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MOLECULAR AUTHENTICATION AND QUALITY CONTROL OF *CROCUS SATIVUS* AND *ALOE BARBADENSIS* IN RAW MATERIAL SOURCE AND POLYHERBAL MEDICINE EMPLOYING SCAR MARKERS

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Abstract

Crocus sativus L. and *Aloe barbadensis* Mill. are well known functional ingredients in health and food products and are known for their medicinal properties as antioxidant, antidepressant, relaxant and cathartic agents. In this study, reliable quality control markers were developed for the quality assurance of *C. sativus* and *A. barbadensis* at raw material source as well as in finished herbal products. DNA based sequence-characterized amplified region (SCAR) markers were developed from randomly amplified polymorphic DNA (RAPD) markers specific for both the species to detect its adulteration in commercial products. Developed RAPD markers were cloned and sequenced and SCAR primers were generated SCAR markers upon PCR amplification with genomic DNA. These specific SCAR markers enabled unequivocal detection of adulterants in the commercially procured polyherbal medicine. *C. sativus* could not be identified through SCAR markers, instead SCAR amplicon of *Carthamus tinctorius* was detected suggesting that although being labelled as one of the constituents, *C. sativus* may not have been used to prepare the polyherbal medicine. This simple analytical strategy could be used for large scale screening of medicinal plants at raw material source as well as in finished polyherbal medicine.

Key words: *Aloe barbadensis*; *Crocus sativus*; Adulteration; Quality control; Molecular markers; SCAR markers.

Introduction

The practice and use of indigenous medicines is as old as our civilization. According to WHO, traditional medicines are the mainstay of about 75-80% of the world population in developing countries and have risen worldwide (Anon., 2008). Molecular technology is increasingly becoming popular as a potent tool for unambiguous identification of medicinal plants at raw material source as well as in medicinal products. This is a critical step for quality assurance and remains an important health and economic issue. The increased demand for medicinal plants is due to their natural origin. The modern notion, particularly in the western world, that natural is better than chemical has resulted in the development of 'neo-western herbalism' (Elvin-Lewis, 2001). However, lack of standardization and verification has led to adulteration and counterfeit products. Consequently, not only the efficacy of these products has been reduced, but also the adulteration and substitution has led to adverse reactions that are sometimes life-threatening or lethal (Khan *et al*., 2009).

The traditional medicine, Habb-e-Mudir used in this study is a Unani polyherbal formulation and in powdered form comprises of *C. sativus* (dried stigma) and *A. barbadensis* (dried leaf latex) (Khare, 2003). The powder is then made into pills, thereafter, used as natural health product used for the treatment of amenorrhoea and dysmenorrhoea. *C. sativus* L. commonly known as saffron is one of the most valuable spice in the food and flavour sector which is obtained from dried stigma of its flowers. The worldwide market of saffron is worth nearly $1 billion and approximately 300 tons were globally produced in 2007 (Ozturk *et al*., 2011; Marieschi *et al*., 2012). Saffron also has significant medicinal properties. In traditional medicine, *C. sativus* and its extracts are used as antispasmodic, antidepressant, expectorant and as emmenagogue (Bhargava, 2011). Several pharmacological studies have demonstrated that *C. sativus* extracts have antitumor (Abdullaev, 2002; Aung *et al*., 2007; Gutheil *et al*., 2012), antioxidant (Soeda *et al*., 2007), cardioprotective (Goyal *et al*., 2010; Imenshahidi *et al*., 2010) properties. Some studies have shown beneficial effects of *C. sativus* in primary dysmenorrhoea (Nahid *et al*., 2009) and premenstrual syndrome (Agha-hosseini *et al*., 2008). As a consequence of its high market value, *C. sativus* has been frequently adulterated with readily available and cheaper bulking agent, *Carthamus tinctorius* L., commonly known as safflower, due to their similar morphological features (Marieschi *et al*., 2012). *Aloe barbadensis* Mill. commonly known as *Aloe vera*, has been extensively used as cathartic agent in indigenous systems of medicines. *A. barbadensis* extracts have shown antioxidant activity (Hu *et al*., 2003), anti-inflammatory (Hutter *et al*., 1996) and anti diabetic properties (Tanaka *et al*., 2006). It is one of the many food products that can be considered as new food or new food ingredient (Rodriguez *et al*., 2010). In the market samples, it is sometimes adulterated with *Senegalia catechu* (L. f.) Willd (Anon., 1999).

Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers. Polymerase chain reaction (PCR)-based genetic markers are widely used for molecular detection, genome mapping, map-based cloning, and analysis of genetic variation (Kathidi *et al*., 2003). Recently, various DNA-based molecular markers viz. RFLP, AFLP and RAPD have been successfully developed and used for authentication of samples of the plant based food and herbal ingredients from the local markets (Ganie *et al*., 2012; Lee *et al*., 2006). RAPD analysis could reveal high degree of polymorphism. However, due to low reproducibility and lower annealing temperature, this marker is converted into more stable and reliable sequence characterised amplified region (SCAR) marker.

SCARs are based on sequencing the polymorphic fragment derived from RAPD primers and designing longer primers that will specifically bind to this fragment. SCAR markers usually detect only a single locus and are, therefore, more specific and have high reproducibility and cost effectiveness. The knowledge of the whole genomic sequence is also not required in the development of SCAR markers. Hence, they are preferred over other molecular markers.

The present study was undertaken to develop molecular tools for accurate identification and authentication of polyherbal medicines. The specific objective of this study was to develop species-specific Sequence Characterised Amplified Region marker for *C. sativus* and *A. barbadensis* as well as their adulterants, *C. tinctorius* and *S. catechu*, and to authenticate laboratory prepared and commercially procured polyherbal medicine, Habb-e-Mudir.

Materials and Methods

Plant material and DNA extraction: Habb-e-Mudir, was procured from Balli Maran market, Delhi, India. The genuine sample of *C. sativus* was obtained from Pampore, Jammu and Kashmir, while that of *A. barbadensis* was obtained from herbal garden, Jamia Hamdard, New Delhi, India. Their adulterants, *C. tinctorius* and *S. catechu* were obtained from Khari Boali, Delhi, India. These samples were identified and authenticated at NISCAIR, New Delhi and voucher specimens were deposited (niscair/rhmd/Consult/-2014/2543/122-1-2). The genomic DNAs from the powdered samples of herbal constituents as well as their adulterants were isolated by modified CTAB method (Khan *et al*., 2007). The pills of polyherbal product, Habb-e-Mudir were thereafter prepared in our laboratory from the genuine herbal constituents as per standard operating procedures (SOPs) of Unani pharmacopeia. The DNAs from the samples of laboratory prepared and commercially obtained polyherbal medicine were also isolated using modified CTAB method (Khan *et al*., 2007).

RAPD analysis: The isolated genomic DNAs of the herbal constituents and their adulterants were subjected to RAPD analysis (William *et al*., 1990). PCR amplification was carried out using random primers of OPK series (Operon Technologies, USA). Amplification was performed with a reaction volume of 25µl containing 2 µL of DNA template (15 ng/µL), 2 µL of dNTPs mix (2.5 mM each), 0.5µL Taq DNA polymerase (3U/ µL), 2.5µL reaction buffer(10 X) and 2 µL Primer set (15 ng/µL). Final volume of the reaction mixture was made up to 25 µL by distilled water. RAPD was performed in an Arktik thermal cycler (Thermo Scientific, USA) with 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, and extension at 72°C for 1 min. The samples were subjected to a final extension at 72°C for 3 min. PCR products were separated by gel electrophoresis (1.2%). Electrophoretic profile was visualized and photographed under UV transilluminator. Analysis of electrophoretic profile for polymorphism was based on presence and absence of DNA bands on agarose gel.

Conversion of RAPD amplicon to the SCAR marker: The unique RAPD amplicons of *C. sativus* and *A. barbadensis* and their adulterants *C. tinctorius* and *S. catechu*, respectively were excised from agarose gel and eluted by gel extraction kit (Fermentas, USA). These purified amplicons were ligated into p-GEMT easy vector and transformed into competent E. coli (DH5α) cells. After plating onto Luria-agar medium supplemented with ampicilin, IPTG and X-gal, the transformed cells were identified by blue-white screening. The presence of inserts was confirmed by restriction digestion of the isolated plasmid DNA with EcoR1. Sequencing of the cloned amplicons was performed by automated sequencer using T7 forward and SP6 reverse primer sets (Bangalore Genei, India).

