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Isozyme markers for the crude drugs of maiden hair ferns from the Western Ghats, South India

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ABSTRACT

Adiantum, commonly called as maiden hair ferns, is a large genus of the non- flowering vascular plants. Most of the species are grown as ornamentals and many species are used in traditional medical practices and they are sold in the market for considerable prices. There is more possibility for the adulteration in the market sample, either intentionally or unintentionally, due to the presence of morphologically similar species with many variants within species. In order to identify the molecular markers, at species level, for the identification and confirmation of crude drugs, isozyme analysis was initiated on six different species of *Adiantum* from the Western Ghats, South India. The isoperoxidase analysis revealed the identity of the selected six species and the number of bands varied from six to eighteen. The present study revealed the presence of interspecific variation in the isozyme pattern for peroxidase. Of which, *Adiantum raddianum* and *Adiantum lunulatum* banding profile showed high percentage of similarity index compared to other species. Next to this, *Adiantum caudatum* and *Adiantum zolingeri* showed the similarity. The band PRX $1^{3 \text{ and } 4}$, PRX 2^{4}, PRX 4^{4} and PRX 7^{1} showed their unique presence in *Adiantum raddianum*, PRX 5^{1} for *Adiantum caudatum*, PRX 6^{4} and PRX 7^{2} for *Adiantum zolingeri*, PRX 3^{1}, PRX $9^{1 \text{ and } 3}$ and PRX 10^{1} for *Adiantum lunulatum*, PRX 2^{3} and PRX 6^{1} for *Adiantum hispidillum* and PRX 4^{3} for *Adiantum incisum.* These unique bands can be used to distinguish and characterize the species and differentiate the original crude drug from the adulterant.

KEYWORDS

Adiantum;
Isozymes;
Peroxidases.

INTRODUCTION

Discovery and development of new therapeutic agents is a continuing process. In spite of the fact that, at present, we have at our command a formidable array of modern drugs, the need to discover and invent new agents is genuine and urgent[1]. But random screening of medicinal plants for bioactivity and bioactive compounds has not proved economically effective method in contrast to the screening of medicinal plants, which are used in traditional medical practices[2]. Plants used in tribal medicines are usually screened for bioactivity and bioactive compounds. *Adiantum,* commonly called as maiden - hair fern, has about 23 species in India

Full Paper

widely distributed in the Indian subcontinent. Maximum number (15 species; 78%) of species occurs on the mountains of south India[3]. All the species are commonly grown as ornamental plants and majority of them are also used in traditional medicines to cure various diseases like cough, fever, skin diseases, catarrhal affection, throat infection, bronchial disorders, dysentery, ulcer, epilepsy, leprosy, biliousness, inflammation, cold, headache, piles, hair growth etc. *Adiantum lunulatum* Burm., *Adiantum capillus – veneris* L and *Adiantum raddianum* C. Presl are sold in the market by the trade names "Hansraj" and "Paroshan" at a rate of Rs.50 per Kg[4]. All the species are collected from the wild and none of the species is under cultivation. *Adiantum caudatum* L. is largely used as substitute for *Adiantum capilllus- ven*eris L. and there is also possibility for the intentional or unintentional adulteration of common species in the place of rare species. Such adulterant may not have the same medicinal property as in the original species. Moreover species like *Adiantum lunulatum* and *Adiantum raddianum* are polymorphic taxa with many morphotypes, cytotypes and ecotypes[5,6]. The availability of such a wide range of variants in these species presents a problem in the selection of suitable variant with good medicinal property. Pharmocognosy i.e. standardization of crude drugs, plays an important role in the identification of crude drugs based on organoleptic, microscopic and physio – chemical standards which have recently been studied[4]. Reliable information on the existing genetic variation is required for selection, breeding and conservation programmes of genetic resources. Genetic variability is the first hand indication of all the heritable characters including the active principles in medicinal plants. Genetic variation that is believed to exist between or within a species can be determined by using morphological, biochemical and molecular markers[7]. Isozyme provides relatively simple and inexpensive method of obtaining genetic information[8]. Isozyme data allow quantification of the similarity or difference within and between populations[9]. Since 1930's, electrophoresis coupled with the zymogram technique have been the tool of choice for studies of heritable variation by geneticists, systematists and population biologist[10,12]. In recent years isozyme analysis and molecular markers are also being used in pharmacognosy[13,14]. With the aim to find out the genetic variation and biochemical markers among these crude drugs from different species, isozyme analysis was carried out, as the first step, on six different species viz., *Adiantum raddianum* C. Presl *Adiantum incism* Forssk., *Adiantum lunulatum* Burm., *Adiantum caudatum* L., *Adiantum zollingeri* Mett.ex.Khun and *Adiantum hispidilum* Sw. from the Western Ghats, south India. The results obtained in this study were thought to be useful for researchers dealing with pharmaceuticals, pteridologist and horticulturist dealing with ferns.

