



Molecular authentication of the medicinal herb *Ruta graveolens* (Rutaceae) and an adulterant using nuclear and chloroplast DNA markers

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ABSTRACT. Dried parts of different plant species often look alike, especially in powdered form, making them very difficult to identify. *Ruta graveolens*, sold as a dried medicinal herb, can be adulterated with *Euphorbia dracunculoides*. The genomic DNA was isolated from the leaf powder (100 mg each) using the modified CTAB method. Internal transcribed spacer sequences of nuclear ribosomal DNA (nrDNA-ITS), and chloroplast spacer sequences (*rpoB* and *rpoC1*) are regarded as potential genes for plant DNA barcoding. We amplified and sequenced these spacer sequences and confirmed the sequences with a BLAST search. Sequence alignment was performed using ClustalX to look for differences in the sequences. A DNA marker was developed based on *rpoB* and *rpoC1* of the nrDNA-ITS for the identification of the adulterant *E. dracunculoides* in samples of *R. graveolens* that are sold in local herbal markets. Sequence-characterized amplified region markers of 289 and 264 bp for *R. graveolens* and 424 bp for *E. dracunculoides* were developed from dissimilar sequences of this

nrDNA-ITS to speed up the authentication process. This marker successfully distinguished these species in extracted samples with as little as 5 ng DNA/ μ L extract.

Key words: Chloroplast spacer sequences; SCAR marker; Herbal drugs; *Euphorbia dracunculoides*; nrDNA; *Ruta graveolens*

INTRODUCTION

The adulteration of medicinal plants from other plant species, which have similar morphology, is a common issue in herbal markets. The adulteration of these herbal drugs may be intentional or non-intentional at any stage of the plant materials. Therefore, authentication of raw medicinal plants is a fundamental requirement for quality assurance in herbal drug markets. Recently, efforts have been made to obtain accurate identification of medicinal plants used in raw drug trade to ensure the purity, quality and safety of the drugs (Jayasinghe et al., 2009). The morphological, biochemical or histological characteristics employed in the identification are less reproducible (Kiran et al., 2010) under different environmental conditions. Limitations of these markers for authentication of herbal drugs have generated a need to develop more reproducible molecular markers for quality control of these medicinal herbs. Similarly, phytochemical-based analysis methods, such as Fourier transform infrared spectroscopy, high-performance liquid chromatography mass spectrometry, nuclear magnetic resonance spectroscopy, and thin-layer chromatography, are generally used in the authentication of plant materials (Fugel et al., 2005; Kurtz et al., 2010). However, in some cases, phytochemical profiles may alter within the same species because of external factors such as temperature, light, humidity, soil composition and pH, storage conditions, etc. Therefore, DNA-based authentication methods are reproducible for any fresh as well as dried old material where a great extent of confusion exists.

Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA) have been extensively used for phylogenetic study among plant species (Choo et al., 2009; Gulbitti-Onarici et al., 2009), molecular discrimination of herbal materials (Zhang et al., 2007) and DNA barcoding (Zuo et al., 2011). The nrDNA-ITS region has frequent insertions/deletions, which can be phylogenetically informative (Baldwin et al., 1995). The conserved regions of ITS region including 18S and 28S genes of nuclear ribosomal transcriptional unit are used to infer phylogenetic relationships at higher taxonomic levels, whereas the more rapidly evolving segments (ITS and IGS) are used for studies at the genic or population levels (Alvarez and Wendel, 2003). Besides nrDNA-ITS sequences, there are other gene spacer sequences and non-coding region in the plant genome, which also have the priority of identification and authentication at inter-specific level and at inter-generic level of the plants. Chloroplast genes are a versatile tool for the plant identification and barcoding to establishing genetic relationship among various plant species (Nock et al., 2011). Chloroplast contains highly conserved genes, which are fundamental for plant life, and more variable regions, which are informative over a broad time scale.

