Detection of Adulteration by *Wedelia calendulacea* in *Eclipta alba* through ISSR and RAPD Markers

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Abstract

In the crude herbal drug market, the highly medicinal *Eclipta alba*, popularly known as "Bhringaraja", is often adulterated with *Wedelia calendulacea*. To evaluate the ability of simple genetic fingerprinting methods to differentiate between the two species, Inter-Simple Sequence Repeat and Random Amplified Polymorphic DNA assays were performed on 30 individuals of *E. alba* and 4 individuals of *W. calendulacea*. Samples of *W. calendulacea* were found to cluster in clades that distinctly separated from *E. alba* and at least one band was identified for each of the two species that can serve as a marker for identification. Despite wide variations and a limited number of samples, phylogenetic analysis helped in differentiating the original from the adulterant plant. The study underscores the importance of simple DNA-based fingerprinting as a cost-effective method for the detection of adulteration in plants, particularly with respect to *E. alba*.

Key words

Eclipta alba · *Wedelia calendulacea* · Asteraceae · phylogenetics · adulteration · molecular markers

Abbreviations

ISSR:	Inter-Simple Sequence Repeat
PCA:	Principal Component Analysis
PCoA:	Principal Coordinate Analysis
RAPD:	Random Amplified Polymorphic DNA

Supporting information available online at http://www.thieme-connect.de/products

Eclipta alba (L.) Hassk, commonly known as "Bhringaraja", is a medicinal herb belonging to the Asteraceae family that is traditionally used as a liver tonic and hair growth promoter in various traditional systems of medicine including Ayurveda. It is used in the treatment of spleen enlargements, uterine hemorrhages, skin diseases, and scorpion bites [1–4]. Other than the use of the whole plant juice as a powerful hepatoprotective and rejuvenative tonic, it is also used for the treatment of respiratory disorders, including coughs and asthma [5]. *Wedelia calendulacea* (L.) Less ("Pithabringi"), which belongs to the same family as *E. alba,* has a similar common name, and has been known for similar therapeutic uses [1,4]. It is often used as a substitute for the latter. It is interesting to note that wedelectone, a coumestan, is one of the major polyphenolic active constituents found in both species and it has been reported for activities against breast and prostate carcinomas [6,7].

Correct botanical identities of several crude drugs used in Ayurveda and in other traditional systems have not been properly established. These drugs have been categorized as "Sandigdha Dravya" or doubtful entities [8]. These are herbs with names that do not precisely indicate the exact botanical identity, but describe the therapeutic utility of the plant. The same is true with "Bhringaraja". Both *E. alba* and *W. calendulacea* are used under the name "Bhringaraja", often for treatment of the same ailment. However, it is reported that "Shweta Bhringaraja" – white-flowered herb (*E. alba*) – is superior in quality [9]. Reports of "*E. alba*" being adulterated with *W. calendulacea* are frequent [2,10,11]. Quality issues, including the genuineness of crude drugs are a major concern plaguing the herbal drug industry, particularly in India. The use of authentic medicinal plant species is a fundamental requirement in herbal medicine [12]. Adulteration, substitution, and mislabelling may cause potential harm to patients [13–17]. Along with safety and toxicity, there is a need to develop methods of profiling medicinal plants for the purpose of authentication. For genetic materials, which are more stable and uniform, DNA-based typing methods are increasingly being used for this purpose [18,19]. Methods like RAPD and ISSR fingerprinting are cost effective, relatively simple, and easy to perform. Although not always robust, they are techniques that yield results that can be interpreted to answer questions on genetic similarities, differences, and relatedness at reasonably lower costs and can be carried out at any laboratory that has a PCR facility. In the present study, such simple fingerprinting methods have been employed to identify and differentiate *E. alba* from its putative adulterant *W. calendulacea.*

Amplification of genomic DNA of 30 individuals of *E. alba* and 4 individuals of *W. calendulacea* using the two most reliable of seven ISSR primers tested yielded 23 scorable loci. Four out of the five RAPD primers tested showed clear band resolution and reproducibility. A total of 58 polymorphic loci were produced by the four RAPD primers. However, an assay with the ISSR primer UBC 880 showed the best discriminatory power and reproducibility amongst the primers employed in ISSR or RAPD assays during the present study (**Fig. 1 S**, Supporting Information).

