Authentication of Herbal Medicinal Plant- \textit{Boerhavia diffusa} L. Using PCR-RFLP

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Abstract

Herbal drugs are integrated part of both modern and traditional systems of medicine and are frequently found to be adulterated with other drugs of same morphological features, thereby requires scientific methods for their authentication and standardization. Various methods like chemo-profiling using chromatographic and spectroscopic techniques are used for the purpose but have their limitations because of the variations in the chemistry of the metabolites due to the age of the plants and their varied geographical distribution. DNA-based techniques have been widely used as a method of authentication for such types of herbal drugs. In the present study, using a DNA based technique known as PCR-RFLP, an attempt has been made to authenticate one such herbal drug \textit{Boerhavia diffusa} which gets adulterated with \textit{Trianthema portulacastrum} and \textit{T. monogyna}. The PCR amplified product of ribosomal ITS gene when subjected to restriction digestion with five different restriction enzymes viz. \textit{MspI}, \textit{HinfI}, \textit{Mbol}, \textit{EcoRI} and \textit{EcoRV} showed varied banding patterns for \textit{Boerhavia diffusa} and adulterated plants. Of the five enzymes \textit{MspI}, \textit{HinfI} and \textit{Mbol} could be used for authentication of \textit{B. diffusa} as they gave unique patterns for this plant and were different from that of adulterants. Rest of the enzymes either could not digest the ITS product or was not showing sufficient polymorphism to assign it to a particular genotype. Our study has established ITS-RFLP based markers for the authentication of \textit{B. diffusa}, \textit{T. portulacastrum} and \textit{T. monogyna} with enzymes \textit{MspI}, \textit{HinfI} and \textit{Mbol}.

Keywords: \textit{Boerhavia diffusa}, \textit{Trianthema portulacastrum}, \textit{T. monogyna}, Adulteration, Authentication, Herbal drugs, ITS-RFLP, Molecular markers and Polymorphism.

Introduction

Plants play a major role in the drug industry – be it synthetic or traditional. As for the synthetic drugs, there exists a standardized authenticated process for its quality control, but no such mechanism exists for raw materials or finished products in the traditional drugs. Hence, it is not possible to check the adulteration in the finished drug in traditional medicine. Adulteration in herbal drugs may be intentional for monetary gains or unintentional due to lack of awareness and knowledge about the authentic plants, confusion in vernacular names between indigenous systems of medicine and local dialect, similarity in morphological and aromatic features of the different plant sources, non-availability of the authentic plants, careless collection and other unknown reasons (1). Due to adulteration, faith in herbal drugs has declined (2). Adulteration in market samples is one of the greatest drawbacks in promotion of herbal products (3). It is invariably found that the adverse event reports are not due to the intended herb, but rather due to the presence of an unintended herb (4). This kind of adulteration is found among three plants,
Boerhavia diffusa (family Nyctaginaceae), Trianthema portulacastrum and Trianthema monogyna (family Aizoaceae) belonging to different families and having similar medicinal properties. These plants differ in their morphology and availability to a certain extent, but since they grow in the same vicinity, it becomes undistinguishable for the pluckers, who are interested in the roots having similar appearance and hence are wrongly supplied to the drug companies. Trianthema portulacastrum has dark green leaves like Boerhavia diffusa but its leaves have a maroon outline, however, not significantly distinguishable by people who are unaware of these differences and tend to confuse between the two causing unintentional adulteration. Trianthema monogyna has light colored leaves, thorns in the axils of branches and different flowers.

Boerhavia is also known by the names of Boerhavia adscendens, B. caribaea, B. coccinea, B. erecta, B. paniculata, B. repens, and B. viscosa. Though considered as a weed, its leaves, seeds and roots are edible and are rich in carbohydrates and proteins. The roots are diuretic, emetic, expectorant, laxative and stomachic. They are used in the treatment of asthma, oedema, anaemia, diabetes (5), jaundice (6), ascites, scanty urine and internal inflammation (7). Trianthema portulacastrum also called as Desert horsepurslane is shown to have anti-carcinogenic potential (8, 9), used in the treatment of oedema in the liver and the spleen (10). It has good hepato-protective activity against paracetamol and thio-acetamide intoxication in mice (11). This plant is shown to be diuretic, locative, analgesic and anti-inflammatory and is used in the treatment of asthma, jaundice, abdominal diseases and fever. These plants although have similar medicinal properties, vary in their active secondary metabolites which may produce different effects and hence the prevention of adulteration is important.