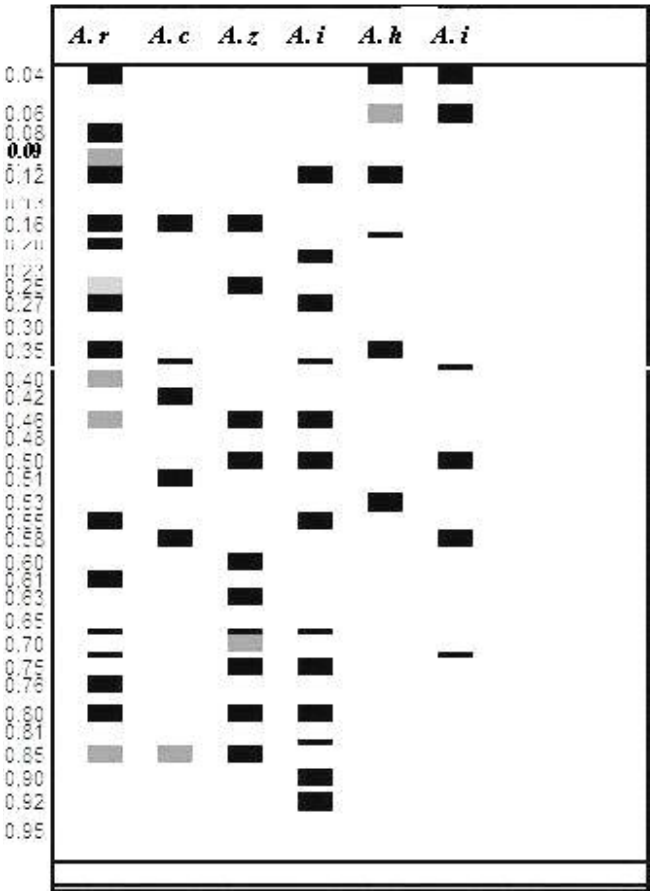
MATERIALS AND METHODS

Adiantum raddianum C. Presl *Adiantum incism* Forssk., *Adiantum lunulatum* Burm., *Adiantum caudatum* L., *Adiantum zollingeri* Mett.ex.Khun and *Adiantum hispidulum* Sw. were used as plant materials. For peroxidase, 500 to 1000 mg of young freshly harvested leaves (Croziers) were taken and homogenized with 3.5 ml of ice cold homogenizing buffer (0.1M Phosphate buffer (pH 7.0)) in a pre-chilled pestle and mortar and centrifuged at 12,000 rpm for 10 min. The supernatant was subjected to electrophoresis as described by Sadasivam and Manickam[15] on PAGE. For the detection of isozymes on the gels, the staining solution, were prepared by Sadasivam and Manickam[15]. After the electrophoresis, the gels were incubated in the staining solution for few minutes under the dark condition till the clear bands appeared. The gels were fixed with 7% acetic acid solution for 30 min. and the gels were washed with distilled water and the bands were documented using the Vilber loubermat gel documentation system. The bands profiles were compared using the Bio-gene software (Germany).

