A review: Importance of utilizing plant authenticating technologies in a herbal hotspot- Sri Lanka

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ABSTRACT

This study evaluated how the conservation of herbal medicinal plants, sustainability of the herbal drug industry and the authentication of medicinal plants were the most critical issues for the last decade. The adulteration of medicinal plants laid with the starting material and may be intentional or non-intentional. Numerous approaches can be implemented to ensure plant-based material authentication for medicinal plants. This review covers a comprehensive and critical analysis of the DNA molecular markers, Isotope Ratio Mass Spectrometry and the various aspects of authentication of medicinal plants, which is currently lacking in Sri Lanka. It is a special emphasis on accurate identification due to the various isotope abundance and genotype of the plants with geographical variation.

Key words: Authentication, DNA markers, IRMS, medicinal plant, plant barcodes.

INTRODUCTION

The world is losing species at a rate that is 100 to 1000 times faster than the natural extinction rate. Mass extinctions of species have occurred five times earlier in the history of the world – the last time was 65 million years ago. Unfortunately, we are now witnessing the 6th mass extinction, which is a result of competition for resources between human and all others (Tiffany et al., 2015).

During past decades, the consumption of herbal products has increased in developed countries. Global population have changed into herbal products resulting from increased health awareness (80%), harmful side effects and product withdrawal of chemical products creating more market opportunities both locally and internationally. The production and marketing of herbal products has experienced a rapid expansion in major markets in the world including Germany, China, Japan, USA, France, Italy, UK and Spain (WHO, 2014-2023).

Islands often have a high proportion of endemic, in effect ‘limited-edition’, species have accounted for the bulk of the world’s recognized extinctions of species in the historical times. Much of the Earth’s terrestrial biodiversity is located at tropical latitudes and rainforests, the accelerating loss of these forests is currently perceived to be a major threat to the rich biodiversity heritage countries and islands in the Southeast Asian region and elsewhere (Lane, 2010).

Sri Lanka is one of the most biologically diverse country in Asia. Sri Lanka has various climate and topography despite its small land size of 6,524,540 hectares (Pathirage, 2012), it has resulted in rich endemism distributed within a wide range of ecosystems. The recent surveys indicated that there are 1432 medicinal plant species in Sri Lanka and out of them 100-200 species are endangered (IUCN data, 2017). Therefore, a need has been raised to rationally utilize medicinal plants for curative purposes with the proper maintenance of biodiversity in Sri Lanka. Present turnover of the herbal and Ayurvedic industry in Sri Lanka is approximately 2.5 billion per year (EDB Sri Lanka data base, 2018).

Ethno medicine evolved from the traditional use of plant parts for treating various ailments as shown Figure 1. Historically, most medical breakthroughs have risen based on the plant sources showing potential for curing many diseases and the ethnic groups have perceived the medicinal properties of the plants with their odour, flavour and shapes of the plant materials (Peter et al., 2009).

The conservation and authentication of biological
diversity is the sustainable use of its components and equitable sharing of the benefits arising from the commercialization and other utilizations of the plant genetic resources. This has inspired the research for ethno pharmacological values in the hope of giving rise to new drug leads or to the discovery of new bioactive compounds (Pathirage, 2012).

This study supports herbal producing and exporting, medium and small business entrepreneurs in Sri Lanka, in their efforts to diversify export through sustainable production. As the specific aims of observing market entry requirements for herbal medicinal products, categorization of herbal produce companies per selected parameter, export volumes and value distribution, identify issues and constraints related to the herbal medicinal industry, strategy option for the development of the herbal produce industry in Sri Lanka.

Nowadays, the discovery of genetic markers and chemical finger printings have been most useful to authenticate the herbal medicinal plants with their conserved medicinal properties. Apart from authentication of traded medicinal plants with these techniques which can be applied in biodiversity monitoring, conservation, impact assessment, monitoring of illegal trading and forensic botany (Jansen et al, 2005). The objective of this study is to discuss the importance of utilizing these techniques for the countries like Sri Lanka where the economy mainly rely on tourism and export of herbal/ natural products.