The nucleotide sequences of RAPD amplicons were used for similarity searches against non-redundant database using basic local alignment search tool program (NCBI BLAST). RAPD amplicons were used to design specific internal primers (SCAR primers) using the NCBI primer3 tool. PCR conditions for amplification of DNA samples of the herbal plants using their specific primers were optimized (Table 1). The amplification products obtained were resolved on 1.2 pecent agarose gel and visualized as well as documented using Gel Doc system (ProteinSimple, USA).

SCAR marker amplification in complex mixtures of DNAs from the polyherbal medicine, Habb-e-Mudir: Complex mixtures of DNA isolated from the commercially procured polyherbal medicine, Habb-e-Mudir were subjected to PCR assay using the designed SCAR primers to authenticate these market samples. Similarly, complex mixtures of DNA isolated from the polyherbal medicine prepared in our laboratory using authenticated genuine herbal constituents were amplified using designed SCAR primers of *C. sativus* and *A. barbadensis* (positive control) and their adulterants, *C. tinctorius* and *S. catechu* (negative control). The conditions were optimized as mentioned in Table 1. The amplified products were resolved on 1.2 pecent agarose gel and visualized as well as documented using Gel Doc system (ProteinSimple, USA)

Table 1. PCR conditions for the development of SCAR markers.

Cycles	Denaturation		Annealing					Polymerization	
	Temperature (°C)	Time (min)	Temperature (°C)				Time (s)	Temperature (°C)	Time (min)
			C. sativus	*C. tinctorius*	*A. barbadensis*	*S. catechu*			
First cycle	94	3	-	-	-	-	-	-	-
30 Cycles	94	1	55.6	54.8	58	59	30	72	**1**
Last cycle	-	-	-	-	-	-	-	**72**	**3**

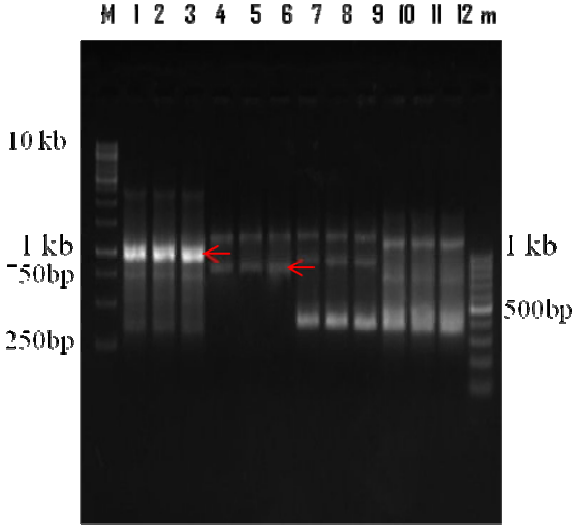

Fig. 1. RAPD profile of *Crocus sativus* (C1) and *Carthamus tinctorius* (C2) with OPK- 02(lane 1- 6), OPK-04 (lane 7-12). Pattern of amplification in *C. sativus* is shown in lanes 1,2,3,7,8,9 and *C.tinctorius* in lanes 4,5,6,10,11,12. Unique bands are marked with red arrows: lanes 1-3 (C1-989bp) and lanes 4-6 (C2-880bp). Lane M: 1kb marker & lane m: 100bp marker.

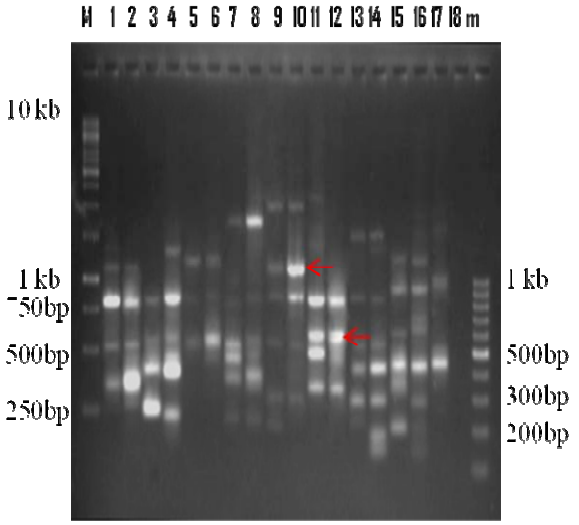

Fig. 2. RAPD profile of *Aloe barbadensis* (A1) and *Senegalia catechu* (A2) with OPK-02(lane 1- 4), OPK-04 (lane 5-8), OPK-14 (lane 9-12), OPK-16 (lane13-16), OPK-18(lane17-18). Pattern of amplification in *S. catechu* is shown in lanes 1, 2, 5, 6, 9, 10, 13, 14, 17 and *A. barbadensis* in lanes 3, 4, 7, 8, 11, 12, 15, 16, 18. Unique bands are marked with red arrows: lanes 9 & 10 (A2-1100bp) and lanes 11&12 (A1-600bp). Lane M: 1kb marker & lane m: 100bp marker.

