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Development of RAPD Markers for Authentication of *Ruta graveolens* (L.) and its Adulterant

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ABSTRACT

Ruta graveolens (L.) is a small aromatic shrub and has been used, as medicinally and magically, since ancient times. In this study, RAPD (random amplified polymorphic DNA) was employed to develop reproducible markers under wide variation of conditions for authentication of *Ruta graveolens* L. from its adulterant *Euphorbia dracunculoides* L. 42 decamer oligonucleotide primers were screened for identification of genuine and adulterant samples using the DNA isolated from the dried leaf, seed and stem of both samples. Out of 42 primers used, of which 20 did not amplify, 10 gave faint fragment and 12 gave species-specific reproducible unique fragments, which could clearly distinguish genuine as well as adulterant samples having similar morphology. RAPD could thus, help to serve as a complementary tool for quality control.

Keywords: *Euphorbia dracunculoides*, herbal drugs, molecular marker, polymerase chain reaction

INTRODUCTION

Medicinal plants are well known for their reputed medicinal properties; however, most of them are empirically used in Indian system of medicine to cure several human ailments. Correct identification and quality assurance is indispensable to ensure reproducible medicinal quality of herbal drugs. Authentication is especially useful in case of those medicinal herbs that are frequently substituted or adulterated with other species or varieties which are morphologically and phytochemically indistinguishable (Kiran *et al.* 2010). Several herbal drugs on the market still cannot be identified or authenticated based on their morphological or histological characteristics. Use of a wrong herb may be ineffective or it may worsen the condition and may even cause death. In Belgium, the use of a traditional Chinese medicine (TCM) contaminated with plants from the *Aristolochia* species resulted in an epidemic of subacute intestinal nephropathy. Many of the affected patients required kidney transplantation. When 19 kidneys and urethras removed from 10 patients were examined histologically, neoplasms were detected in 40% (Cosyns *et al*. 1999). *Aristolochia* is now banned in UK but it is still widely available via the internet. A Google search carried out in early April generated no fewer than 86000 hits using the search term "*Aristolochia*" and 60,600 for "snakeroot", the common name.

Several plants of Rutaceae family are used in traditional medicine world-wide. The most common medicinal plant of this family is *Ruta graveolens* L., known as rue and native to Europe. It is an ornamental, aromatic, culinary and medicinal plant, and available all over the world. The rue shrubs are 0.6-0.9 m tall, robust, semi-woody and perennial. This species contains more than 120 compounds of different classes of natural products such as acridone alkaloids, coumarins, essential oils, flavonoids and furoquinolines. The plant is widely being used for medicinal purpose in various clinical conditions from very ancient time but rationality of its use is still controversial. In homeopathy, rue is an important remedy for deep aching pain and rheumatism besides being used for eyestrain-induced headache (San Miguel 2003). It has also been used as a remedy for gastric disorders, stiff neck, dizziness, headache and so on (Conway and Slocumb 1979). Besides this, plant is widely used as sedative and antihelmintic (Skidmore-Roth 2001), hypotensive (Chiu and Fung 1997; Trovato *et al*. 2000), anti-inflammatory, antiviral, and antiplasmodial activity (Yamamoto *et al*. 1989; Queener *et al*. 1991; Raghav *et al*. 2006), antimicrobial, cytotoxic (Ivanova *et al*. 2005), fungicide (Oliva *et al*. 2003; Meepagala *et al*. 2005), herbicide (Hale *et al*. 2004), and contraceptive (Maurya *et al*. 2004). Its potent female antifertility and abortive effects have been reported from countries like Brazil (de Freitas *et al*. 2005), India (Gandhi *et al*. 1991), Peru (Gutierrez-Pajares *et al*. 2003) and Mexico (Conway and Slocumb 1979). The other species of different genus with similar morphology such as *Euphorbia dracunculoides* L. is being sold in local markets or used clinically as a replacement of *Ruta graveolens* L. at a relatively reduced cost under the name of suddaba (Rahman *et al.* 2003). This may results in compromising the therapeutic value. The adverse effect of *E. dracunculoides* was studied as epistaxis, nausea/vomiting and haematuria respectively (Rahman *et al*. 2003). Limitations of chemical and morphological markers for authentication have generated a need for newer methods in quality control of *R. graveolens.* DNA-based molecular markers however, are important tools in quality assurance and preservation of germplasm of medicinal plant species. Genuine sample specific markers are needed to maintain the quality of this medicinal plant for herbal formulations. Our major objective therefore, was to develop a DNA-based molecular tool for accurate identification of *R. graveolens* in local markets to differentiate and authenticate *R. graveolens* and *E. dracunculoides*.

