Original Article

Development and detection efficiency of sequence characterized amplified region markers for authentication of medicinal plant *Ruta graveolens* and its adulterant *Euphorbia dracunculoides*

Abstract

Background: With the increase in demand of herbal medicines, adulteration in these drugs is also gaining momentum and remains an indispensable problem in domestic and export markets. Correct identification is the first step toward assuring quality, safety, and efficacy of indigenous herbal medicines. **Materials and Methods:** In this study, sequence characterized amplified region (SCAR) markers were developed to discriminate *Ruta graveolens* from its adulterant *Euphorbia dracunculoides*. Random amplified polymorphic DNA (RAPD) was performed and subsequently converted into SCAR markers. **Results:** After performing RAPD, SCAR primers were designed from the selected unique RAPD amplicons of the genuine drug as well as its adulterant. These primers produced 670 bp and 750 bp SCAR markers with genomic DNA sample of *R. graveolens* and *E. dracunculoides*, respectively. **Conclusion:** Development of these markers will help in the quality control of herbal drugs and monitoring widespread adulteration of these drugs by pharmaceutical industries and government agencies.

Key words:
Euphorbia dracunculoides, Ruta graveolens, sequence characterized amplified region marker

Introduction

The use of traditional herbal medicines and their preparations is as old as our civilization. According to World Health Organization, about 70–80% of the world population is still using traditional medicines[1] due to their significant healing powers and lesser side effects. Traditional herbal medicines are often deemed to be safe due to their natural origin. However, due to the lack of accurate inspection system, adulterated, and spurious herbal drugs pose serious threats to consumers/patients.[2]

The dried leaves of *Ruta graveolens*, known as barg-e-sudab, are crude indigenous drug of high therapeutic value. The drug is prescribed to the patients suffering with gastric disorders and dizziness.[3] It is used as a sedative and antihelminthic.[4] It is also used as a remedy for a severe headache and rheumatism.[5] The drug also possesses antiinflammatory, antiviral, and antiplasmodic properties.[6-8] Total extract (70% ethanol) of this plant showed *in vitro* cytotoxicity against tumor cell lines.[9]

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Euphorbia dracunculoides belonging to different genus has morphological characters similar to *R. graveolens* and is thus used in lieu of *R. graveolens*. The negative effects of *E. dracunculoides* were studied as epistaxis, nausea/vomiting, and hematuria,[10] which were not observed in the case of *R. graveolens*. Hence, its substitution/addition in Berg-e-sudab results into reduced quality and efficacy of the drug and even may cause negative effects on the consumer's health.

Molecular marker technology is increasingly becoming popular as a potent tool for detection of adulteration in traditional herbal medicines.[11] Various molecular markers, such as amplified fragment length polymorphism, restriction fragment length polymorphism and random amplified polymorphic DNA (RAPD) have been used widely for authentication of various plant species.[12] However, RAPD markers are difficult to reproduce and are, therefore, preferentially converted into more stable and reliable marker by cloning and sequencing. Under stringent conditions, the designed specific oligomers from cloned RAPD amplicon, amplify a single band analogous to the genetically defined locus, sequence characterized amplified region (SCAR).[13] This is called SCAR marker and favored over other molecular markers due to its distinct characteristics such as high sensitivity, high reproducibility, and cost-effectiveness.

In this study, DNA fingerprints for *R. graveolens* and *E. dracunculoides* were developed using RAPD-polymerase chain reaction (PCR) and subsequently converted into reproducible SCAR markers using genuine samples of these herbs. They were further validated using the mixed powdered samples of the genuine drug as well as its adulterant.

Materials and Methods

DNA isolation

The sample of *R. graveolens* was collected from the Central Council for Research in Unani Medicine (CCRUM) and Khari Baoli, New Delhi and identified by Dr. H. B. Singh, National Institute of Science Communication and Information Research, New Delhi. After identification, the sample from CCRUM was found to be of the genuine medicinal herb, *R. graveolens* and the market sample was found to be its adulterant *E. dracunculoides*. The genomic DNAs from these powdered samples of *R. graveolens* and its adulterant *E. dracunculoides* were isolated by modified cetyltrimethylammonium bromide method.[14]

Random amplified polymorphic DNA-polymerase chain reaction analysis of genomic DNAs

The amplification of genomic DNAs extracted from powdered samples of *R. graveolens* and its adulterant was performed with operon primer C (OPC) kit (Operon life Technologies Pvt. Ltd, USA) using RAPD-PCR.[15] RAPD assays were performed in a final volume of 25 μL containing 2.0 μL of DNA template (15 ng μL^{-1}), 2.5 μL reaction buffer (10X), 2 μL of deoxynucleotide triphosphate mix (2.5 mM each), 0.5 μL Taq (*Thermus aquaticus*) DNA polymerase (3.0 U μL^{-1}) and 2.0 μL primer set (15 ng μL^{-1}). Amplification was performed in a Techne Thermal Cycler TC (Touchgene) using the following conditions: Initial denaturation at 94°C for 3 min, 45 cycles of denaturation at 94°C for 1 min, annealing at 35.5°C for 30 s, extension at 72°C for 1 min, and the last cycle of extension at 72°C for 3 min. PCR products were separated with electrophoresis in 1.2% agarose gel, visualized, and photographed under UV transilluminator (UV Tech, UK).