Therefore, to get the most beneficial effect, adulteration has to be checked for which a precise method should be applied to authenticate these plants apart from morphological and histological methods. One method, which can be relied on is the use of molecular markers which are generally referred to biochemical constituents, including primary and secondary metabolites (phenotype) and other macromolecules such as nucleic acids (genotype). In medicinal plants, majority of active chemicals acting as drugs are secondary metabolites which vary with the age and different geographic locations thereby not reliable as molecular markers. Also there is a potential for adulteration of plants with extracts from plants that have lower drug content. DNA based molecular markers have several advantages over typical phenotypic markers and 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 (12). DNA can be extracted from fresh or dried organic tissue (13) of the botanical material hence the physical form of the sample for assessment does not restrict detection. Based on the specificity of the genotype of a system, a particular DNA profile being unique can be ascribed to a particular organism. Hence, various DNA marker based methods can be used for species characterization and adulteration detection in medicinal plants.

Various types of DNA based molecular techniques utilized to evaluate DNA polymorphism include PCR based methods which include random amplified polymorphic DNA (RAPD) (14, 15) and amplified fragment length polymorphism (AFLP) (16), minisatellites and microsatellites (17), restriction fragment length polymorphism (RFLP), PCR-RFLP and sequencing based markers. Due to their high level of polymorphism, they have been extensively used for DNA fingerprinting as well as for genetic markers. RFLP of the internal transcribed spacer (ITS) gene is one of the methods to detect polymorphism to establish species specific patterns. The internal
transcribed spacer is a sequence of RNA in a primary transcript that lies between precursor ribosomal subunits. These sequences are coded by ribosomal DNA. Eukaryotic organisms have two internal transcribed spacers; ITS-1 located between the 18S gene and the 5.8S gene, while ITS-2 is located between the 5.8S and the 28S gene. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races) because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA). Variation among individual rDNA repeats can sometimes be observed within the ITS. In the ITS region, restriction digestion shows specific patterns for a species and a variation in the pattern within a species, which can be detected as polymorphism. Ribosomal DNA – ITS-RFLP of Vigna mungo var silvestris, V. trilobata, V. glabrescens and diverse cultivars of V. mungo, have been used for species identification (18). We have made an attempt to establish fingerprinting pattern for Boerhavia diffusa, Trianthema portulacastrum, and T.monogyna using ITS-RFLP and thus authenticate these plants and prevent the adulteration between the three.

Material and Methods

Collection of plant material: Leaves of Boerhavia diffusa, Trianthema portulacastrum and Trianthema monogyna were collected from various areas of Chandigarh, India; Panchkula, Ambala, and Yamunanagar, Haryana, India; Mohali, Amritsar and Patiala, Punjab, India; Delhi, India; Mumbai, Maharashtra, India; and Baroda, Gujrat, India. Five samples from each region were collected for the experiments.

Reagents for Restriction Fragment Length Polymorphism: Enzymes MspI, Eco RI, Hinf I, Eco RV, Mbol, Tango buffer (Fermentas), Buffer R (Fermentas), Buffer Eco RI (Fermentas) were used in the experiments.

Plant DNA extraction: A modified method of Biswas and Biswas (13) was used to isolate genomic DNA from plants without liquid nitrogen.

Amplification of the Internal Transcribed Spacer region by Polymerase Chain Reaction: PCR was set up for 50 µl reaction mixture containing 5.0 µl 10X Taq buffer with 15 mM magnesium chloride, 1.0 µl magnesium chloride (25 mM), 5.0 µl dNTPs (2 mM), forward primer (ITS F) (5’ TCC TCC GCT TAT TGA TAT GC 3’) 1.0 µl, reverse primer (ITS 1F) (5’ AAG TCG TAA CAA GGT TTC CGT AG 3’) 1.0 µl, milli Q autoclaved water 33.5 µl, 0.5 µl Taq polymerase (1.25 U), and template DNA 3.0 µl. The PCR mixtures were exposed to denaturation at 95º C for 5 min followed by 30 cycles of denaturation at 95º C for 30 sec, annealing at 52º C for 30 sec, extension at 72º C for 1 min, final extension at 72º C for 7 min in the Eppendorf Mastercycler Personal Thermal cycler. After the completion of the cycles, the sample was tested for amplification by electrophoresing on 1% agarose gel using 3 µl ITS amplified product and 1 µl loading dye at a voltage of about 70 V for 60 to 90 min. The marker used for size determination was 100 bp ladder.