Results

DNA isolation and RAPD: High molecular weight genomic DNA was isolated from *C. sativus* and *A. barbadensis* as well as their adulterants *C. tinctorius* and *S. catechu*. The yield of DNA was 169ng/µl and 163ng/µl in case of *C. sativus* and *A. barbadensis,* respectively, while amount of DNA isolated from *C. tinctorius* and *S. catechu* was 168ng/µl and 160ng/µl, respectively. The quality and quantity of the isolated DNA, determined by taking OD at 260/280, ranged from 1.74-1.76. Twenty random oligonucleotide primers were used for RAPD analysis of genomic DNA samples of *C. sativus* and *A. barbadensis* as well as their adulterants *C. tinctorius* and *S. catechu*. Out of 20, only 11 primers showed polymorphism. However, highest degree of well reproducible polymorphic bands were observed with only five primers (OPK-02, 04, 14, 16, and 18). Out of these primers, OPK-02 produced unique amplicons of size 989bp and 880 bp in *C. sativus* and *C. tinctorius*, respectively (Fig. 1); whereas, primer OPK-14 generated unique amplicons of 600bp and 1100bp in *A. barbadensis* and *S. catechu*, respectively (Fig. 2).

SCAR marker development: BLAST analysis revealed that the nucleotide sequences of the unique amplicons have no similarity with the sequences present in the database. These unique sequences of *C. sativus, C. tinctorius, A. barbadensis* and *S. catechu* were thereafter, submitted to the genBank (Accession numbers KU297238, KU297239, KU297240, KU297241, respectively).

Based on the characterized sequences, SCAR primers for *C. sativus, A. barbadensis*, *C. tinctorius* and *S. catechu* were designed and are given in Table 2. SCAR primers of *C. sativus* yielded 900 bp amplicon with genomic DNA of *C. sativus* (Fig. 3) while *C. tinctorius* gave 800 bp SCAR marker upon amplification with its SCAR primer (Fig. 4). Similarly, amplification products of 550bp in *A. barbadensis* and 1000bp in *S. catechu* were detected, when their genomic DNAs were amplified with their SCAR primers (Figs. 5 and 6).

Table 2. SCAR primers used for SCAR marker development.

SCAR primer	Sequence
Crocus sativus forward primer	5'- GCAACCGCCTGATTGGGCAG-3'
Crocus sativus reverse primer	5'- AGTGCTAATGAAATCATGCA-3'
Aloe barbadensis forward primer	5'-GTGCGACCGCCTTGTTATGCA-3'
Aloe barbadensis reverse primer	5'- AGCCCGACGGCGATGATGAA-3'
Carthamus tinctorius forward primer	5'-CATGCCATTTAAGAGTGATGC-3'
Carthamus tinctorius reverse primer	5'- GTTCTAAGAGCCGTGAAATA-3'
Senegalia catechu forward primer	5'- GAACGACTCTCAGGTCAGGC-3'
Senegalia catechu reverse primer	5'- ATAGCCCAAATAGATTACAA-3'

Authentication of polyherbal medicine: The amplification of DNA sample extracted from genuine polyherbal medicine prepared in the laboratory with SCAR primers of *C. sativus* and *A. barbadensis* produced SCAR amplicons of 900 bp and 550bp, respectively (positive control) (Fig. 7a), while SCAR primers of *C. tinctorius* and *S. catechu* gave no amplification (negative control) (Fig. 7b). Further, these SCAR primers were used to authenticate the market sample of the herbal product. Amplification product of 550bp corresponding to *A. barbadensis* was detected while no amplification was observed in *C. sativus* (Fig. 8a). Instead, SCAR amplicon of size 800bp corresponding to *C. tinctorius* was detected. Amplification with SCAR primers of *S. catechu* has not given amplification for its corresponding SCAR marker (Fig. 8b).