MATERIALS AND METHODS

A genuine sample of *R. graveolens* was provided by the Central Council Research for Unani Medicine (CCRUM) Hyderabad. The samples for authentication were purchased from local markets of Khari Baobli, Delhi, India. The material was identified at National Institute of Science Communication and Information (NISCAIR)

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Table 1 The unique amplicons specific to *R. graveolens* and *E. dracunculoides* samples with 12 decamer oligonucleotide primers obtained after PCR amplification.

Name of plant species	Fig. 2				Fig. 3				Fig. 4			
	Size of unique fragments (bp)				Size of unique fragments (bp)				Size of unique fragments (bp)			
	P-11	P-12	P-13	P-14	OPC-1	OPC-2	OPC-3	OPC-4	OPC-5	OPC-6	OPC-7	OPC-8
R. graveolens	2400, 2200	1200, 800, 550	1800, 1300, 800	2300, 1300, 900	2400, 2000, 1800, 1500, 1400	2500, 1900, 1400, 1000	2800, 1200	2400, 1200, 450	2500, 1900, 1500	2000, 1100, 900, 850	1900, 1600	2600, 2200, 2000, 1200
E. dracunculoides	1200	650	-	1600	600	-	950	-	-	-	-	-
Total no of unique fragments/primer	3	4	3	4	6	4	3	3	3	4	2	4

by Dr. H. B. Singh and voucher (NISCAIR/RHMD/consult/-2007-08/937/121) was kept in Herbarium. Leaf powder (100 mg) was used for DNA extraction in accordance with modified CTAB method (Khan *et al*. 2007). The total genomic DNA was used in RAPD-PCR to develop fingerprints for the authentication of genuine as well as adulterant samples.

Reagents and chemicals

The stock solution concentration were: CTAB 3% (w/v), 1 M Tris-Cl (pH 8), 0.5 M EDTA (pH 8), 5 M NaCl, absolute ethanol (AR grade), chloroform-IAA (24:1 [v/v]), polyvinylpyrrolidone (PVP) (40 000 mol wt) (Sigma) and β-mercaptoethanol. All the chemicals used in the experiments were of analytical grade. The extraction buffer consisted of CTAB 3% (w/v), 100 mM Tris-Cl (pH 8), 25 mM EDTA (pH 8), and 2 M NaCl, respectively. PVP and β-mercaptoethanol were added freshly prepared.

RAPD analysis

RAPD reaction was performed according to the method developed by McClelland *et al*. (1995). The total volume of reaction was performed in 25 µl. PCR reaction for RAPD analysis consisted of 15 mM $MgCl_2$, 2.5 µl 10X buffer, 2 µl 2 mM dNTPs (mix), 1.5 U *Taq* polymerase in buffer, 25 ηg/µl of each primer, 30 ηg/µl plant DNA and sterile water up to 18 µl. All reagents were supplied by Bengalore Genei Pvt. Ltd. (Bangalore, India) except for primers, which were supplied by Genetix Biotech Asia Ltd. (Bangalore, India). For DNA amplification a Techne thermal cycler was programmed for 1 cycle of 3 min at 94°C, 30 s at 35.5°C, and 1 min at 72°C followed by 45 cycles of 1 min at 94°C, 30 s at 35.5, and 1 min at 72°C, then terminating with 5 min at 72°C. The RAPD fragments were separated on 1.2% agarose gel by electrophoresis in 1X TAE buffer and stained with ethidium bromide.