Restriction Fragment Length Polymorphism (RFLP) of the amplified ITS regions of the plant DNA: The amplified ITS products, enzyme buffers and autoclaved distilled water were thawed from -20º C to 4º C by placing on ice for 1 to 2 h. Twenty µl mixtures were made for the enzymes to act on the templates with the respective buffers. Amplified ITS (template) 10 µl (1 µg), buffer (10X) -2 µl (1X), enzyme (10 U/ µl) – 1 µl (0.5 U/µl), and 7 µl of autoclaved distilled water were added. Following mixtures were made: enzyme Eco RI, amplified ITS (template) 10 µl (1 µg), buffer (10X) - 2 µl (1X), enzyme (10 U/µl) - 0.75 µl (0.375 U/µl) and autoclaved distilled water – 7.25 µl; enzyme Mbo I: amplified ITS (template) 10 µl (1 µg), buffer (10X) - 2 µl (1X), enzyme (10 U/µl) - 0.5 µl (0.25 U/µl) and autoclaved distilled water - 7.5 µl; enzyme HinfI: amplified ITS (template) 10 µl (1 µg), buffer (10X) - 2 µl (1X), enzyme (10 U/µl) - 0.3 µl (0.15 U/µl) and autoclaved distilled water - 7.7 µl; enzyme Hinfi: amplified ITS (template) 10 µl (1 µg), buffer (10X) - 2 µl (1X), enzyme (10 U/µl) - 0.3 µl (0.15 U/µl) and autoclaved distilled water - 7.7 µl; enzyme Eco RV: amplified ITS (template) 10 µl (1 µg), buffer (10X) - 2 µl (1X), enzyme (10 U/µl) - 0.3 µl
(0.15 U/µl) and autoclaved distilled water - 7.7 µl. The mixtures were placed in the water bath (Tanco India) at 37º C for 16 – 20 h according to the time required by the enzymes for digestion. The mixtures were then placed in water bath at 65º C for 20 min to inactivate the enzymes. These were then electrophoresed in 2% agarose gel with 5 µl sample and 1 µl loading dye (as in DNA isolation procedure) and 100 bp ladder was used as marker at 66V for 60-90 min to check the RFLP patterns obtained by restriction digestion of the enzymes on the ITS regions of the plant genome. The bands were analyzed for their size variation by comparing them with the standard marker.

**Results and Discussion**

DNA of high quality was obtained from all the samples. All the samples of each plant collected from different regions showed same results, therefore representative data is presented in the article. A product of 700 bp of ITS region was obtained after PCR amplification for *Boerhavia diffusa* samples. The same molecular weight products (700 bp) of ITS were also observed for *Trianthema portulacastrum* and *Trianthema monogyna* samples (Fig. 1). All the obtained ITS products of *B. diffusa* plants from different regions, when subjected to restriction digestion with five enzymes *MspI*, *Eco RI*, *Hinf I*, *Eco RV*, *MboI* separately to study the polymorphism, each individual enzyme showed unique pattern. The digested products of ITS of both *T. portulacastrum* and *T. monogyna* showed exactly same banding pattern with the five enzymes but were different from that of *B. diffusa*. With *MspI*, ITS of *B. diffusa*, *T. portulacastrum* and *T. monogyna* gave four fragments. However, the four fragments obtained in *B. diffusa* were different from that of *T. portulacastrum* and *T. monogyna* which showed similar patterns (Fig. 2, Table 1). When digested with the enzymes *Eco RI* and *MboI*, a single band of ITS was obtained for all the samples of *B. diffusa* which shows that there was no restriction site for these enzymes. In *T. portulacastrum* and *T. monogyna*, similar results were obtained with *Eco RI* but with *MboI* two fragments of 470 bp and 220 bp were obtained. On digestion of the ITS with the enzyme *HinfI*, five fragments were observed with four restriction sites in *B. diffusa*, while *T. portulacastrum* and *T. monogyna* displayed three restriction sites each for same enzyme (Fig. 3, Table 1). *Eco RV* digestion of the ITS produced two fragments of 300 bp and 400 bp for all the