RESULTS AND DISCUSSION

A total of eighteen bands from different positions were observed in *A. raddianum,* fourteen in *A. lunulatum,* nine in *A. zollingeri,* six in *A. caudatum, A. incisum* and *A. hispidulum* and their Rf values ranged from 0.04 to 0.95 (Figure 1). Multiple regions of activity were observed for this enzyme system (PRX 1 to 10). Region 1 showed four bands (PRX $1^{1 \text{ to } 4}$), difference in bands position was also observed (Figure

1). The first band (PRX 1^1) was common to *A. raddianum, A. incisum* and *A. hispidulum*. The second band (PRX1^2) was observed in *A. lunulatum* and *A. hispidulum*, while the band PRX1^3 and PRX1^4 were restricted to *A. raddianum*. So, the third and fourth bands showed their unique presence in this region, it can be used to distinguish *A. raddianum* from other species (Figure 1). Similar to Region1, region 2 also contained bands in four different positions. The first band (PRX 2^1) showed their presence in *A. raddianum, A. lunulatum* and *A. hispidulum*. The second band (PRX 2^2) was shared by *A. raddianum, A. caudatum* and *A. zollingeri*. But the third and fourth bands showed the variation and unique presence in *A. hispidulum* (PRX 2^3) and *A. raddianum* (PRX 2^4) respectively.



A. r - ***Adiantum raddianum* C.Presl**
A. c - ***Adiantum caudatum* L**
A. z - ***Adiantum zollingeri* Mett.ex.Khun**
A. l - ***Adiantum lunulatum* Burm.**
A. h - ***Adiantum hispidulum* Sw**
A. i - ***Adiantum incisum* Forssk.**

Figure 1 : Isoperoxidase profiles of *Adiantum* sps.

In region 3, a total of three bands were observed in different positions (PRX 3^{1to3}). The first band was restricted to *A. lunulattum*. This unique banding profile can be used as identification marker for this species. The second band (PRX 3^2) was observed in *A. raddianum* and *A. zollingeri*. But the intensity of the band was varied between these two species. *A. raddianum* showed only lightly stained band, but *A. zollingeri* showed very dark stained band. Similar to second band, the third band (PRX 3^3) was shared by *A. raddianum* and *A. lunulattum*. Region 4 contained bands in four different positions; the first band (PRX 4^1) showed their presence in *A. raddianum* and *A. hispidulum*. The second band (PRX 4^2) was common to two species such as *A. caudatum* and *A. zollingeri*. The third (PRX 4^3) band was showed their expression in *A. incisum, A. caudatum* and *A. lunulatum*. The fourth (PRX 4^4) band showed their expression only in *A. raddianum*. Region 5, showed three bands in different positions. *A. caudatum* was showed the unique distribution in their position and the first band was restricted (PRX 5^1). The second band (PRX 5^2) was common to *A. raddianum, A. zollingeri* and *A. lunulatum*. The third band (PRX 5^3) showed their presence in *A. zolingeri, A. lunulatum* and *A. incisum*.

Region 6 contained bands in four different positions. The first band (PRX 6^1) and fourth band (PRX 6^4) were showed their unique presence in *A. hispidulum* and *A. zollingeri* respectively, while the other two bands were shared by other species. The second band (PRX 6^2) was common to *A. raddianum* and *A. lunulattum*. The third band (PRX 6^3) was showed their presence in *A. caudatum* and *A. incisum*. Region 7 also showed the unique banding profile, the first band (PRX 7^1) was restricted to *A. raddianum*, the second one (PRX 7^2) to *A. zollingeri*. The third band was shared by *A. raddianum, A.zollingeri* and *A. lunulatum*. Region 8 failed to show the unique features of the species, in this region the selected plants showed their similarities in this enzyme system. The first band (PRX 8^1) was observed in *A. raddianum* and *A. incisum*, the second band (PRX 8^2) was shared by *A. zollingeri* and *A. lunulatum*, the third band (PRX 8^3) showed their presence in *A. raddianum, A. zolingeri* and *A. lunulatum*.

Region 9 contained bands in three different positions. The first band showed the unique presence in *A. lunulatum*. The second band was common to *A.*

Full Paper

raddianum and *A. caudatum*, the position of the band showed the similarity but the intensity of the bands varied between these two species (Figure 1). The third band also showed the unique presence in *A. lunulatum*. Region 10 contained a single band, whose position was restricted to *A. lunulatum*, while other species did not show any profile (TABLE 2).

TABLE 2 : Isoperoxidase pattern of the *Adiantum* sps.