RESULTS

R. graveolens was chosen to test the reliability of quality control using RAPD technique. In the local market samples, *E. dracunculoides* was found as adulterant when later identified at NISCAIR, New Delhi (voucher no, NISCAIR/RHMD/consult/-2007-08/937/121). The dried leaf, fruit and stem of *E. dracunculoides* is more or less similar in morphology with those of *R. graveolens* (**Fig. 1**). The RAPD technique carried out in replicate on both plants for reproducibility of this technique using 42 decamer primers. Of the 42 primers, 12 generated reproducible, unique, monomorphic and polymorphic fragments in PCR amplification using the genomic DNA extracted from leaf, seed and stem, respectively. The genuine and adulterant samples were discriminated by the presence or absence of unique fragments in RAPD profile. The RAPD profile generated from genomic DNA isolated from dried leaf, fruit and stem was same with all decamer primers. The total number of unique fragments specific to genuine as well as adulterant samples with different primers is summarized in **Figs. 2-4** and **Table 1**.

DISCUSSION

Among the genera of Ruteae, *R. graveolens* is characterized by strong-smelling of ethereal oils in its leaves, slightly toxic and bitter in taste (Townsend 1968; Chiej 1984). The bruised leaves have a pleasant orange-like fragrance (Genders 1994). Since, dried parts of this scented plants such as leaf, fruit and stem are used in herbal formulations, and morphological characteristics of dried parts of both plants are more or less similar, and difficult to discriminate by conventional methods (**Fig. 1**) and thus, *E. dracunculoides* easily adulterated under by name 'suddaba' with *R. graveolens* in local herbal markets (Rahaman *et al.* 2003). The leaves of *R. graveolens* are bipinnate or tripinnate and obviate-oblong. The fruit is capsule globosely with 4-5 lobed containing numerous seeds having tiny size, blackish and triangular in shape. The leaves of *E. dracunculoides* are lanceolate or linear oblong, subacute, base rarely rounded or sub-cordate; odour and taste not distinct. The fruit is capsule with subglobose shape, 3-celled with or without an attached pedicel, smooth or obscurely reticulate and glabrous. Seeds are ovoid-terete and gray or dark gray respectively. These taxonomical characteristics sometimes deviate for precise authentication of these medicinal plants which have more or less similarity in morphology.

In our study, 12 decamer primers clearly discriminated genuine and adulterant samples by unique fragments generated in RAPD reaction. Each fragment in RAPD is derived from a region of the genome that contains two short segments in inverted orientation on opposite strands that are complementary to the primer and sufficiently close together for the amplification to work (Hon *et al*. 2003). The primer OPC-1 amplified more unique fragments (2400, 2000, 1800, 1500 and 1400 bp) than the other primers used in the study (**Table 1**). These unique fragments act as markers for their authentication as well as for future study. The single primer in our study was found to discriminate the genuine as well as adulterant sample. The medicinal plant *R. graveolens* has different established medicinal effects, different from *E. dracunculoides* both according to their traditional usage and in animal studies. The adulteration of *R. graveolens* with *E. dracunculoides* in herbal markets reduces the medicinal efficacy of this scented medicinal plant. The RAPD analysis has been widely used for differentiation of a large number of medicinal species from their close relatives or adulterants, including *Panax* species (Shaw and But 1995), *Coptis* species (Cheng *et al.* 1997), *Astragalus* species (Cheng *et al*. 2000), *Lycium barbarum* (Cheng *et al.* 2000), *Panax ginseng* species (Um *et al.* 2001), *Echinacea* species (Nieri *et al.* 2003), turmeric (Sasikumar *et al.* 2004), *Astragali radix* (Na *et al.* 2004), *Dendrobium officinale* (Ding *et al.* 2005), *Typhonium* species (Acharya *et al.* 2005), *Dendrobium* species and its products (Zhang *et al.* 2005), *Tinospora cordifolia* (Wild.) Miers ex Hook & Thomas (Rout 2006), *Mimosae tenuiflorae cortex* (Rivera-Arce *et al.* 2007), *Rahmannia glutinosa* cultivars and varieties (Qi *et al.* 2008), *Desmodium* species (Irshad *et al.* 2009), *Piper nigrum* (Khan *et al*. 2010) and *Cuscuta reflexa* and *Cuscuta chinensis* (Khan *et al*. 2010). The RAPD technique has been used for deter-

