polarizing microscope.

3. **Refractive Index (RI):** It is defined as ratio of velocity of light in media to that in vacuum. It varies with wavelength. Immersion method is used to determine the RI. For example Alum and Garnet are isotopic, Calcite, Quartz, Sapphire and Ruby are anisotropic – uniaxial, and Mica and Gypsum are anisotropic – biaxial.

4. **Chemical properties:** The Chemical properties include effect on heat, solubility, reaction with acids like HCl, HNO_3, H_2SO_4, assays which include Volumetric/ Gravimetric/ AAS/ ICPA/ Flame photometry for Na, K, qualitative test for carbonate and sulphate, analysis of heavy metals like Arsenic and other elements.

5. **Microscopic methods:** It is a simple, inexpensive and widely used method for the authentication of minerals [12]. Light microscopy and polarized microscopy are the common microscopes used for authentication. Light microscopes have a wide application in mineralogy [62]. A polarizing microscope is a microscope that is mainly used in geological studies to study geological specimens. For this reason, it is also known as a petrography microscope. A comparative study was carried out with microscopy for the Chinese patent medicine Bo Ying Compound.

The micro morphological characteristics of its 22 components and in crude constituents have been documented and compared with each other. Their corresponding features were described and documented with color digital micrographs, so as to authenticate the presence of genuine crude constituents in the medicine. Another study focused on the authentication of four kinds of mineral arsenicals, including orpiment, realgar, arsenolite and arsenic trioxide.

The macroscopic and microscopic characteristics of the minerals were examined and they found that the all can be easily identified and authenticated by using light microscopy coupled with polarized microscopy [63].

6. **Spectroscopic methods:**

a. **Near Infrared Spectroscopy (NIRS):** Near Infrared Spectroscopy has received much attention for chemical quality and process control because of its speed and attribute of requiring little or no sample preparation [64]. NIRS uses the near infrared region of the electromagnetic spectrum(from about 800 nm to 2500nm).

b. **Electron Spectroscopy for Chemical Analysis (ESCA):** ESCA otherwise known as X- ray Photoelectron Spectroscopy (XPS) involves only the top 20-50A° of the sample, making it an extremely surface sensitive technique. ESCA spectra can also provide information about an element's chemical environment or oxidation state. The chemical environment of an atom affects the strength with which electrons are bound to it. Atoms associated with different chemical environments produce peaks with slightly different binding energies which are referred to as chemical shift.

c. **Inductively Coupled Plasma Mass Spectrometry (ICP-MS):** ICP-MS is a type of mass spectrometry that is highly sensitive and capable of the determination of a range of metals and several non metals at concentration below one part in 10^{12} (parts per trillion). Samples are decomposed to neutral elements in high temperature argon plasma and analyzed based on their mass to charge ratios. It is an automated, simple and unique quantitative and qualitative analysis. It measures elemental isotopes ratio.

d. **Atomic Absorption Spectrometry (AAS):** AAS is based on the light absorption of elements. Nearly 30-40 elements can be detected. AAS is used for the quantitative estimation of inorganic minerals in plant drugs/poly herbal formulations, drugs of mineral/ metals and animal origin. The estimation can be made at ppm (parts per million) level and still lower levels by graphite furnace method.

e. **X-ray Diffraction Analysis (XRD):** The X-ray diffraction pattern of a pure substance is like a fingerprint of the substance. The powder diffraction method is ideally suitable for characterization and identification of polycrystalline phases. Today about 50,000 inorganic and 25,000 organic single components, crystalline phases, and diffraction patterns have been collected and stored on magnetic or optical media as standards. The main use of powder diffraction is to identify components in a sample by a search/match procedure. Further more, the areas under the peak are related to the amount of each phase present in the sample.

f. **X-ray Fluorescence Analysis (XRF):** X-ray fluorescence (XRF) is the emission of characteristic "secondary" (or fluorescent) X- rays from a material that has been excited by bombarding with high energy X- rays or gamma rays. The phenomenon is widely used for elemental analysis and chemical analysis particularly in the investigation of metals, and for research in geochemistry, forensic science and archeology.

C. **Authentication of Marine and Animal Products:** Animal and marine products have constituted part of the inventory of medicinal substances used in various cultures since ancient times. In Indian systems of medicine nearly 15-20 percent is based on animal derived substances [65]. Although research on marine natural products started only about 50 years ago, marine organisms have been used in traditional systems of medicine in India much before that [66]. The following techniques would help in the authentication methods of these products.