**Fig. 1.** ITS products after PCR amplification Lane 1- 100 bp ladder, lane 2- *B. diffusa* from Chandigarh, lane 3- *B. diffusa* from Patiala, lane 4- Delhi, lane 5- *B. diffusa* from Baroda, lane 6- *T. portulacastrum* from Chandigarh, lane 7- *T. portulacastrum* from Patiala, lane 8- *T. monogyna* from Chandigarh, lane 9- *T. monogyna* from Patiala, lane 10- 100 bp ladder

**Fig. 2.** Restriction digestion of the ITS with *Msp I*; Lane 1-100 bp ladder, lane 2- *B. diffusa* from Chandigarh, lane 3- *T. portulacastrum* from Chandigarh, lane 4- *T. monogyna* from Chandigarh, lane 5- 100 bp ladder
samples of three plants. Polymorphisms among plant varieties are widely found and they have been studied based on biochemical markers (19) as well as DNA markers (18, 20) mostly with respect to geographical distribution and to establish their phylogenetic relationships (18, 20, 21). DNA based techniques have also been 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 and/or phytochemically indistinguishable (22).

The use of biochemical markers like isozymes showed limited polymorphism which was found to be insufficient to distinguish closely related cultivars in *Musa* species (19). DNA based polymorphisms provide a broader arena to establish markers for different species and/or varieties of plants (23, 24). ITS-RFLP is among some of the reliable methods to establish markers for the authenticated identification of plants (23) as ITS has both conserved (5.8S) and variable (ITS 1 and ITS 2) regions. The ITS obtained in all the three plants of *B. diffusa*, *T. portulacastrum* and *T. monogyna* were of 700 bp, and hence their RFLP was necessary to establish variation. The restriction enzymes used for RFLP were selected on the basis of previous reports showing their use on plant genomic DNA (18, 19).

In the present study, it has been observed that the enzyme *Eco RI* could not restrict the ITS of all the three plants and *MboI* could not cut the ITS of only *B. diffusa*. The inability of these two enzymes to cut the rRNA unit of all these plants can be attributed either to lack of a restriction site or the restriction site in the DNA may be methylated. While restriction sites were observed with the enzymes *MspI*, *HinfI* and *Eco RV* in all the three plants, restriction sites were seen only in *T. portulacastrum* and *T. monogyna* with the enzyme *MboI*. The RFLP pattern revealed that only *MspI*, *HinfI* and *MboI* could be used to establish markers for *B. diffusa* and *T. portulacastrum* as they gave totally different patterns. No intra-species variation was found among the *B. diffusa* plants. As reported earlier, *HinfI* and *MspI* generally have sites in plant ITS (18, 19). *T. portulacastrum* and *T. monogyna* did not show variation among them with any enzyme, but the RFLP patterns were exactly similar with the enzymes used in this study. Therefore, it will be necessary to use a number of other enzymes to establish a variation as it is unexpected to have no variation among the two different species. Our
study has established ITS-RFLP based markers for the authentication of *B. diffusa* and *T. portulacastrum* using the enzymes *MspI*, *HinfI* and *MboI*. These restriction enzymes can be applied to find out authenticity of plants used in drug preparations and avoid adulteration. Similar works on the authentication of medicinal plants of *Dendrobium* species by the internally transcribed spacer of rDNA has been reported earlier (23). RAPD technique has been used to identify eight types of dried *Coptis* rhizomes and one type of *Picrorrhiza* rhizome, a substitute for the former in the Chinese herbal market (24). AP-PCR, RAPD and RFLP have been successfully applied for differentiation of *Panax ginseng* and *Panax quinquefolius* (20) and to detect substitution by other closely related species (25). The present techniques have allowed an effective and reliable differentiation of *B. diffusa* from its adulterants. Proper combination of molecular techniques and analytical tools will lead to the development of comprehensive systems for the characterization of herbal medicinal plants and can be conveniently applied at the industrial level for quality control of these drugs. The authentication of plants based on genetic/molecular markers is important for quick identification in crude, semi-processed and processed herbal formulations wherever they are used.

**Conclusion**

Among the three plants *Boerhavia diffusa*, *T. portulacastrum* and *T. monogyna*, no variation was observed in the size of the ITS region. However, ITS-RFLP with experimental restriction enzymes showed absolutely similar patterns for *T. portulacastrum* and *T. monogyna* which varied from that of *B. diffusa*. Thus, restriction enzymes *MspI*, *HinfI* and *MboI* can be used to develop molecular markers that can differentiate these plants, authenticate them and prevent adulteration.

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**References**


**PCR-RFLP based authentication of Boerhavia diffusa**