MW- Rf values	*A. raddianum*	*A. caudatum*	*A. zollingeri*	*A. lunulatum*	*A. hispidulum*	*A. incisun*
PRX 1^{1} - 0.04	*				*	*
PRX 1^{2} - 0.06					*	*
PRX 1^{3} - 0.08	*					
PRX 1^{4} - 0.09	*					
PRX 2^{1} - 0.12	*			*	*	
PRX 2^{2} - 0.16	*	*	*			
PRX 2^{3} - 0.18					*	
PRX 2^{4} - 0.20	*					
PRX 3^{1} - 0.23						
PRX 3^{2} - 0.25	*		*			
PRX 3^{3} - 0.27	*			*		
PRX 4^{1} - 0.35	*				*	
PRX 4^{2} - 0.36		*	*			
PRX 4^{3} - 0.37		*		*		*
PRX 4^{4} - 0.40	*					
PRX 5^{1} - 0.42		*				
PRX 5^{2} - 0.46	*		*	*		
PRX 5^{3} - 0.50			*	*		*
PRX 6^{1} - 0.53			*		*	
PRX 6^{2} - 0.55	*			*		
PRX 6^{3} - 0.58		*				*
PRX 6^{4} - 0.60			*		*	
PRX 7^{1} - 0.61	*					
PRX 7^{2} - 0.63			*			
PRX 7^{3} - 0.66	*		*	*		
PRX 8^{1} - 0.74	*					*
PRX 8^{2} - 0.75			*	*		
PRX 8^{3} - 0.80	*		*	*		
PRX 9^{1} - 0.81				*		
PRX 9^{1} - 0.85	*	*				
PRX 9^{1} - 0.90				*		
PRX 10^{1} - 0.92				*		

The present study revealed the interspecific difference in the isoperoxidase banding profile of *Adiantum* by PAGE. Each and every species showed the similarities and difference in their banding profiles. Practically, identifying Pteridophytes using morphological characters has number of problems. Characterization of pteridophyte depends on spore texture, colour, shape and arrangement (spore formation is seasonally influenced), development of tissue (Croziers), venation pattern, rhizomes types etc. As a general biologist / botanist, identifying the pteridophytes is a difficult task; in addition, differentiation of each and every character requires the minute microscopical observation and depth analysis. In Angiosperm also, morphological markers have several disadvantages. To overcome this problem, botanist and pteridologist depend on the biochemical and molecular markers for the classification and identification. Of which, isozymes are practical and useful genetic and biochemical marker as well as good estimators of the genetic variability in plant populations. The present study revealed that the selected six species were easily separable isozymically and strengthens the isozymic usage in the taxonomical studies as a criterion. Accuracy in recording or observing the medial use of a plant, selection of the superior genotypes of the plant species, chemical characterization of the compound(s) and the role of the effect are important issues that need to be verified in the development of drugs of plant origin. Genetic marker provides to superior genotype, species among individuals and populations[16].

Adiantum incisum, Adiantum lunulatum, Adiantum caudatum and *Adiantum zollingeri* are simply pinnate fronds while *Adiantum raddianum* is with tripinnate or quadripinnate fronds and *Adiantum hispidulum* is with bipartite pedately divided tripinnate fronds. Such morphological similarities or dissimilarities are correlated with the bands in isozyme (Figure 1). The presence or absence of chemical constituent has been found useful in the placement of the plant in their taxonomic categories[17]. Each isozymes has a specific role in the metabolic pathway and functions in harmony with other enzymes with in the organizational framework of cells. Isozymes often exhibit tissue or cell specificity[18]. Isozymic variation has been chosen here to reveal the diversity existing at molecular level in *Adiantum* species. The present study confirmed the role of isozymes in variation and similarity between the selected species. Each zone occupied by a particular isozyme in the form of band and is representative of the expression of a particular gene locus coding for that

isozyme. In certain species, in a particular zone more than one distinct band is resolved. These bands could represent allelic isozymes, coded by different alleles of the same gene at locus and thus occupy that particular zone on the gel[19]. In the present study also the similar kind of banding profiles observed in all enzyme system indicating the presence of multiple alleles. Electrophoretic studies and similarity index indicated that *A. raddianum* and *A. lunulatum* had more alleles in common with each other than they had with other species. Next to this, *A. caudatum* and *A. zollingeri* showed the similarity. The band PRX $1^{3 \text{ and } 4}$, PRX 2^4, PRX 4^4 and PRX 7^1 can be used as marker for *A. raddianum*, PRX 5^1 for *A. caudatum*, PRX 6^4 and PRX 7^2 for *A. zollingeri*, PRX 3^1, PRX 9^{1and3} and PRX10^1 for *A. lunulatum*, PRX 2^3 and PRX 6^1 for *A. hispidulum* and PRX 4^3 for *A. incisum*.