Several plants of the Rutaceae family are being used as a traditional medicine worldwide. A scented medicinal plant named *Ruta graveolens* L. [(‘rue’ or ‘sudab’ in Hindi (India)], native to Europe is distributed throughout the world. This species has a

more or less similarity to *Euphorbia dracunculoides* in fresh as well as in a dried state. The leaves are small, oblong, deeply divided, pinnate and glandular dotted with an unpleasant odor. The flower has four petals except for the central flower, which has five petals. The fruits are round, brown, small, and lobulated. The taste is slightly stinging but is masked by the strong bitter odor. *E. dracunculoides* Lam. (Euphorbiaceae) has a similar morphology to *R. graveolens* and is being sold as a substitute or adulterated with the above plant species (Khan et al., 2011; Al-Qurainy et al., 2011). The aqueous extract of *E. dracunculoides* has shown adverse effects such as epistaxis, nausea/vomiting and hematuria (Rahman et al., 2003), which were different from *R. graveolens*. The authentication of *R. graveolens* has been carried out in previous study with RAPD markers and ITS sequences of nrDNA (Khan et al., 2011; Al-Qurainy et al., 2011); however, due to less reproducibility of RAPD marker, it has generated a need to develop a more reproducible marker for the identification of this species in the herbal markets. Based on one marker, we cannot accurately authenticate the genuine as well as adulterant samples; therefore, a combined study should be performed for more precise authentication. Further, sequences from single chloroplast or nuclear genes have been useful for the identification and authentication of these plants, which have a similarity in morphological markers as well as in other markers. Therefore, we employed chloroplast spacer sequences (*rpoB* and *rpoCI*) and nrDNA-ITS for the authentication of *R. graveolens* and its adulterant *E. dracunculoides*. Since they have a number of valuable characteristics, i.e., the availability of conserved regions, there is an ease in their amplification, and sufficient variability to distinguish even closely related species. Furthermore, a sequence-characterized amplified region (SCAR) marker was developed for both genuine as well as adulterant samples from nrDNA-ITS sequence to avoid further sequencing stage, so that these plant species could easily be identified.

MATERIAL AND METHODS

Sample collection from local herbal market

Samples of *R. graveolens* were collected under the vernacular name 'sudab' from different shops of the local herbal market Khari Baoli, Delhi, India. All samples collected were identified by a taxonomist.

Genomic DNA extraction and polymerase chain reaction (PCR) amplification

The genomic DNA was isolated from the leaf powder (100 mg each) using the modified CTAB method (Khan et al., 2007). Total genomic DNA was employed in the PCR for the amplification of the ITS region of nrDNA, *rpoB* and *rpoCI* from *R. graveolens* (genuine) as well as *E. dracunculoides* (adulterant), respectively. The universal primers, sequence and their PCR conditions used in the amplification are given in Table 1.

The nrDNA-ITS was amplified from total genomic DNA according to the protocol by White et al. (1990) and Song et al. (2009). The PCR bead (GE Healthcare, UK) was employed for amplification and single reaction consisted of 20 μ L deionized sterile water, 25 ng DNA per reaction volume and 10 pm of each forward and reverse primer.

Table 1. The universal gene specific primers, sequences and their reaction conditions.

Gene	Primer	Primer sequence 5'-3'	Reaction conditions
nr ITS	Forward	GTCCACTGAACCTTATCATTAG	94°C 5 min
	Reverse	TCCTCCGCTTATTGATATGC	94°C 1 min, 49°C 1 min, 40 cycles 72°C 5 min
rpoB	Forward	ATGCAACGTCAAGCAGTTCC	94°C 1 min
	Reverse	GATCCCAGCATCACAATTCC	94°C 30 s, 53°C 40 s, 40 cycles 72°C 5 min
rpoC1	Forward	GTGGATACACTTCTTGATAATGG	94°C 1 min
	Reverse	CCATAAGCATATCTTGAGTTGG	94°C 30 s, 53°C 40 s, 40 cycles 72°C 5 min

PCR product purification and sequencing

The amplified PCR products of the above genes were directly sequenced at Macrogen Inc., South Korea, using dye terminator chemistry. Cycle sequencing was conducted using the same primers used in amplification and BigDye version 3 reagents and an ABI PRISM 3100 DNA Analyzer (Perkin-Elmer, Applied Biosystems). Cycling conditions included an initial denaturing set at 94°C for 5 min, followed by 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The sequencing product was precipitated with 17 µL deionized sterile water, 3 µL 3 M sodium acetate, and 70 µL 95% ethanol. Polyacrylamide gel electrophoresis was conducted with long ranger single packs (FMC BioProducts) and an ABI 3100 automated DNA sequencer (Perkin-Elmer, Applied Biosystems). Each sample was sequenced in the sense and antisense direction and analyzed with the ABI Sequence Navigator software (Perkin-Elmer/Applied Biosystems).

