CHAPTER 1: INTRODUCTION
Introduction

1.1 Plant based medicines: global demand, emphasis on standardization

Botanicals have a growing global market due to their demand as nutraceuticals and herbal products. Herbal products are a very diverse category of plant products and extracts that are known as dietary supplements (United States), natural health products (Canada), phytomedicines (Europe), and traditional medicines (developing countries). The world market for herbal medicine, including herbal products and raw materials has been estimated to have an annual growth rate between 5 and 15%. Total global herbal drug market is estimated as US $62 billion and is expected to grow to US $5 trillion by the year 2050. India has a wealth of traditional knowledge and wisdom. Ayurveda contributes Rs 3500 crores (US $813 million) annually to the internal market. The Indian medicinal plants-based industry is growing at the rate of 7–15% annually. The value of medicinal plants-related trade in India is estimated at Rs 5000 crores per annum. Increased demand of medicinal plants for pharmaceuticals, phytochemicals, nutraceuticals, cosmetics and other products is an opportunity sector for Indian trade and commerce. Global trends in favour of botanicals have brought concerns over the quality, safety and efficacy of these products. Various pharmacopoeias and regulatory agencies on botanicals such as WHO Guidelines on Good Agricultural and Collection Practices (GACP), Research and Evaluation of Traditional medicine, US-FDA, European Scientific Cooperative On Phytotherapy (ESCOP), AYUSH (Department of Ayurveda, Yoga, Unani, Siddha and Homeopathy, Govt. of India), Therapeutic Goods Administration (TGA), Australia, Complementary Healthcare Council of Australia, National Center for Complementary and Alternative Medicine, Health Canada Natural...
Health Products Regulations\textsuperscript{12} emphasize the need for establishing and verifying botanical identity of raw materials as a first step in ensuring quality, safety and efficacy. In a recent review Rader et al have reinforced the need for development of better tools for botanical authentication, extraction, and characterization of bioactive constituents and the detection of hazardous adulterants or contaminants as these have a bearing on quality and safety\textsuperscript{13}.

1.2 Quality control of starting material
In the context of botanicals, the term standardization generally means all the measures taken to ensure its reproducible quality. In the guidance documents published by the American Herbal Products Association, definition of standardization is delineated as: "Standardization refers to the body of information and controls necessary to produce material of reasonable consistency. This is achieved through minimizing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing processes"\textsuperscript{14}. Standardization of botanicals is a complex issue. Unlike modern pharmaceutical products that are usually single or combinations of purified compounds prepared from synthetic materials using reproducible manufacturing procedures, botanicals are usually whole herbs themselves, or their formulations or extracts consisting of several bioactive compounds and hence are difficult to standardize\textsuperscript{15}. Consistency in composition and biological activity of botanicals is further limited by problems in identifying plants, genetic variability, variable growing and harvesting conditions, differences in extracts and lack of information about active pharmacologic principles\textsuperscript{16}. In the absence of authentication as a first quality control step, problems may arise in the final product due to misidentification of the collected plant materials, or adulteration or contamination with
other species. Thus authentication of the starting/raw material is important for ensuring reproducible quality, safety and efficacy\textsuperscript{17}.

1.3 Problems in quality control of starting/raw material

Several problems are encountered in authentication of starting material. Often, it is difficult to establish the identity of certain species that may be known by different binomial botanical names in different regions. Shankhapushpi, an important medhya Rasayan in Ayurveda is known by different botanical names viz., \textit{Canscora decussata}, \textit{Evolvulus alsinoides} and \textit{Clitoria ternata} in different regions of India. \textit{Boerhaavia diffusa} is used widely as a ‘quality of life enhancer’ in the traditional system of medicine. However, both \textit{B. diffusa} and the plant \textit{Trianthema portulacastrum} are known as ‘Punarva’, and so both the plants may be collected at the same time. Such wrong identification of medicinal plants could cause adverse effects\textsuperscript{18}. Also morpho-taxonomic confusion exists regarding the identity of closely related species. Wild collection of plants for the use in botanicals can also affect quality. Plants collected in the wild may include non-targeted species either by accidental substitution or by intentional adulteration. For example there is confusion regarding the identity and relationship of \textit{P. amarus} and its close relatives \textit{P. debilis}, \textit{P. urinaria} and \textit{P. fraternus} which grow together in the natural habitat. \textit{Mucuna pruriens} is the best example for unknown authentic plant and similarity in morphology. It is adulterated with other similar papilionaceae seeds such as \textit{M. utilis} (sold as white variety) and \textit{M. deeringiana} (sold as bigger variety). Apart from this, \textit{M. cochinchinensis}, \textit{Canavalia virosa} and \textit{C. ensiformis} are also sold in Indian markets. Substitution of \textit{Periploca sepium} for \textit{Eleutherococcus senticosus} has been widely documented, and is regarded as responsible for the “hairy baby” case involving maternal/neonatal androgenization\textsuperscript{19}. Other
examples include substitution of the bark of *Holarrhena antidysenterica* by *Wrightia tictoria*, and *Saraca indica* by *Trema orientalis*.