**REVIEW METHODOLOGY**

The authentication techniques reviewed in this study includes a literature search in the world's acknowledged databases including PubMed, Medline, Scopus, Embase and Springer. The search was based on the key words; Authentication, DNA Markers, IRMS, medicinal plant and plant barcodes in the period of 2003-2018. Based on the keywords, 270 publications were identified. The titles of these studies as well as their duplicity were then checked to discover whether they focus on the research topic. There were 200 studies remaining for further analysis after excluding 70 duplicate articles. After that, the author checked the content of the abstracts whether the study examined the research topic. 120 articles were selected for the full-text and abstracts analysis, out of which only 80 full-text studies could have been then used for the detailed analysis of the research topic. The detailed review procedure has been illustrated in Figure 2.

**AN OVERVIEW OF METHODOLOGIES**

Morphological, chemical and DNA molecular marker detection methods were used to solve the problems by differentiating the genuine material from the adulterants, substitutes and spurious herbal drugs. Although the conventional analytical tools to detect the synthetic adulterants of medicinal plant products; these methods are hardly powerful enough to identify the biological adulterants. Therefore, the usage of chemo metrics has been applied in biological herbal adulterant detection and authentication of a wide range of herbal medicines (Willcox, 2004). Each of these methodologies has limitations and more analytical methods are needed to assist the authentication process. Molecular biology offers
an assortment of techniques that can be very useful for authentication of medicinal plants while the chemical fingerprinting is one of the most corroborating and reliable quality control and isotope profiling which ensures quality and reproducibility of herbal medication (Taverase et al., 2004). The growth potential of the herbal medicine industry can only be achieved if the composition of herbal medicine is standardised to ensure proper quality control and accountability. This study covers the application of analytical chemistry techniques along with the combination of different molecular markers analysis (REL F, AFLP, ISSR, SSR, LAMP, and RAPD) to authenticate herbals (Figure 3).
MOLECULAR BIOLOGY METHODS BASED ON GENETIC FINGERPRINTS

Molecular markers are based on the differences in the genetic sequences existing between individuals and species. The knowledge of the whole DNA sequence is not necessarily needed because a number of protocols are based only on the differences in size or on matching of flanking sequences that do not require the knowledge of the sequence in between. Nevertheless, databases comprising the patterns obtained for different species are often indispensable for fraud detection.

Types of molecular markers

A vast numbers of molecular markers (Table 1) have been developed and commercially applied to increase the marketability in importing and exporting facility of herbal market. The available common markers are identified by 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) and Single Nucleotide Polymorphisms (SNPs). DNA barcoding, microarrays-based markers and Next Generation Sequencing (NGS) based markers are relatively new developments which present highly accurate and cost effective methods. Each of these techniques that target a particular component of the genome or is completely arbitrary faces unique methodological, technical, and material challenges. Therefore, no DNA marker can today be considered ideal. The use of a particular marker will therefore depend on the objectives.

Issues of DNA markers towards authentication

DNA based technology though superior has certain drawbacks. One requirement is high quality DNA to analyse samples, which might be problematic for dried or processed materials (Table 1). The low content of secondary metabolites in plants such as polysaccharides, essential oils, phenolics, alkaloids and tannins may inhibit PCR or might affect DNA isolation by increasing with the age of the plant (Ali et al., 2013). These reactions become severe as the material ages. Therefore, it is prone to be contaminated which minimises the activity of numerous enzymes in the PCR (Al-Shaqa et al., 2013). The market samples (herbal raw materials) might be contaminated with endophytic fungi that could obscure the results of dominant markers like RAPD, AFLP and ISSR and might also influence DNA sequencing (Moon et al., 2015). However, this can be overcome with a plant specific primer design. DNA related methods totally fail when the herb is in capsule form or an extract. Consequently, doning of PCR products is sometimes inevitable. In order to develop a marker for identifying closely related species and varieties and common herbal contaminants and adulterants is necessary, which is a costly and time-consuming process.
Table 1: A vast numbers of molecular markers.