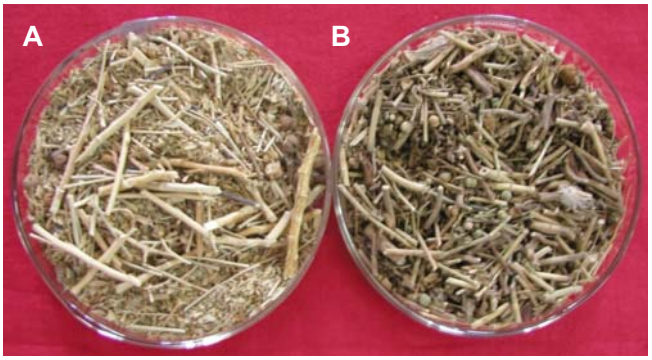

Fig. 1 Morphological slides of genuine and adulterated samples. (**A**) Genuine samples of *Ruta graveolens* (L.) provided by the Central Council for Research in Unani Medicine (CCRUM) Hyderabad. (**B**) Adulterated samples of *Ruta graveolens* (L.) with *Euphorbia dracunculoides* (L.) purchased from Khari Baoli, New Delhi, India.

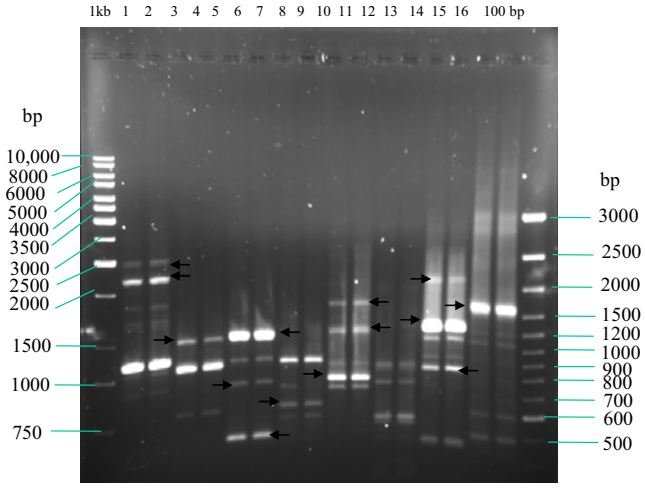

Fig. 2 RAPD analysis carried out with primers (P). P-11 (lanes 1-4), P-12 (lines 5-8), P-13 (lanes 9-12), P-14 (lanes 13-16) on genomic DNA extracted from leaf of *Ruta graveolens* L. (lanes 1, 2, 5, 6, 9, 10, 13, 14) and *Euphorbia dracunculoids* L. (lanes 3, 4, 7, 8, 11, 12, 15, 16). 1 kb and 100 bp are DNA ladders.