Plantain (*Plantago ovata*) is often found to be contaminated by *D. lanata*. Certain rare and expensive medicinal plant species are often adulterated or substituted by morphologically similar, easily available or less expensive species. For example *Swertia chirata* is frequently adulterated or substituted by the cheaper *Andrographis paniculata* and *Capsicum minimum* is substituted by *Capsicum annuum*. Further in order to establish botanical identity, whole plants in flowering or fruiting stages are preferred. This is possible in case of plants collected directly from the wild or field grown plants, but is difficult in case of plant materials purchased from the market. Moreover, confidence in botanical identification varies, depending in part on the preservation of the specimen and the level of experience of the individual analyst. Thus the tools needed for authentication depend on the plant and process involved. These could be as simple as botanical/morphological identification or as elaborate as genetic or chemical profiling or a combination of both.

1.4 **Conventional methods of crude drug identification and their limitations**

Conventionally, macroscopic and microscopic examination and chemical profiling of crude herbal materials are carried out for authentication and quality control. Macroscopic identity of medicinal plant materials is based on parameters like shape, size, color, texture, surface characteristics, fracture characteristics, odor, taste etc which are compared to a standard reference material. Microscopy involves comparative microscopic inspection of broken as well as powdered test materials with the reference material. Microscopic and macroscopic analysis is not a definite means of crude drug
identification as these parameters are judged subjectively and closely related species, substitutes and adulterants may resemble the genuine material. Moreover, other factors including environment, growth period and storage conditions of the herbs may affect the fine structure of the material. In chemical standardization analysis of one or more specific chemical markers that can easily distinguish varieties is a preferred option. Such metabolites being used as markers may or may not be therapeutically active but should ideally be neutral to environmental effects and management practices. The routinely used chemoprofiling techniques are Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and gas Chromatography (GC). Here chromatographic fingerprints are used as reference standards, to indicate the purity, identity and stability of the herbal drug. Spectroscopic methods such as infrared (IR), nuclear magnetic resonance (NMR), and ultraviolet-visible (UV-vis) may also be used for identification. In addition, other techniques such as volumetric analysis and gravimetric determinations are frequently employed (quality control mts, WHO, Geneva). Increased sensitivity has been achieved by using hyphenated techniques, coupling HPLC or GC with other analytical systems such as mass spectrometry. These include HPLC-MS, LC-MS, LC-MS-MS, GC-MS and such. Recently, capillary electrophoresis has been used to infer botanical sources and to assess the quality of herbal material and also to detect and quantitate various phytochemicals. The limitation of using chemoprofiling data for confirming botanical identity is that the chemical composition of a plant varies with environment (geographic location, weather, soil conditions), physiology, genetic predisposition of a plant population toward certain metabolic pathways, plant part used,
season, storage and processing conditions\textsuperscript{45, 46}. Moreover, closely related plant species may contain similar components from which a definite botanical identification may not be possible. Further, complications arise if commercial preparations have been “spiked” with other components (e.g. chemicals, pharmaceuticals) to mimic the authentic material or to produce a desired effect. Attempts to chemically standardize crude materials, extracts or other preparations in the absence of botanical authentication and characterization could be misleading\textsuperscript{13}. Thus although chemical standardization is important, its utility is limited when the starting material is not well characterized botanically\textsuperscript{24}.