Sequence analysis

The sequence alignment was performed between genuine and adulterant samples to observe the sequence variations for their discrimination to each other. The ITS sequences of nrDNA, *rpoB* and *rpoC1* of *R. graveolens* and *E. dracunculoides* were obtained using the Basic Local Alignment Search Tool (BLAST) through <http://blast.ncbi.nlm.nih.gov/Blast.cgi> to confirm our sequence from related genus or species sequences available on NCBI database. All sequences generated from *R. graveolens* and *E. dracunculoides* in the present study had to be submitted to the GenBank®. All three gene spacer sequences of both species were aligned using ClustalX version 1.81 (Thompson et al., 1997) to establish the degree of differences.

Development of SCAR marker and its validation

The nrDNA-ITS region has been used in phylogenetic analysis in the plants at generic and intra-generic levels. The length and sequences of nrDNA-ITS regions of ribosomal RNA repeats are believed to be quickly evolving and therefore may vary in the genome. Therefore, nrDNA-ITS region is believed to be a preferred choice for molecular typing of plant species. Moreover, in recent years, ITS sequence of nrDNA has been employed as genetic markers in various plant species (Zhang et al., 2007; Choo et al., 2009; Gulbitti-Onarici et al., 2009). These nrDNA-ITS fragments amplified from the genomic DNA samples of *R. graveolens* and *E. dracunculoides* were sequenced and sequence differences were observed through the

Clustal-X software. For the development of SCAR marker, the species-specific primer was designed from dissimilar sequences of nrDNA-ITS using primer 3 tool. The higher dissimilarity of the sequence of nrDNA-ITS between genuine as well as adulterant samples has made development of SCAR marker successful. The sequence of the designed primers for the development of SCAR marker for *R. graveolens* and *E. dracunculoides* is shown in Table 2.

Table 2. SCAR primer sequences and their reaction conditions for the amplification of the SCAR marker.

	Primer	Primer sequence 5'-3'	Product size (bp)	Reaction conditions
<i>SCAR289F</i>	Forward	GATATCTCGGCTCTCGCATC	289	94°C 5 min 94°C 1 min, 48°C 45 s, 25 cycles 72°C 5 min
<i>SCAR289R</i>	Reverse	GCTTTGTTTTACCACCGAT		
<i>SCAR264F</i>	Forward	AGAACGTAGCGAAATGCGAT	264	94°C 5 min 94°C 1 min, 48°C 45 s, 25 cycles 72°C 5 min
<i>SCAR264R</i>	Reverse	GCTTTGTTTTACCACCGAT		
<i>SCAR424F</i>	Forward	CGCAGCCTCATAACAAAACC	424	94°C 5 min 94°C 1 min, 48°C 45 s, 25 cycles 72°C 5 min
<i>SCAR424R</i>	Reverse	AGCGAGGGTCTTACAACCAC		

Furthermore, the marker was validated using the designed specific primer of one species with DNA of both samples in the PCR. The marker sensitivity was also checked using the serial dilution of DNA, which ranged from 5-25 ng/25 µL reaction volume.

RESULTS AND DISCUSSION

DNA-based molecular tools are urgently needed to authenticate raw herbal materials in order to maintain their quality and efficacy in the herbal markets. These markers are more reproducible under wide variables as compared to other markers, whereas *R. graveolens* (sudab) has more similarities in morphological markers with *E. dracunculoides* and is easily adulterated and/or replaced in the local markets (Rahman et al., 2003; Khan et al., 2011). The bipinnate or tri-pinnate and obviate-oblong leaves of *R. graveolens* have more similarities with leaves of *E. dracunculoides*, which are lanceolate or linear oblong. *R. graveolens* has a strong-smell in dried as well as in fresh leaves, slightly toxic and a bitter in taste, whereas odor and taste are not distinct in the case of *E. dracunculoides*. The fruits of both species have extensive similarities and create more confusion, for example, *R. graveolens* has a capsule, 4-5 lobed globosely containing numerous seeds, tiny size, blackish and triangular in shape while *E. dracunculoides* also has capsule with subglobose shape, 3-celled, seeds ovoid-terete and gray or dark gray (Khan et al., 2011). *R. graveolens* was previously authenticated from its adulterant *E. dracunculoides* based on unique bands using RAPD markers (Khan et al., 2011). However, authentication of a medicinal herb should not depend on single marker, but it should be based on a combined study with the aim of producing a reproducible marker.