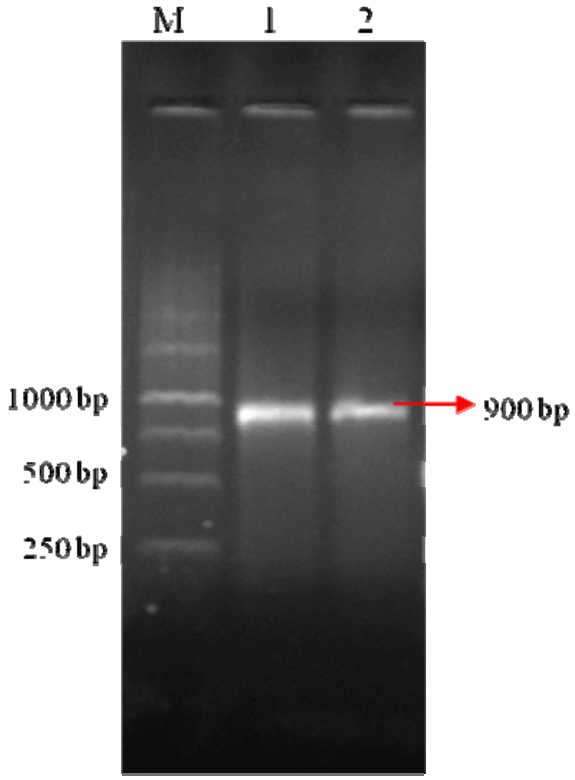

Fig. 3. Genomic DNA sample of *Crocus sativus* amplified using SCAR primer specific to *C. sativus.*

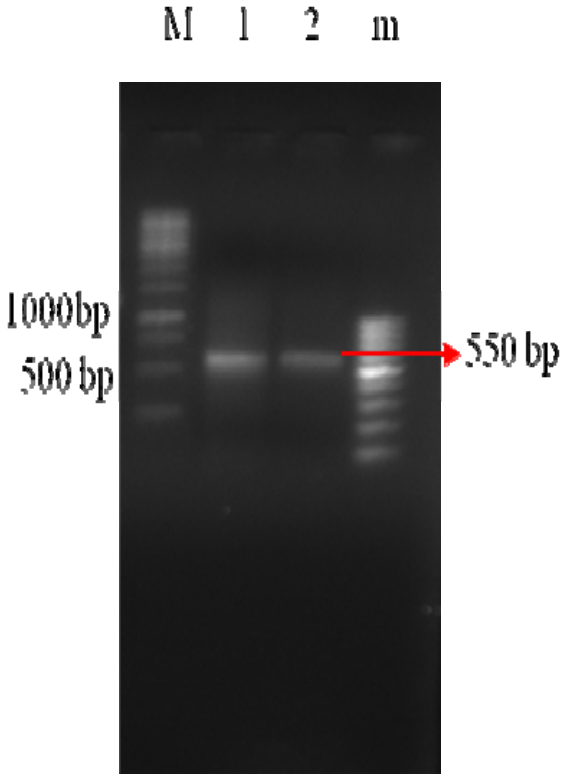

Fig. 5. Genomic DNA sample of *Aloe barbadensis* amplified using SCAR primer specific to *A. barbadensis.*

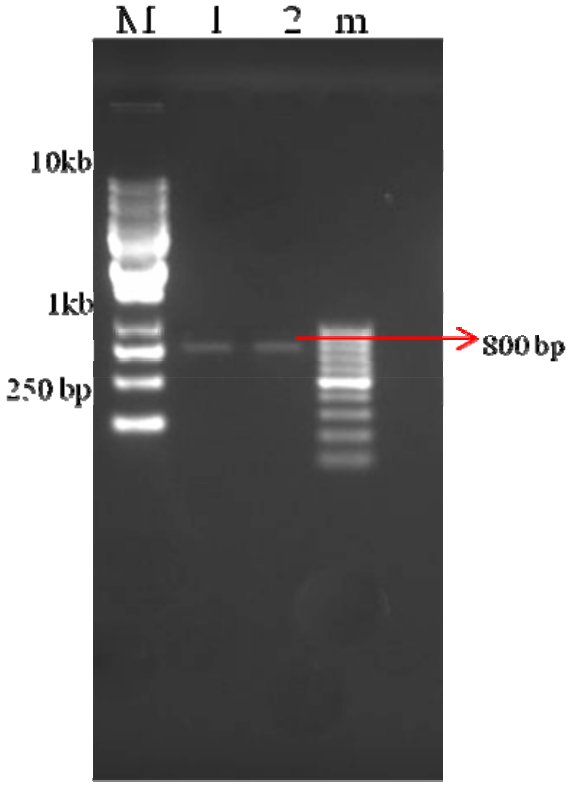

Fig. 4. Genomic DNA sample of *Carthamus tinctorius* amplified using SCAR primer specific to *C. tinctorius.*