Cloning and sequencing of the random amplified polymorphic DNA markers

Unique RAPD amplicons from *R. graveolens* and *E. dracunculoides* were excised and eluted by gel extraction kit (Fermentas) and cloned into p-GEMT Easy Vector. Thereafter, the recombinant plasmid was used to transform *Escherichia coli* (DH5α) competent cells. After transformation, the white colonies were picked up from the Luria Agar plate containing ampicilin, X-galactosidase and isopropyl-beta-D-thiogalactopyranoside and recombinant plasmid was isolated from each overnight grown colony. The presence of insert was confirmed by restriction digestion of the plasmid DNA with EcoRI. The cloned DNA inserts were sequenced by automated sequencer using T7 forward and SP6 reverse primer sets (Bangalore Genei Pvt. Ltd., Bengaluru, India).

Designing sequence characterized amplified region primers and validation

A similarity search against nonredundant database was performed for nucleotide sequences of RAPD amplicons using Basic Local Alignment Search Tool (BLAST) program.[16] Specific primer pairs (SCAR primers) were designed from these nucleotide sequences using Primer BLAST[16] and used for PCR amplifications of genomic DNAs from both *R. graveolens* and *E. dracunculoides*. PCR conditions for amplification using SCAR primers were optimized [Table 1]. The amplified products were resolved on 1.2% agarose gel.

To check the detection efficiency of these SCAR primers, the powdered samples of both *R. graveolens* (R1) and *E. dracunculoides* (R2) were mixed in different ratios and their genomic DNAs were amplified using SCAR primers specific to *R. graveolens* and *E. dracunculoides* [Table 2].

Results

DNA isolation and random amplified polymorphic DNA

The yield of genomic DNA for *R. graveolens* and *E. dracunculoides* was 167 ng μL^{-1} and 162 ng μL^{-1}, respectively. The quality and quantity of the isolated DNA, determined by taking OD at 260/280, ranged from 1.75 to 1.78.

Table 1: Polymerase chain reaction conditions for the development of sequence characterized amplified region markers

Cycles	Denaturation		Annealing			Polymerization	
	Temperature (°C)	Time (min)	Temperature (°C)		Time (s)	Temperature (°C)	Time (min)
			Ruta graveolens	*Euphorbia dracunculoides*			
First cycle	94	3	-	-	-	-	-
30 cycles	94	1	55.9	54.5	30	72	1
Last cycle	-	-	-	-	-	72	3

Table 2: Powdered samples of *Ruta graveolens* and *Euphorbia dracunculoides* mixed indifferent ratios to check the sensitivity of sequence characterized amplified region primers

Ruta graveolens (g)	*Euphorbia dracunculoides* (g)	Ratio
5.0	0.0	10:0
4.5	0.5	9:1
4.0	1.0	8:2
3.5	1.5	7:3
3.0	2.0	6:4
2.5	2.5	5:5
2.0	3.0	4:6
1.5	3.5	3:7
1.0	4.0	2:8
0.5	4.5	1:9
0.0	5.0	0:10

RAPD fingerprints obtained with four decamers (OP-01, OP-02, OP-05, and OP-06) showing the highest degree of polymorphism is reported in this study. Of these primers, OP-02 generated unique amplicons of 827 bp in *R. graveolens* (R1) and 1000 bp in *E. dracunculoides* (R2), respectively [Figure 1]. These unique bands can differentiate the two herbs, *R. graveolens* and *E. dracunculoides*.

Analysis of sequences and sequence characterized amplified region primer designing

After cloning and sequencing of unique RAPD amplicons specific to *R. graveolens* (R1) and *E. dracunculoides* (R2), the DNA sequence of R1 and R2 were submitted to gene bank (Accession number: 883977 and 883978). BLAST analysis revealed that there was no similarity between these sequences of *R. graveolens* and *E. dracunculoides* and known nucleotide sequences in the gene bank. SCAR primers were designed from short internal sequences of RAPD amplicons using NCBI primer BLAST tool and used in further amplification experiments [Figure 2a and b].