1. **Chromatographic methods:**

a. **Gas Chromatography /Mass Spectrometry (GC-MS):** GC-MS is the marriage of two analytical methods into a versatile technique for the identification of complex volatile materials. Gas Chromatography (GC) effectively separates the constituents of the sample for subsequent analysis and identification by Mass spectrometry (MS). The first result obtained is the compiled data of total ion chromatogram (TIC), which is a plot of the total mass eluting from the GC and detected by MS as a

function of time. In a study, effective extraction and GC/MS protocols were established for the detection of authentic and counterfeit components found in allegedly musk containing samples collected from various sources in Taiwan [67].

b. **Liquid Chromatography-Mass Spectrometry (LC-MS):** Liquid chromatography mass spectrometry (LC-MS or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and specificity.

Generally its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals. Raw materials like honey and animal fats can be authenticated by this technique and the spectral fingerprints of them can be generated. A qualitative analysis of carotenoid composition was performed by HPLC/UV on samples of *Corallium rubrum* to generate a chromatogram profile [68].

2. **Immunoassays:**

a. **Enzyme linked Immunosorbent Assay (ELISA):** The use of immunological methods for the detection of adulteration in milk and milk products resulted in the development of sensitive, reliable assays capable of detecting low levels of adulteration in milk and milk products. The use of monoclonal antibodies, careful selection of target antigen, and suitable ELISA format has greatly increased the good analysis ability to distinguish between species in milk and milk products [69].

3. **Non Immunological protein based methods:**

a. **Poly Acrylamide Gel Electrophoresis (PAGE):** Polyacrylamide gel electrophoresis (PAGE) provides a versatile, gentle and high resolution method for fractionation and physical-chemical characterization of molecules on the basis of size, conformation and net charge. The polymerization reaction can be rigorously controlled to provide uniform gels of reproducible, measurable pore size over a wide range. This makes it possible to obtain reproducible relative mobility (R_f) values as physical-chemical constants [70].

b. **Isoelectric Focusing (IEF):** Isoelectric focusing (IEF), also known as electro focusing, is a technique for separating different molecules by their electric charge differences. It is a type of zone electrophoresis, usually performed on proteins in a gel, that take advantage of the fact that overall charge on the molecule of interest is a function of the pH of its surroundings. The above said non immunological protein based methods is focused for milk speciation especially for milk caseins and whey proteins [70].

c. **DNA Finger Printing Methods:** Genetic fingerprinting, DNA testing or DNA profiling is a technique to distinguish between individuals of the same samples using only samples of their DNA. DNA fingerprints depend on the genetic differences between individuals, the so called DNA markers. DNA fingerprinting is a powerful tool in poultry for investigating genetic diversity within stocks and establishing relationship among stocks and characterizing individuals or populations genotypically. The different techniques of DNA profiling include Restricted Fragment Length Polymorphism (RFLP), PCR- Based techniques; Random amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP) and Real time PCR (RT-PCR) [71].

4. **Spectral methods:** The spectral methods used to authenticate animal include UV-Vis spectrophotometer, Near Infrared and Fourier Transform Infrared spectrometer etc. These methods are specifically employed in the authentication of honey, which is one of the most common adjuvant used in Indian system of medicine.

CONCLUSION: A significant number of methods to authenticate ASU drugs have been addressed here. A simple method like organoleptic characteristics may hold good to authenticate certain drugs but some may require highly sophisticated techniques too, based on the adulterants and similarity in the chemical constituents.

So, it is in the hands of the researchers to choose the right method suitable for the drug of interest which would match the reference standard. Even before choosing the methods the raw materials should be confirmed with traditional practitioners/ vaidhyas.

In addition, while utilizing chemical methods and other analytical tools it is mandatory to stick on to the latest validated techniques that suit the study. One needs to understand what type of raw material/formulation one is dealing with and the type of preparation to be evaluated. The evaluation of the adulterants simultaneously along with study drug will pave way for the researchers working on the same drug to eliminate the adulterants and identify the standard and pure raw material.

In spite of all these factors, the microbial contamination, pesticide residue and heavy metal analysis should be considered before processing the raw material for drug preparation. This is regarding the safety issue of drugs. This type of analysis is required when evaluating the authenticity of botanicals because these extraneous contaminants can cause undesired physiological effects.

To conclude, more basic research should be carried out and many individuals should be trained with these authentication techniques to address this issue prevailing in ASU drugs.

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