1.5 \textbf{Alternative approaches for botanical identification}

The limitations of routine pharmacognostic techniques have stimulated the search for alternative methods of medicinal plant characterization. Molecular markers such as protein and DNA have been explored for authentication and differentiation of plant species. Protein markers have been applied for species identification\textsuperscript{47} and inter- and intra-species differentiation\textsuperscript{48, 49}. The most commonly used protein markers are isozymes, which are variant forms of the same enzyme\textsuperscript{50, 51}. However the use of protein markers is limited, as protein patterns vary in different tissues, developmental stages and environmental conditions and are subject to degradation on long-term storage of herbs. Also distinguishable markers may not be easily identified in closely related species. Molecular markers that reveal polymorphisms at the DNA level are called DNA markers. Recently, DNA based markers have become versatile tools and have found applications in various fields like taxonomy, plant breeding, population genetics, genome mapping, genome evolution, marker-based gene tags, map based cloning of \textit{agronomically important genes}, \textit{marker-assisted selection of desirable genotypes} etc.
Introduction

DNA markers reveal genetic differences that are more informative, specific and reliable than phenotypic differences such as morphology and chemical composition since the genetic composition is unique for each individual and is neutral to physiological and environmental factors. DNA marker technology can be very useful in identifying and differentiating closely related medicinal plant species. Advantages of DNA marker based techniques are that DNA can be isolated from fresh or dried parts of a plant such as leaves, stems, roots and small amount of sample is sufficient for analysis. Further, DNA markers are unaffected by environment. These techniques have been widely used for standardization of Traditional Chinese Medicine and their application for quality control of Ayurvedic medicinal plants needs to be exploited.

1.6 Types of DNA markers used in plant genome analysis

Various types of DNA based molecular techniques are utilized to evaluate DNA polymorphism and are generally classified as hybridization-based, Polymerase Chain Reaction (PCR)-based and Sequencing-based methods. Hybridization-based methods include Restriction Fragment Length Polymorphism (RFLP) and Variable Number Tandem Repeats (VNTR). PCR based techniques include Random Amplified Polymorphic DNA (RAPD), Arbitrarily Primed PCR (AP-PCR), DNA Amplification Fingerprinting (DAF), Inter Simple Sequence Repeats (ISSRs) polymorphisms and Amplified Fragment Length Polymorphism (AFLP). Sequencing based strategies include analysis of single nucleotide polymorphisms (SNPs), polymorphisms in nuclear ribosomal genes (rDNA) such as ITS1, ITS2 and IGS and plastid genes such as rbcL, matK, trnL-trnF, ndhF, psal-accD.

DNA based techniques have found a wide range of applications in commercially important plants such as food crops, horticultural crops and more recently in medicinal
plants. The range of applications in medicinal plants include; genotyping, assessment of genetic variation among closely related species and varieties, authentication of species, detection of adulteration or substitution, medicinal plant breeding and selection of desirable chemotypes.

The different types of DNA based markers used in plant genome analysis, their scope of application particularly in medicinal plants and limitations are reviewed elaborately in chapter 2.

1.7 The present study

1.7.1 Aim and Objectives

The aim of this thesis was to explore the possibility of using DNA based molecular markers for correct identification of Ayurveda based medicinal plants and further evaluate their suitability for use in crude and semi-processed materials and formulations. The objectives of the study were:

i. To select suitable medicinal plant for the study

ii. To identify putative species-specific RAPD amplicons

iii. To develop species-specific DNA marker for selected medicinal plant

iv. To study applicability of the developed marker for authentication of crude and semi-processed commercial products of the selected medicinal plant.

1.7.2 Rationale of work

Z. officinale Roscoe (ginger), which is one of the twenty top selling ayurvedic medicinal plants, was chosen for the study. It is an official drug in Ayurvedic, Indian Herbal, Chinese, Japanese, African and British Pharmacopoeia. Various methods including macroscopy, microscopy and chemoprofiling are reported for quality control of crude ginger and its products. These methods have limitations in correctly identifying
and distinguishing it from closely related species. Hence, newer complementary methods for correct identification of ginger are useful. Two closely related Zingiber species; Z. cassumunar and Z. zerumbet are used in this study since they are similar to Z. officinale Roscoe in morphology and medicinal uses\textsuperscript{71, 72} and are known contaminants or adulterants of Z. officinale\textsuperscript{73}. While Z. cassumunar Roxb. is mentioned in Thai herbal pharmacopoeia\textsuperscript{74}, Z. zerumbet is not mentioned in any of the official pharmacopoeias\textsuperscript{75}. Although all three plants are reported to have anti-inflammatory action, the chemical compounds to which these effects are attributed are different: 6-, 8- and 10-gingerol from Z. officinale, (E)-1-(3,4-dimethoxyphenyl)but-1-ene and (E)-1- (3,4-dimethoxyphenyl) butadiene from Z. cassumunar; and zerumbone from Z. zerumbet. Further, the mechanism of action of these compounds has not been established and substitution of any one species for another could lead to unwanted effects. With this background, development of DNA based markers that can be applied for identification and differentiation of Z. officinale Roscoe from closely related species; Z. zerumbet and Z. cassumunar was undertaken. Table 1. (next page) gives a comparative account of the three Zingiber species used in this study.
Table 1. Zingiber species comparison