In the present study, three gene spacer sequences, viz, ITS sequence of nrDNA, *rpoB* and *rpoCl*, were used for the authentication of *R. graveolens*, which is being sold under the vernacular name 'sudab' in the Indian herbal markets. However, *E. dracunculoides* was found to be an adulterant when later very carefully identified at the species level at the National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (voucher No. NISCAIR/RHMD/consult/-2007-08/937/121) (Khan et al., 2011). In the BLAST search, both samples showed a similarity with respective species or genus as nrDNA-ITS sequences

with a genuine sample of 'sudab', i.e., *R. graveolens* showed 99% similarity to the *R. graveolens* (accession No. EU591989) available in the GenBank®, whereas nrDNA-ITS sequences of adulterant sample showed 100% similarity with species of *Euphorbia* but did not show any similarity with genuine sample.

The chloroplast gene spacer sequences, viz, *rpoB* and *rpoC1*, amplified from *R. graveolens* showed a 96 and 99% similarity with *Citrus reticulata* (*rpoB2*) (accession No. EF590436) and *Ruta chalepensis* chloroplast genome (accession No. EU531646) in the BLAST search, respectively. Similarly, *rpoB* and *rpoC1* spacer sequences amplified from *E. dracunculoides* showed 95 and 98% similarity with the *Jatropha curcus* (accession No. FJ695500) and *Euphorbia lathyris* (accession No. GQ435927), respectively. However, the phylogenetic relationship of *E. dracunculoides* together with *Euphorbia* species available in the GenBank® has clearly shown its complete nesting within the *Euphorbia* tree based on nrDNA-ITS sequences.

All gene spacer sequences were aligned and analyzed from comparable regions between genuine as well as adulterant samples. The insertions/deletion (indels) was counted between *R. graveolens* and *E. dracunculoides* sequences for their discrimination. The nrDNA-ITS region from 1-613 bases was found to show 199 indels (Figure 1). However, chloroplast gene spacer sequences *rpoB* from 32-489 bases showed 55 insertions and *rpoC1* region from 17-504 bases showed 47 insertions alone (Figures 2 and 3).

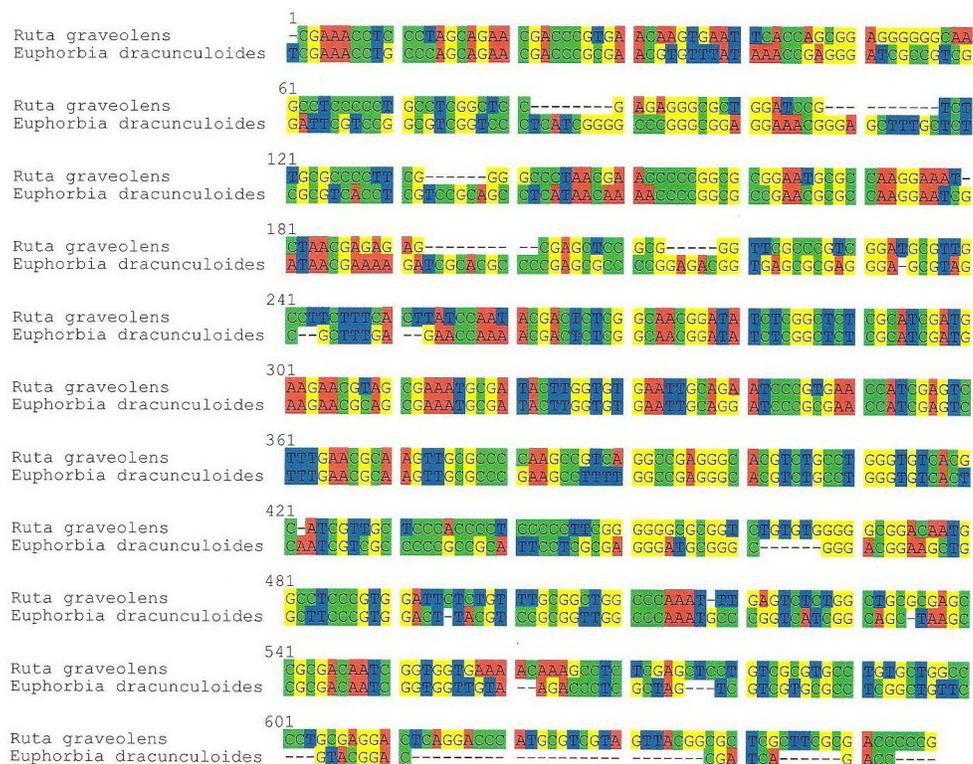


Figure 1. Complete alignment matrix of the internal transcribed spacer (ITS) gene sequences between *Ruta graveolens* and *Euphorbia dracunculoides*.