Jaccard's coefficient of similarity between all of the 34 individual plants ranged from 0.10 to 0.91 in the ISSR assay and from 0.04 to 0.97 in the RAPD assay. For cluster analysis, a similarity matrix was used to construct an unrooted phenetic dendrogram using the program PhyloWidget. It showed two main clades, one for *E. Alba* (EA) and the other for *W. calendulacea* (WC) in both the ISSR and RAPD assays (**Fig. 1**). The WC group separated out from the EA group very early. Interestingly, in the ISSR assays, within the EA group, five minor clades were observed, in which EA 30 (clade IV) appeared as distinct (**Fig. 1 b**). Among the remaining minor clades, clade I consisted of 12 individuals (EA1, EA2, EA6, EA8, EA3, EA4, EA5, EA7, EA9, EA12, EA15, EA10), clade II consisted of 8 individuals (EA11, EA14, EA13, EA16, EA17, EA18, EA19, EA23), clade III of 5 individuals (EA21, EA25, EA27, EA28, EA29), and clade V of 4 individuals (EA20, EA26, EA24, EA22). Similarly, with the RAPD assays, EA and WC appeared as separate major clades (**Fig. 1 a**). Within the EA group, 9 minor clades appeared, in which EA1 (clade I) and EA10 (clade VIII)

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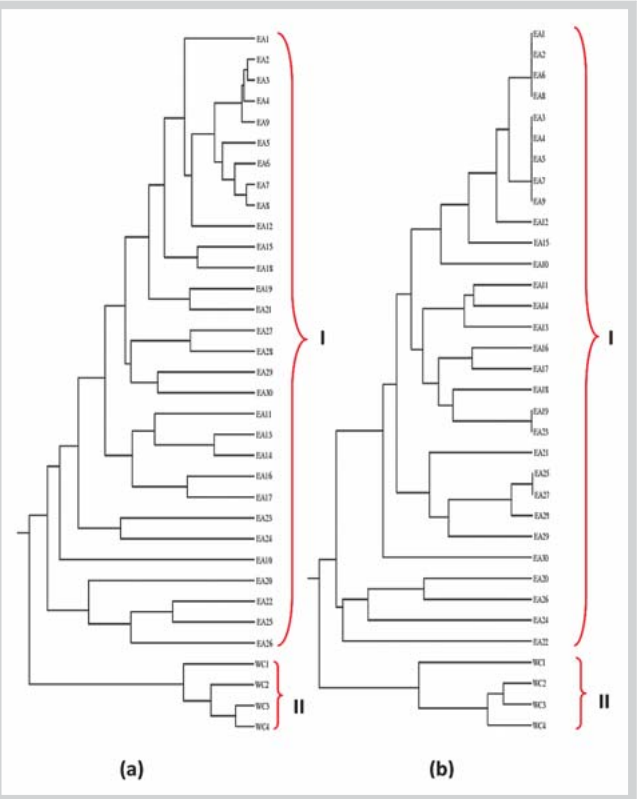

Fig. 1 **a** RAPD- and **b** ISSR-based UPGMA phenogram showing similarities amongst 30 individuals of *E. alba* (EA1 to EA30) and 4 individuals of *W. calendulacea* (WC1 to WC4).

were grouped as distinct clades. Among the minor clades, clade II consisted of 9 individuals (EA2, EA3, EA4, EA9, EA5, EA6, EA7, EA8, EA12), clade 3 consisted of 2 (EA15, EA18), clade IV of 2 (EA19, EA21), and clade VII of 2 (EA23, EA24) individuals, while clade V had 4 individuals (EA27, EA28, EA29, EA30), clade VI had 5 (EA11, EA3, EA14, EA16, EA17), and clade XI had 4 individuals (EA20, EA22, EA25, EA26). Both unrooted phenograms showed genetic divergence within the EA, while EA differentiated from WC distinctly.