Validation and detection efficiency of sequence characterized amplified region primers

The genomic DNA of *R. graveolens* and *E. dracunculoides* were amplified using the SCAR primer pairs (R1P and R2P). SCAR primer, R1P specific to *R. graveolens* gave amplification only with genomic DNA of *R. graveolens* generating 670 bp bands,

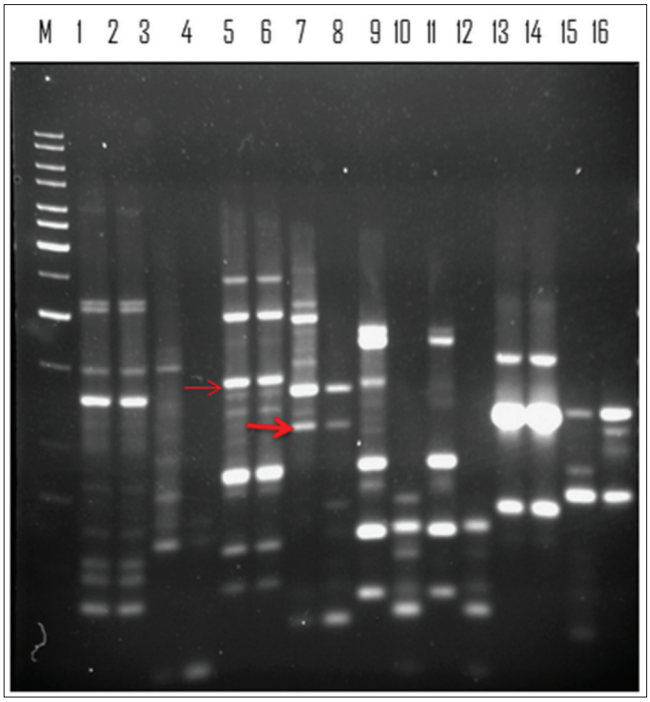

Figure 1: Random amplified polymorphic DNA profile of *Ruta graveolens* and *Euphorbia dracunculoides* with OPC-01 (Lane 1–4), OPC-02 (Lane 5–8), OPC-05 (Lane 9–12), OPC-06 (Lane 13–16). Pattern of amplification in *Ruta graveolens* is shown in Lanes 1, 2, 7-10, 13, 14 and *Euphorbia dracunculoides* in Lanes 3–6, 11, 12, 15, 16. Unique bands are marked with red arrows: lanes 7 and 8 (R1-827 bp) and Lanes 5 and 6 (R2-1000 bp). Lane M – 1 kb marker

while no amplification was observed in *E. dracunculoides* [Figure 3a]. Similarly, amplification product of 750 bp was detected with genomic DNA of *E. dracunculoides* using SCAR primer R2P specific to *E. dracunculoides*, but no amplification with genomic DNA of *R. graveolens* [Figure 3b]. Validity and specificity of SCAR primers were confirmed by the above results and thus, can be used for authentication and quality control of genuine drug *R. graveolens* from its adulterant *E. dracunculoides*.

The detection efficiency of SCAR primers was tested by mixing the powdered samples of *R. graveolens* and *E. dracunculoides* in different ratios [Table 1]. SCAR primer sets, R1P and R2P were used for amplifying the genomic

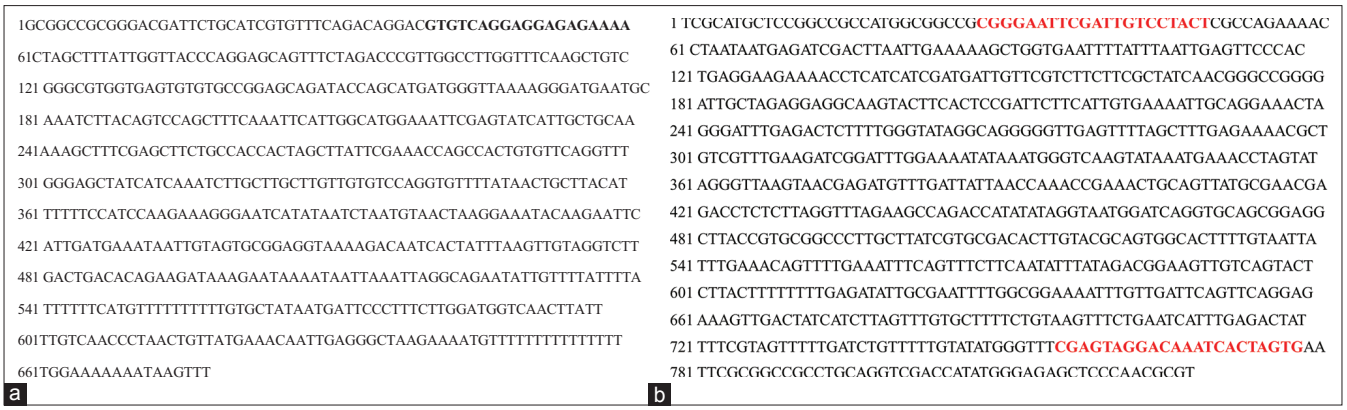

Figure 2: (a) Nucleotide sequence of the cloned amplicon of *Ruta graveolens,* (b) nucleotide sequence of the cloned amplicon of *Euphorbia dracunculoides*