<table>
<thead>
<tr>
<th></th>
<th>DNA Bar-coding</th>
<th>AFLP</th>
<th>LAMP</th>
<th>RFLP</th>
<th>ISSR</th>
<th>RADP</th>
<th>SSR</th>
<th>SCAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Availability</td>
<td>Limited</td>
<td>Unlimited</td>
<td>unlimited</td>
<td>Limited</td>
<td>Medium</td>
<td>Unlimited</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>Genomic abundance</td>
<td>High</td>
<td>Very High</td>
<td>High</td>
<td>High</td>
<td>Medium</td>
<td>Very High</td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td>Quality of DNA</td>
<td>Pure</td>
<td>Pure</td>
<td>Moderate</td>
<td>Pure</td>
<td>Moderate</td>
<td>Moderate to pure</td>
<td>Low</td>
<td>Pure</td>
</tr>
<tr>
<td>Skill Require</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Genomic DNA required</td>
<td>30-50 ng</td>
<td>200-300 ng</td>
<td>10-20 ng</td>
<td>2-5 µg</td>
<td>15-30 ng</td>
<td>15-30 ng</td>
<td>30-50 ng</td>
<td>30-50 ng</td>
</tr>
<tr>
<td>Probes used</td>
<td>Specific</td>
<td>Specific to adapter sequence</td>
<td>Specific</td>
<td>Specific to repeats</td>
<td>Random</td>
<td>Specific</td>
<td>Specific</td>
<td>Specific</td>
</tr>
<tr>
<td>Radio Activity requirement</td>
<td>No</td>
<td>Yes/No</td>
<td>Yes/No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Specificity and sensitivity</td>
<td>High</td>
<td>Very High</td>
<td>Very High</td>
<td>Low</td>
<td>Medium</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td>Type of polymorphism</td>
<td>Nucleotide changes in universal genes.</td>
<td>Nucleotide base changes that affect specificity of restriction endonucleases and presence/absence of nucleotide complementary to selective nucleotides</td>
<td>Complete presence/absence of DNA fragment</td>
<td>Nucleotide base change that affect specificity of restriction endonucleases</td>
<td>Nucleotide base change at primer binding sequences</td>
<td>Nucleotide base change at primer binding sequences</td>
<td>Complete presence/absence of DNA fragment</td>
<td>Complete presence/absence of DNA fragment</td>
</tr>
<tr>
<td>Reliability</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>High</td>
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<tr>
<td>Reproducibility</td>
<td>Very high</td>
<td>Medium</td>
<td>Very high</td>
<td>Very high</td>
<td>Very Low</td>
<td>Very Low</td>
<td>Very high</td>
<td>Very high</td>
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<tr>
<td>Cost</td>
<td>Very high</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
</tr>
</tbody>
</table>
Table 1 Continuation: A vast numbers of molecular markers.

<table>
<thead>
<tr>
<th></th>
<th>DNA Bar-coding</th>
<th>AFLP</th>
<th>LAMP</th>
<th>RFLP</th>
<th>ISSR</th>
<th>RADP</th>
<th>SSR</th>
<th>SCAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applicability in</td>
<td>Easy</td>
<td>Difficult if radio</td>
<td>Easy</td>
<td>Not Easy</td>
<td>Easy</td>
<td>Very Easy</td>
<td>Very easy</td>
<td>Easy</td>
</tr>
<tr>
<td>Plant authentication</td>
<td></td>
<td>activity is used (Licor system is easy)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major Uses</td>
<td></td>
<td></td>
<td>Genetic Diversity</td>
<td>Map construction, genome mapping,</td>
<td>Genetic diversity</td>
<td>Genetic diversity</td>
<td>Genetic diversity</td>
<td>Genetic diversity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>genome mapping, Evolution; Occasionally</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>for genetic diversity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Vassou et al., 2015</td>
<td>Misra et al., 2010</td>
<td>Gani et al., 2010</td>
<td>Horn et al., 2014</td>
<td>Wang,2011</td>
<td>Khan et al., 2010</td>
<td>Shaqha et al., 2013</td>
<td>Choo et al., 2009</td>
</tr>
</tbody>
</table>

(Hung et al., 2014).