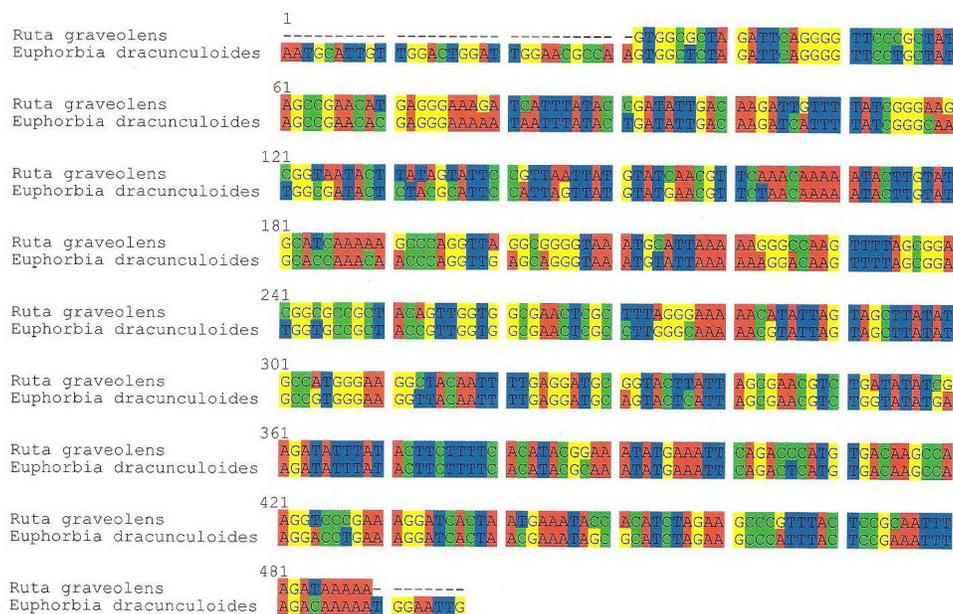


Figure 2. Complete alignment matrix of the chloroplast rpoB spacer sequence between *Ruta graveolens* and *Euphorbia dracunculoides*.

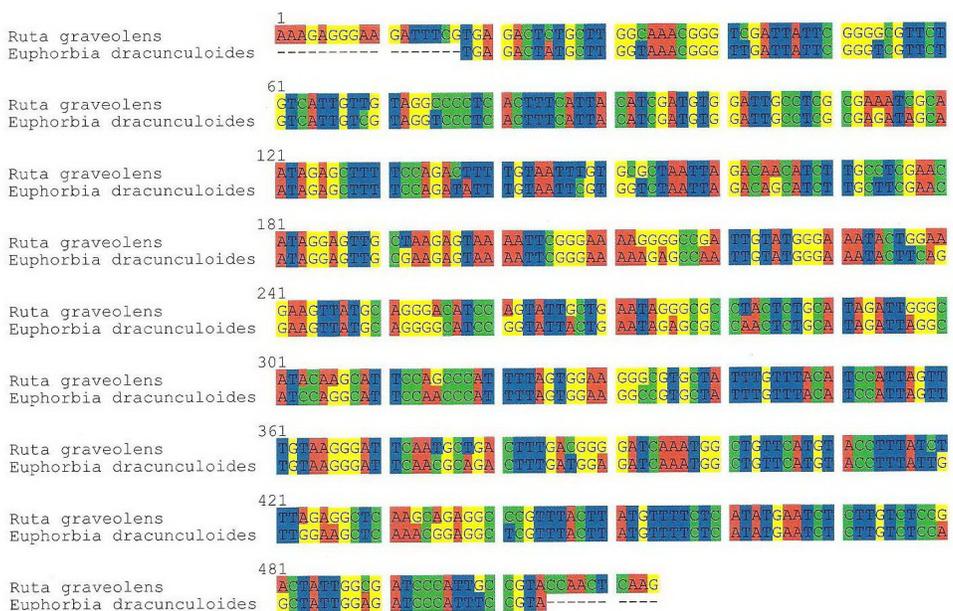


Figure 3. Complete alignment matrix of the chloroplast rpoC1 spacer sequence between *Ruta graveolens* and *Euphorbia dracunculoides*.