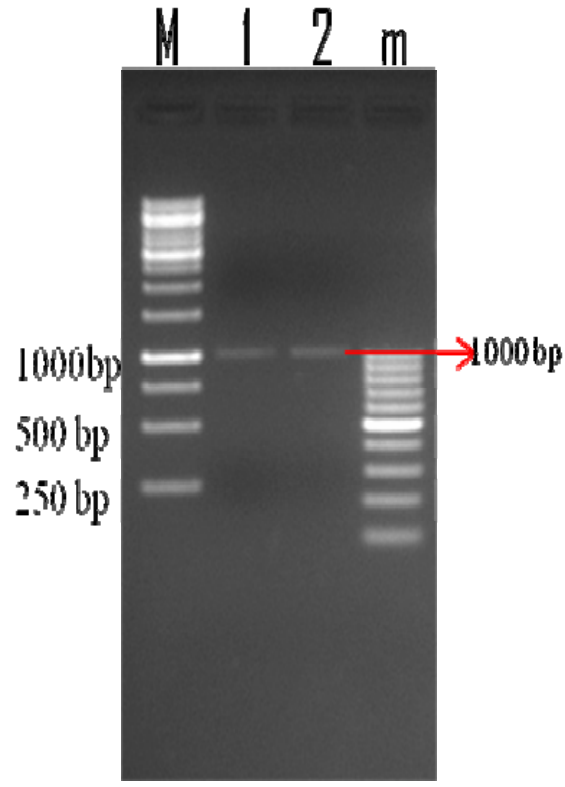

Fig. 6. Genomic DNA sample of *Senegalia catechu* amplified using SCAR primer specific to *S. catechu.*

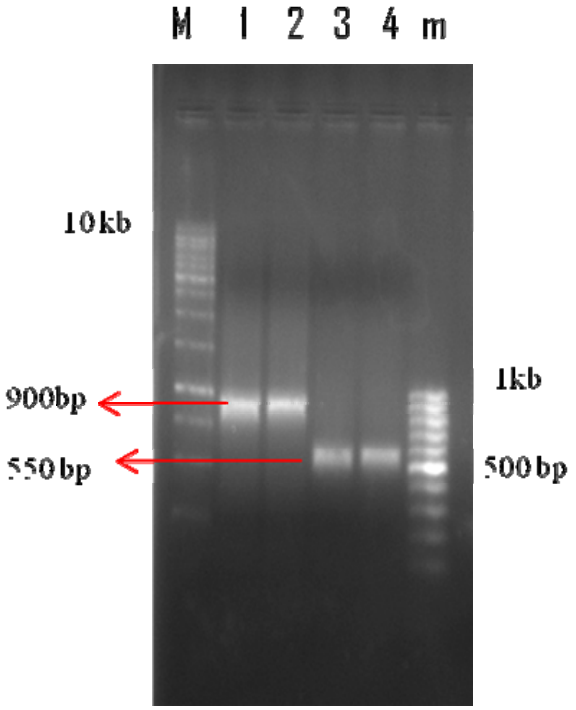

Fig. 7a. Genomic DNA from the herbal product (prepared in lab) amplified using SCAR primer specific to *Aloe barbadensis* and *Crocus sativus* (Positive control). Amplification was observed with SCAR primer of *C. sativus* (lanes 1-2) as well as with SCAR primer of *A. barbadensis* (lanes 3-4).