If one of the major advantages of DNA markers is that they are not tissue-specific, it can also become a problem when possible adulterants come from another part of the same plant, but other parts of it may be mixed in, either voluntarily to increase the mass for profit or unintentionally during harvest. In this type of situation, only a chemical analysis will make it possible to ensure the chemical fingerprint.

**ANALYTICAL CHEMISTRY TECHNIQUES BASED ON BIOCHEMICAL PATTERNS**

These techniques allow for the discrimination of chemo types within species, as well as maturity stages and origins. They are often quantitative analyses able to detect frauds by adulteration or substitution, but also too low contents in active components due to bad maturation state nowadays, the best authentication protocols are based on analytical chemistry, such as NMR (Bailey et al., 2012), HPLC (Gani et al., 2015), capillary electrophoresis, gas chromatography and isotope mass spectrometry, which is associated with powerful detection methods. Once identified, some of the main components of the sample will be selected as phytochemical markers which should be representative of the species, which is responsible for the plant biological activity. For a valid method standard, availability and powerful identification methods, such as Isotope, Ratio mass spectrometry is most required. Standards should be commercially available and their purity guaranteed, the method must also be selective and linear, in the required range, and present low limits of detection and limits of quantization.

Isotope Ratio Mass Spectrometry (IRMS) is an analytical technique which is robust, non-destructive, reliable and feasible, thus ensuring that herbal medicine can be verified and quality controlled and more accurate. It covers a much wider dynamic range and sample preparation is simpler and feasible, though it has some limitations which are the evidence that it could contribute quite significantly to the quality control of herbal medicine (Ward et al., 2016).

**Future perspective of Isotope Ratio Mass Spectrometry**

In the commercial industry of herbal medicine, the preparations are typically very complex and have a multitude of compounds. The relationship of the content and the pharmacological effect of herbal medicine is unclear, but these herbal medicine formulations are usually standardised by using single marker compounds or a group of related compounds which do not include information on other seemingly unimportant constituents present in abundance in the herbal preparation. Georgie et al. (2005) investigation showed that the possibility of IRMS and multivariate analysis along with the DNA marker being used to distinguish different variations in batches from the same supplier. No clustering was observed between the tablets and capsules, which indicates that the variability among samples is
mainly contributed by plant extract variability and not the manufacturing processes. IRMS is the most convenient method to differentiate the geographical location, making it possible to detect if the subjected herbal product has been adulterated with different species (Ali et al., 2013).

**Economic outlook with molecular markers and Isotope Ratio Mass Spectrometry**

Molecular markers 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 as an ideal marker. The use of a molecular marker depends on the objectives of the researcher.

IRMS is an ideal companion for quality control of economically important samples. It has combined with multivariate statistical analysis software which provides new opportunities for conducting sound and reliable quality control analyses on botanical samples. These samples can range from botanical to pharmaceutical (Handa et al., 2004).

Despite major suppliers of global herbal medication escalating prices and health budgets being tightened in the developed world consumers are still driven towards safer herbal medication. The driving forces of this exodus towards herbal medications are the increase of consumer awareness, reduced risk of detrimental side effects and the introduction of value-added raw material based products to the market. This increased demand of herbal medicinal products has been met with a negative response as there is insufficient research and standardization in the field as well as poor legal and regulatory framework resulting in issues related to patents and the introduction of low quality products to the market (Marshell et al., 2007).