In general, ISSR showed 86.96% polymorphic loci and RAPD showed 91.38%. Tangentially, the number of effective alleles (*Ne*), Shannon's information index (*I*), and diversity (*h*) were also analyzed (see Supporting Information), whereby it was found that EA exhibited a wide genetic divergence even though this particular study was limited in the geographical range of the plants covered.

In PCoA, EA appeared separated from WC, although they were observed to be close to each other (**Fig. 2 a, b**). PCA also revealed the genetic relatedness between EA and WC (**Fig. 2 c, d, e**, and **f**). Similar to PCoA, in PCoA also the WC and EA were found to be close to each other. This is perhaps due to the fact that both EA and WC belong to the same family and share some common characters. It may be noted that due to this closeness, there might be some similarity in their chemo profiles, which might in turn account for similar biological activity and similar medicinal use. However, in both PCoA and PCA analyses, individual samples showed clustering into two groups of distinct genetic pools of respective taxa (**Fig. 2**) facilitating differential identification.

Adulteration and substitution of drugs may be intentional or unintentional. Misidentification of a particular herb results in substitution unknowingly, while intentional adulteration is done with less expensive herbs instead of the actual herb, which is generally more expensive [20]. Among the various available methods of quality control, pharmacognostic assays are time-consuming and laborious and therefore are a deterrent for wide use. Development of phytochemical markers are dependent on various factors like environmental conditions, age of plant, soil condition, harvesting time, etc., which tend to fluctuate the chemical constituents and therefore lack uniformity, which is necessary to be used as a tool. Therefore, DNA-based technologies, which are more stable and reliable, have clear advantage over these traditional methods. Although DNA barcoding, extreme PCR, or high-resolution melting analysis provide more authentic and accurate results, cost-effective and reliable identification tools like ISSR and RAPD markers have been considered advantageous since they can be carried out in reasonably equipped laboratories [19, 21–23]. In the present study, phylogenetic inference coupled with PCA and PCoA effectively resolved and clearly differentiated *W. calendulacea* from *E. alba*. Although limited in number and scope, the present study highlights the usefulness of simple DNA-based techniques in the differentiation of the important medicinal plants *E. alba* and *W. calendulacea.*

Materials and Methods

Leaves from 30 individual plants of *E. alba* and 4 plants of *W. calendulacea* were collected from the Western Ghats region of Karnataka, India (latitudes 15°42′N to 16°7′N, longitudes 74°3′E to 74°41′E; **Table 1 S**, Supporting Information). The plant samples were identified by a qualified taxonomist and voucher specimens for *E. alba* (voucher number: RMRC – 982) and *W. calendulacea* (voucher number: RMRC – 983) were deposited in the herbarium at the Regional Medical Research Centre, Belagavi, India. DNA from all leaf samples were extracted by a modified CTAB method. The stock DNA thus obtained was diluted to 30 ng/µL as a working dilution. ISSR and RAPD assays were performed at least twice with each primer to confirm the reproducibility of the bands. Only the consistent and reproducible bands were considered for scoring and subsequent analysis. Negative controls that consisted of reaction mixtures without a DNA template were used during both of the fingerprinting assays. For each genotype, the presence (1) or absence (0) of bands were scored as a binary matrix. Phenetic dendrograms were generated and genetic variations exhibited were derived by PCA and PCoA by using a binary matrix (see Supporting Information).

Supporting information

Detailed information of genetic diversity and materials and methods are provided as Supporting Information.