There was no deleted base found in *rpoB* and *rpoC1* spacer sequences, whereas nrDNA-ITS sequences of both genuine and adulterant samples had insertions as well as deletions. Thus, molecular authentication based on these spacer sequences, i.e., ITS, *rpoB* and *rpoC1*, may represent the best markers to differentiate the genuine as well as adulterant samples of sudab. Thus, in future nrDNA-ITS and chloroplast spacer sequences could be utilized for the accurate identification of these species from commercial herbal products, which ultimately would ensure the reproducibility and the efficacy of genuine herbal drugs. Furthermore, nrDNA-ITS sequences showed more sequence variation as compared to chloroplast spacer sequences. Therefore, an SCAR marker was developed to speed up the authentication process and further avoid the sequencing stage whenever applied to these market samples. It is a monolocus dominant marker and uses specific primers, which was designed from dissimilar sequences of nrDNA-ITS. The development of this marker is easy, fast and reliable, less sensitive to reaction conditions, and easy to perform in any laboratory (Kethidi et al., 2003). Therefore, results are more reliable with this marker as compared to other markers. Similarly, the three species of orchid including *Paphiopedilum armeniacum*, *P. micranthum* and *P. delenatii*, native to southwestern China and Vietnam are endangered and currently listed as Convention on International Trade in Endangered Species of Wild Fauna and Flora (Liu et al., 2006). The SCAR marker was developed for these species from nrDNA-ITS sequences, which clearly differentiated them having a similar morphology (Sun et al., 2011). The designed SCAR primer from nrDNA-ITS sequences was employed with genomic DNA of both samples, which led to amplification of sizes (289 and 264 bp) for a genuine sample and 424 bp for adulterant samples, respectively (Figure 4).

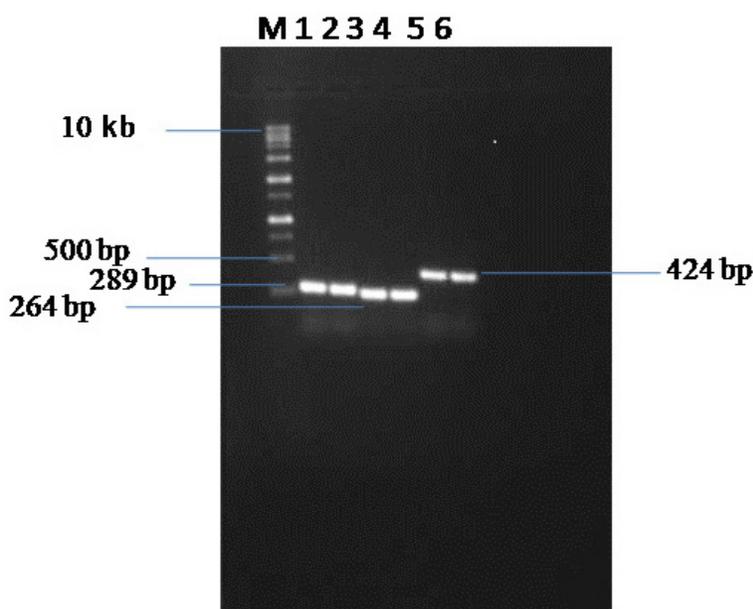


Figure 4. Sequence-characterized amplified region (SCAR) marker specific to *Ruta graveolens* amplified with SCAR289 (lanes 1 and 2) and SCAR264 primer (lanes 3 and 4). SCAR marker specific to *Euphorbia dracunculoides* amplified with SCAR424 primer (lanes 5 and 6).

The sensitivity of developed SCAR marker was detected at different concentrations of genomic DNA, which ranged from 5-25 ng/25 μ L PCR volume. As the concentration of genomic DNA decreased in the reaction mixture, the intensity of band also decreased (Figure 5A,B). This suggests that the DNA of above concentration, if present in the extracted genomic DNA of 'sudab', may have a limited amount of adulterant or totally replaced by adulterant samples. The developed SCAR marker will help in the authentication of these samples.