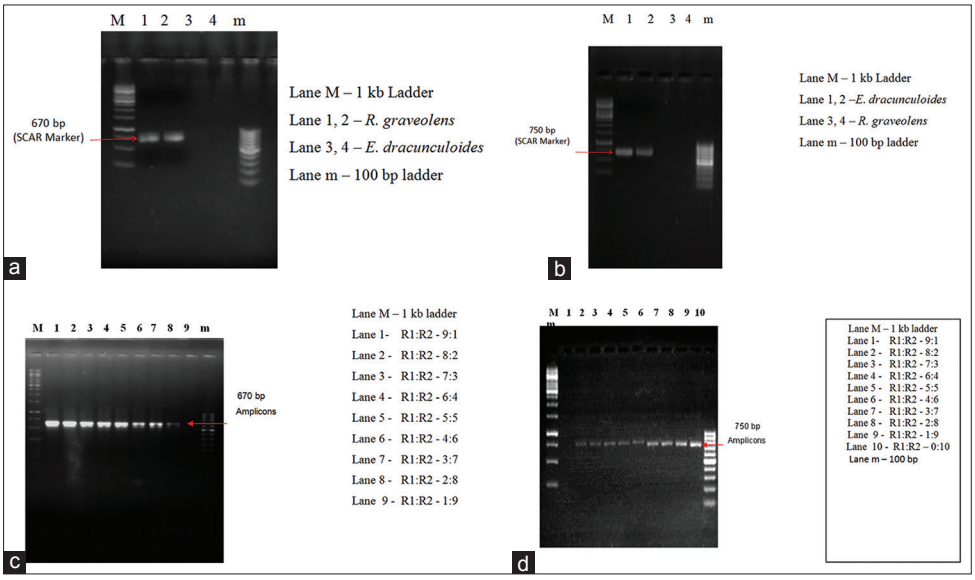

Figure 3: (a) Genomic DNA samples from the leaves of *Ruta graveolens* and *Euphorbia dracunculoides* amplified using the sequence characterized amplified region primers of *Ruta graveolens,* (b) genomic DNA samples from the leaves of *Euphorbia dracunculoides* and *Ruta graveolens* amplified using sequence characterized amplified region primers of *Euphorbia dracunculoides,* (c) genomic DNA samples from the mixtures of *Ruta graveolens* (R1) and *Euphorbia dracunculoides* (R2) amplified using sequence characterized amplified region primers of *Ruta graveolens,* (d) genomic DNA samples from the mixtures of *Ruta graveolens* (R1) and *Euphorbia dracunculoides* (R2) amplified using sequence characterized amplified region primers of *Euphorbia dracunculoides*

DNA extracted from the mixed powdered samples. SCAR primer R1P showed amplification with a gradual decrease in the intensity of the amplicons with respect to decrease in the amount of *R. graveolens* in the mixture of powered samples. No amplification was, however, observed in lane 10, where the *R. graveolens* powder was completely substituted with *E. dracunculoides* powder [Figure 3c]. Similarly, SCAR primer R2P showed amplification with the DNA extracted from the mixed powdered sample, but no amplification in lane 1 was observed, where its powder was completely replaced by that of *R. graveolens* [Figure 3d]. This confirms that these SCAR markers are specific for *R. graveolens and E. dracunculoides,* respectively and are capable of detecting as low as 10% of the genuine or adulterant material present in the mixture.

Discussion

A number of DNA-based molecular markers are used for authentication of herbal medicines.[17] RAPD is a simple and rapid technique. Species-specific RAPD fragments were generated showing polymorphism between the two medicinal herbs. RAPD bands are converted to a more stable and reproducible SCAR markers. SCAR markers have many advantages over RAPD markers as the conditions for annealing are stringent and only a single locus is detected.[18] They are more specific as only one species-specific DNA fragment is amplified in PCR amplification.[19] Validity and specificity of SCAR primers can be used for authentication and quality control

of genuine drug *R. graveolens* from its adulterant *E. dracunculoides*.

Development of these markers will help in the quality control of Unani drugs and monitoring widespread adulteration of these drugs by government agencies. This will not only improve the therapeutic efficacy of these drugs but also help in protecting the consumers/patients from the negative effects of the adulterants. It will, therefore, be a significant step toward quality assurance of the indigenous systems of medicine.

Conclusion

Development of these markers will help in the identification of medicinal plants used in traditional drugs and could be a useful tool to supplement the distinctness, uniformity and stability analysis for plant samples to maintain their identity for the protection in the future.

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Conflicts of interest

There are no conflicts of interest.

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