Isozymes such as esterase, peroxidase have been utilized to trace the genetic lineage of various rice varieties, sugar cane, pulses, medicinal plants and somoclonal variants[20-28]. Similarly in the present study also, the isozymes are used as the biochemical marker for the systematic study of *Adiantum* species. Unique banding profile of peroxidase was observed in the six selected species, which represent the finger print of that particular species. Such finger printing is useful in differentiating the species and act as biochemical markers for these species in plant systematic studies. Molecular marker and isozyme sequencing are considered excellent for population structure analysis but the data obtaining through isozyme are relatively inexpensive compared to DNA. In addition, large number of samples can be processed with far less training and time per sample, where as DNA analysis require more time and sophisticated instruments. Furthermore, in most cases the new DNA based markers provide the same type of information as isozymes.

Preliminary phytochemical screening of different species of *Adiantum* from the south India shows the presence of phenolic group and steroids and absence of alkaloids, saponins and catachin in all the species. Although there is similarity in the distribution of such major chemical groups, the genetical differences, as expressed by peroxidase isozyme, indicates the possibility for the differences in the type of chemical compounds in the same chemical group. It is true by the occurrence of different positions with different Rf value (TABLE 1).

TABLE 1 : Cytological and thin layer chromatography analysis of *Adiantum* sps.,

Name of the species	Chromosome numbers	Rf values from TLC
Adiantum caudatum L		0.08,0.29,0.52
Adiantum hispidulum Sw	n=2n=1716x agamosporous	Bands are not visualized
Adiantum incisum Forssk.	n=30 2x sexual	0.11,0.56,0.831
Adiantum lunulatum Burm.	n=30, n=60 2x, 4x, sexual	0.08,0.41,0.56
Adiantum raddianum C.Presl	n=57, n=114 4x, 8x sexual	0.9,0.74,0.69,0.57, 0.46,0.17,0.11
Adiantum zollingeri Mett.ex.Khun	n=29 2x sexual	Bands are not visualized

Adiantum incisum and *Adiantum zollingeri* are diploid while other species are either with polyploid alone or polyploidy along with diploid cytotypes. Usually diploid plants have a highly conserved minimal number of isozyme for many of the enzymes routinely included in electrophoretic studies. In the present study also the diploid *Adiantum incisum* has only six bands for peroxidase in contrast to fourteen bands in *Adiantum lunulatum* for which both diploid and tetraploid cytotypes are in record from south India. The presence of minimum number of bands i.e. six in the polyploidy *Adiantum hispidulum* is due to its agamosporous nature of reproduction. Among the six species of *Adiantum* in the present study, *Adiantum hispidulum* is the only agamosporous species while other species are either sexual or sexual with agamosporous cytotypes also. Since each and every species of *Adiantum* have their own isozyme pattern, particularly in peroxidase isozyme they can be successfully used as molecular markers in the identification of the crude drugs at species level, in addition to the classical pharmacognostical standards. Identification of molecular markers for the variants with in the species like tetraploid and octaploid cytotypes in *Adiantum raddianum* and diploids and tetraploid cytotypes in *Adiantum lunulatum* is in progress. The preliminary RAPD analysis in different cytotypes of *Adiantum raddianum* shows close relationship between the two cytotypes and thus it indicates the autopolyploid origin of octaploid cytotypes from the tetra ploid cytotypes. From the preset study it is concluded that it is not advisable to use any species of *Adiantum* in the place of another *Adiantum* species, either as a

substitute or as adulterant, since they differ genetically and phytochemically. If any rare species, like *Adiantum capilllus veneris* in south India, is required in large amount, mass cultivation can be practiced without destroying the natural populations.

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