mination of the components in an Ayurvedic herbal prescription, Rasayana Churna to identify three Ayurvedic medicines, dried stem of *Tinospora cordifolia*, dried fruit of *Emblica officinalis* and dried fruit of *Tribulus terestris* (Shinde *et al.* 2007). The marker with 600 bp is specific to *Tinospora cordifolia*; the marker 500 bp is specific to *Emblica officinalis* and the remaining marker >1000 bp was present in *Tribulus terestris* (Shinde *et al.* 2007). The medicinal species of *Selaginella* and variation of the same species from different habitats was authenticated by RAPD analysis (Li *et al.* 2007). The roots of *Glycirrhiza glabra* is adulterated with *Abrus precatorious* (adulterant) in local market samples, a RAPD marker was developed for authentication of genuine sample of *G. glabra* from its adulterant (Khan *et al*. 2009). The advantages of RAPD techniques include their simplicity, rapidity, the low amount of genomic DNA required and the fact that isotopes and prior genetic information are not required (Micheli *et al.* 1994). Our RAPD marker proved to be extremely reproducible under a wide variation of amplification conditions; it is clearly visible up to an annealing temperature of 38ºC (data not shown), and changes in the origin of the primer, the *Taq* polymerase and the thermal cycler used do not affect the result. There were numerous unique fragments found which were specific to genuine as well as adulterant samples in RAPD-PCR. In this study, we suggest that RAPD method is convenient for identifying *Ruta graveolens* (L.) and its adulterant *Euphorbia. dracunculoides* (L.) in the local markets.

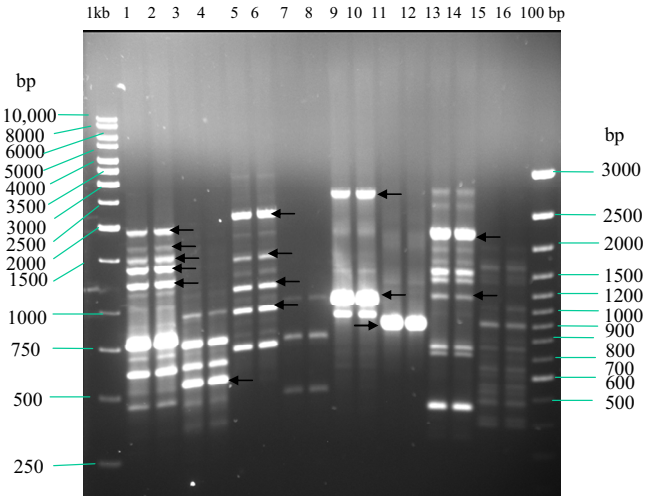

Fig. 3 RAPD analysis carried out with several primers. OPC-1 (lanes 1-4), OPC-2 (lanes 5-8), OPC-3 (lanes 9-12), OPC-4 (lanes 13-16) on genomic DNA extracted from leaf of *Ruta graveolens* L. (lanes 1, 2, 5, 6, 9, 10, 13, 14) and *Euphorbia dracunculoids* L. (lanes 3, 4, 7, 8, 11, 12, 15, 16). 1 kb and 100 bp are DNA ladders.

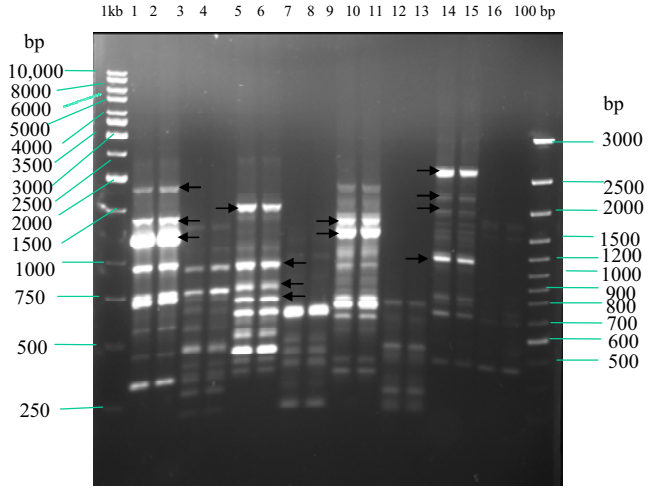

Fig. 4 RAPD analysis carried out with several primers. OPC-11 (lanes 1-4), OPC-12 (lanes 5-8), OPC-13 (lanes 9-12), OPC-14 (lanes 13-16) on genomic DNA extracted from leaf of *Ruta graveolens* L. (lanes 1, 2, 5, 6, 9, 10, 13, 14) and *Euphorbia dracunculoids* L. (lanes 3, 4, 7, 8, 11, 12, 15, 16). 1 kb and 100 bp are DNA ladders.

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