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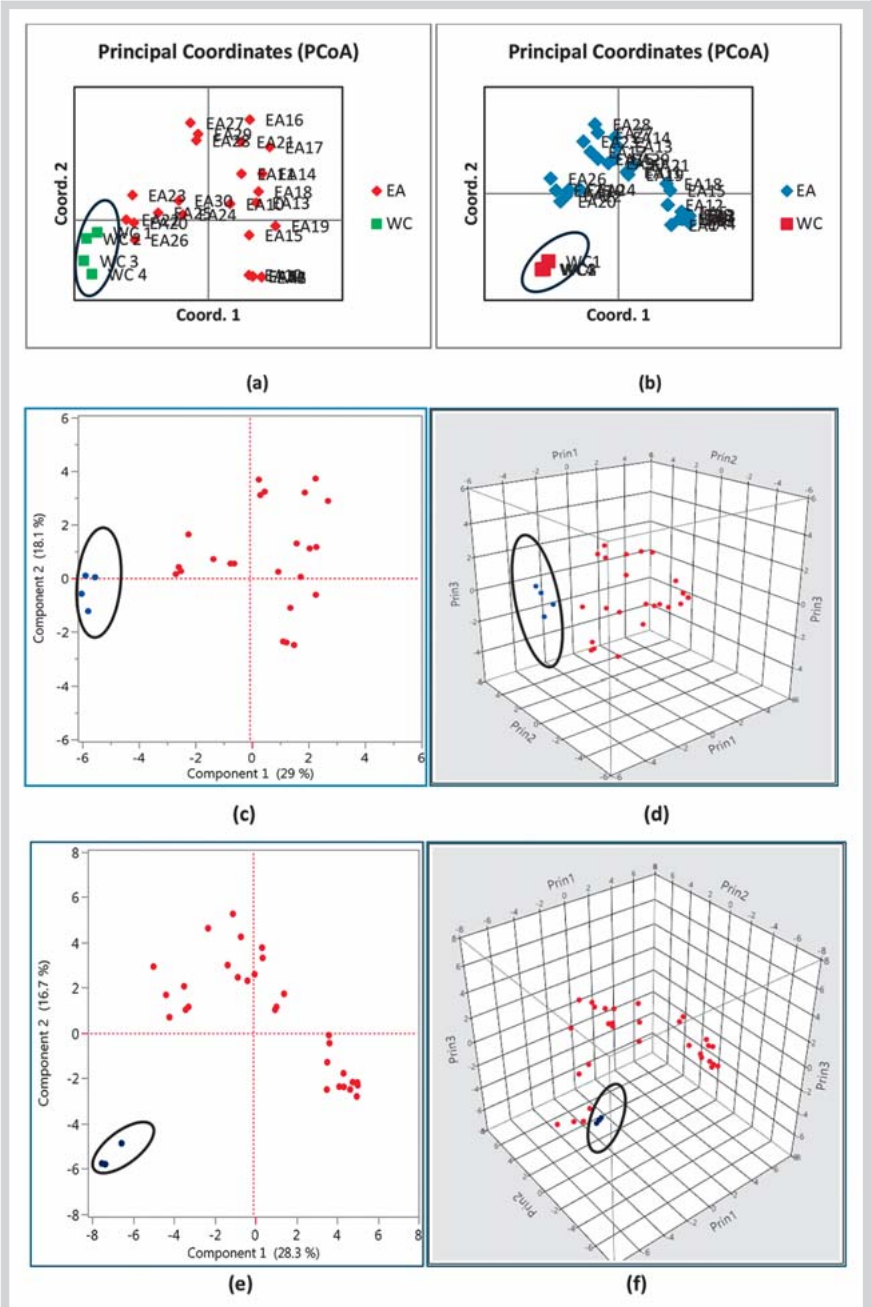

Fig. 2 **a** ISSR-based Principal Coordinate Analysis (PCoA), **b** RAPD-based Principal Coordinates Analysis (PCoA), **c** Score plot, and **d** 3D scatter plot of Principal Component Analysis (PCA) from ISSR markers. (**e**) Score plot and (**f**) 3D scatter plot of principal component analysis (PCA) from RAPD markers. In the diagrams, WC samples are circled.

Conflict of Interest

The authors declare no conflict of interest.

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