<table>
<thead>
<tr>
<th>Description</th>
<th>Z. officinale Roscoe</th>
<th>Z. cassumunar Roxb.</th>
<th>Z. zerumbet (L.) J. E. Smith</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common name</td>
<td>Ginger</td>
<td>cassumunar ginger or Plai (Thai)</td>
<td>bitter ginger</td>
</tr>
<tr>
<td>Part used</td>
<td>rhizome</td>
<td>rhizome</td>
<td>rhizome</td>
</tr>
<tr>
<td>Distribution</td>
<td>SE-Asia</td>
<td>SE-Asia</td>
<td>SE-Asia</td>
</tr>
<tr>
<td>Pharmacopoeia</td>
<td>Ayurvedic, Indian Herbal, Chinese, Japanese, African and British Pharmacopoeia</td>
<td>Thai</td>
<td>Not mentioned</td>
</tr>
<tr>
<td>Identification</td>
<td>Macroscopy, microscopy, organoleptic characters, Volatile oil content, TLC/HPLC/GC (6-, 8-, 10-gingerol; shogaols, α-zingiberene, -bisabolene, sesquiphellandrene,</td>
<td>Chemical tests, TLC as described in Thai Herbal Pharmacopoeia</td>
<td>Not reported</td>
</tr>
<tr>
<td>Traditional use</td>
<td>Gastro-intestinal disturbances, vomiting, colds, flu, inflammation</td>
<td>anti-emetic, Gastro-intestinal</td>
<td>inflammation</td>
</tr>
<tr>
<td>Major reported activities</td>
<td>anti-inflammatory\textsuperscript{76,77} anti-emetic\textsuperscript{78,79,80}</td>
<td>anti-inflammatory\textsuperscript{81,82,83} cytotoxic\textsuperscript{84}, anti-tumor promoter activity\textsuperscript{85}</td>
<td>cancer chemopreventive\textsuperscript{86} 87, 88, 89, 90</td>
</tr>
</tbody>
</table>

1.7.3 Methodology

RAPD analysis was used to identify putative species-specific amplicons for *Z. officinale*. RAPD technique was chosen as it is easy to perform, requires no prior sequence information and is less time consuming as compared to other methods reviewed in chapter 2. RAPD analysis has been widely used for population genetic studies, genetic mapping, genotyping, marker assisted selection of desirable traits and such applications\textsuperscript{69}. However, RAPD analysis is sensitive to reaction conditions, sometimes leading to non-reproducible results. Further, non-stringent annealing temperatures can result in non-specific amplification which may not be reproducible thereby undermining their direct use as markers. Therefore the putative species-specific RAPD amplicons were further cloned and sequenced to develop SCAR (sequence characterized amplified region) markers. SCAR markers have been applied for species
identification in various organisms such as plants, insects, microbes and animals. SCAR primers being longer in length (usually 18-22 nucleotides), have higher annealing temperatures and thus are specific and reproducible. The applicability of the developed SCAR markers to identify *Z. officinale* Roscoe was studied by testing them in several *Zingiber* and non-*Zingiber* species as well as different types of samples such as fresh ginger rhizomes, dried rhizome powders, semi-processed rhizome powder (*shunthi*) and formulations (*laghu soot shekhar* and *trikatu*). The developed SCAR markers P1, P2, P3, P4 and P5 were tested in several non-zingiber species commonly used in ginger containing formulations. Detailed materials and methods are provided in chapter 3.

1.7.4 Summary
Results indicate that markers P1 and P2 could be used for detection of *Z. officinale* by gradient-PCR analysis; P5 could be specific for genus *Zingiber*; P3 was found to be specific for *Z. officinale* and was successfully applied for detection of *Z. officinale* from crude and semi-processed powders and multi-component formulation, *Trikatu*. This is the first report of species-specific SCAR marker development in ginger.