Herbal medicinal products are used as a popular alternative to synthetic drugs and the natural supplement industry, it is becoming extremely popular in the world as botanical supplements, herbal medicines and several compounds for pharmaceutical development every year (Ali-Shaqha et al., 2013). The demand for pure herbal products exceeds the supply in the major international markets in Europe, Japan and USA. Thus, the developing countries have a comparative advantage to export herbal product for the international market. Therefore, the medicinal plant authentication is most important. Unfortunately, there is no single or superior method to assure 100 percent authenticity throughout the entire process, but this can be achieved through the application of a variety of different methods (Ali-Shaqha et al., 2014).

In the future, the standardisation of herbal medicine will possibly play an important role for approval of complex herbal medicine as a treatment and require robust and rapid analytical methods to ensure the quality of these products is retained without the tedious preparation normally necessary for quality control on complex mixtures. Therefore, the quality control analysis will probably not be done on a few selected constituents but will be done on the whole composition of the sample in the future.

In combination with functional genomics, IRMS can assist in investigating the deeper components of the complex nature of the networks operating in plants and how these networks change due to genetic manipulation. It is not only possible to determine the genetic changes but also to establish the fundamental of the plant phenotypes in relation to the development, physiology and environment. IRMS will contribute significantly to research in many biological fields and to the economic development of these fields in the future.

These authentication methods described require knowledge on how the plants can be identified in order based on comparisons of molecular markers and Isotope Ratio Mass Spectrometric criteria. It affirms a sample’s identity or its dissimilarity with the species to which it is supposed to belong. Therefore, it is important to have an accessed database for genomic and biochemical which obtained with its own identity to protect and conserve for each identical medicinal plant and establish as a service.

**DISCUSSION**

In the context of diversity analysis, codominant RFLP markers are of limited utility, unless dominant multi-loci RFLP probes such as microsatellites are employed. The application of hybridization based probes in detecting diversity or for genetic mapping are limited by several associated drawbacks, including the requirement of large amounts of highly pure DNA, use of radioactivity for detection, the need for highly skilled manpower and the low rate of detecting polymorphism. These features make hybridization-based markers less efficient than those based on PCR. Comparatively the cost for the RFLP and Microsatellites primer detection is less.

PCR based markers are relatively easy to use and thus have gained popularity for both mapping and genetic diversity studies, but the cost is very high. PCR based markers can be targeted towards a specific locus or can amplify many loci in a random manner. The co-dominant markers are generally used for the construction of genetic maps and the latter types are more useful for genetic diversity analysis. Target PCR relies on the availability of prior sequence information that enables the design of locus specific primers. Sequence characterized amplified region, is termed as a sequence characterized amplified region or SCAR.

In case these fail to detect polymorphism or distinguish alleles across genotypes, then the PCR products can be restricted with an enzyme for polymorphism that may be present in the amplified region. Such a class of marker is
termed as a cleaved amplified polymorphic sequence. Prime examples of such classes of markers are RAPD, AFLP, selectively amplified microsatellite polymorphic loci, and modifications thereof.

CONCLUSIONS

After considering the various authentication methods mentioned in the study, microsatellite identification technique and IRMS can be used in unison to identify the different strains of the same medicinal plant as well as their adulterants. These tools are beneficial for the conservation of the biodiversity of medicinal endemic plants in the region as well as the taxonomic discrimination and the assessment of the quality of products. The authentication methods can further differentiate the geographic origin of the products. The ability to differentiate products is essential for the maintenance of consistency among products which is required for producers to maintain a favourable reputation among consumers. The good reputation will reflect with a boost in profits gained through international trade in sectors including pharmaceuticals, agriculture, nutraceuticals, toiletries and cosmetics. Investing in genetic fingerprinting and chemical fingerprinting system will have a positive impact on the economy of the region as it will attract more customers because of the transparency it can provide to potential investors.

REFERENCES


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