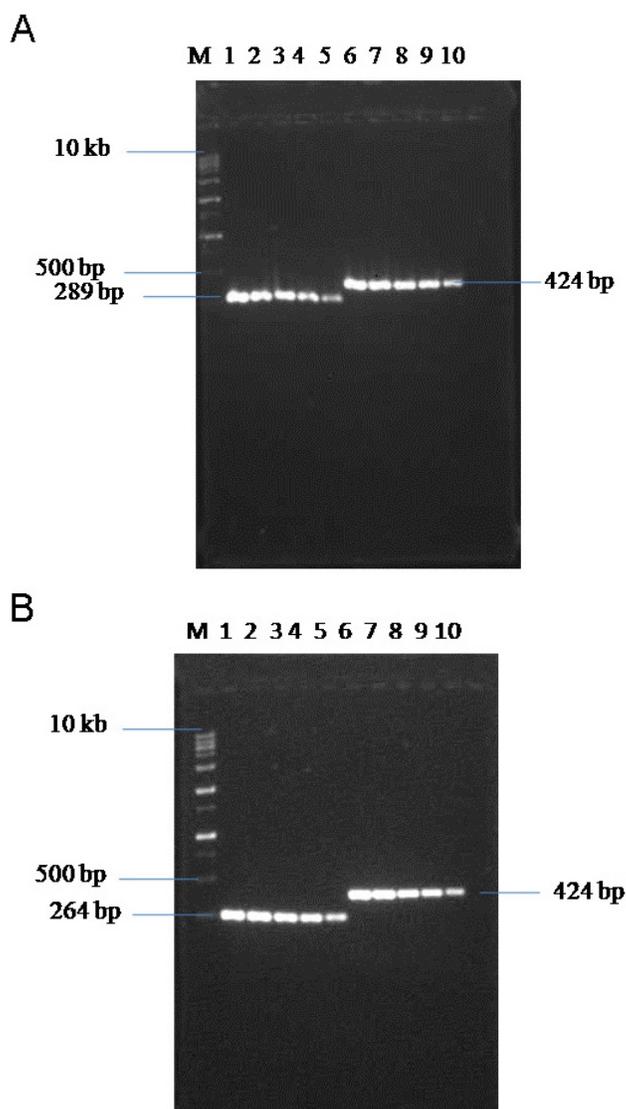


Figure 5. Detection of sequence-characterized amplified region (SCAR) marker sensitivity at various concentrations of genomic DNA. **A.** Amplification of genomic DNA of *Ruta graveolens* with SCAR289 Primer (lanes 1, 2, 3, 4, 5) and *Euphorbia dracunculoides* with SCAR 424 bp (lanes 6, 7, 8, 9, 10). **B.** Amplification of genomic DNA of *R. graveolens* with SCAR264 Primer (lanes 1, 2, 3, 4, 5) and *E. dracunculoides* with SCAR 424 bp (lanes 6, 7, 8, 9, 10).

Further, sequences of developed SCAR marker were BLAST on NCBI (<http://www.ncbi.nlm.nih.gov/>) and found exact respective plant species for which a marker was developed. The generated results in our study were considered to be reliable and have not been affected by physical form, physiological conditions of the plant samples and chemicals used in the study. However, compared with spacer sequences of chloroplast, the nrDNA-ITS region had more sequence variability. Therefore, ITS sequences of nrDNA show a wider prospect in authenticating the medicinal plants available in the herbal markets. The SCAR marker developed from nrDNA-ITS sequences has many advantages such as being easy to apply on dried old samples from which extracted genomic DNA may be sheared and/or obtained in small amounts. Nevertheless, DNA-based authentication of old and dried plant samples is a major challenge that is normally encountered in the raw drug trade. We isolated genomic DNA from dried powder form of genuine as well as adulterant samples and extracted genomic DNA was employed for PCR amplification (Khan et al., 2007). Once the SCAR marker is developed for such samples, which have high content of adulteration, it can be easily authenticated. Thus, the combined study of such DNA markers and further development of SCAR marker from dissimilar sequences of nrDNA-ITS can help in accurate authentication of 'sudab' sold in the market and can be applied on the other plant species, which have high morphological similarities.

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