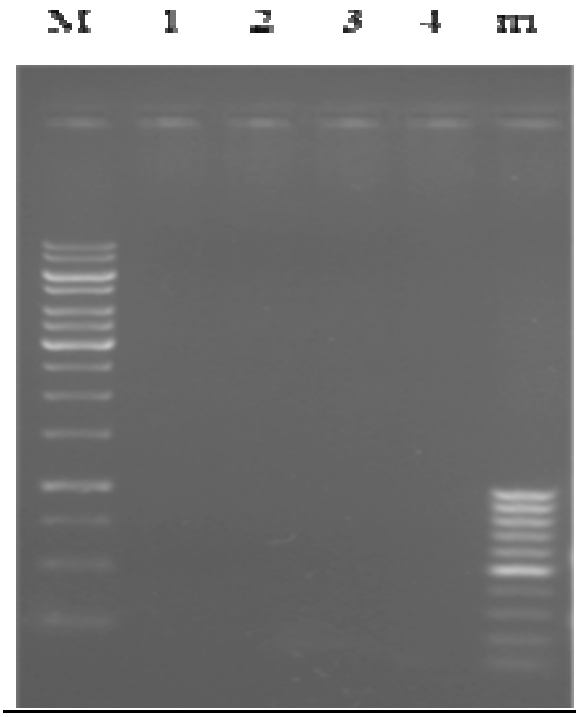

Fig. 7b. Genomic DNA from the herbal product (prepared in lab) amplified using SCAR primer specific to *Carthamus tinctorius* and *Senegalia catechu* (Negative control). No amplification was observed with SCAR primer of *C. tinctorius* (lanes 1-2) as well as with SCAR primer of *S. catechu* (lanes 3-4).

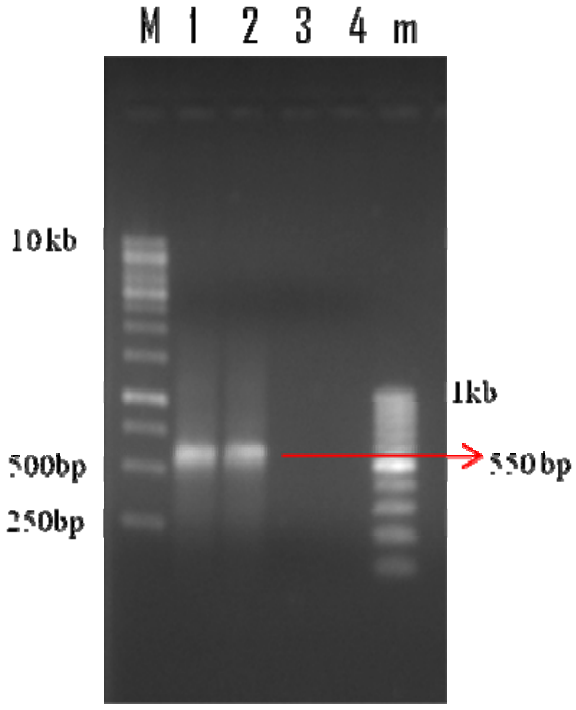

Fig. 8a. Genomic DNA from the herbal product (procured from market) amplified using SCAR primer specific to *Aloe barbadensis* and *Crocus sativus.* Amplification was observed only with SCAR primer of *A. barbadensis* (lanes 1-2) and no amplification was seen with SCAR primer of *C. sativus* (lanes 3-4).

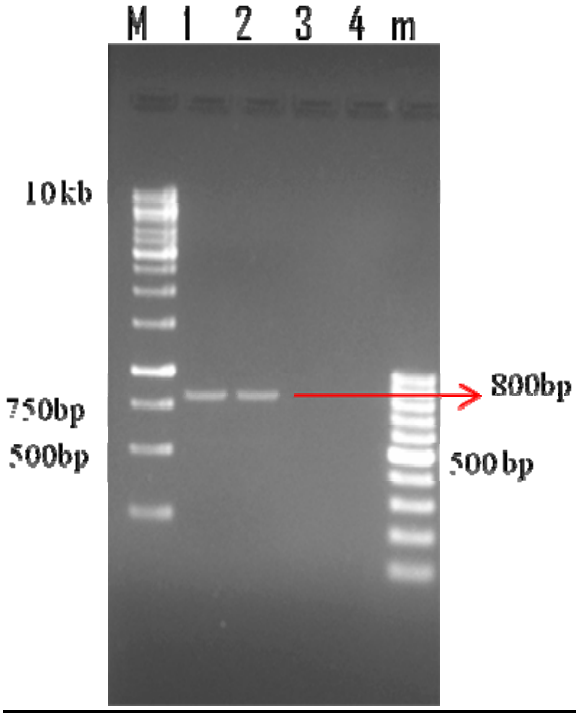

Fig. 8b. Genomic DNA from the herbal product (procured from market) amplified using SCAR primer specific to *Carthamus tinctorius* and *Senegalia catechu.* Amplification was observed only with SCAR primer of *C. tinctorius* (lanes 1-2) while no amplification was seen with SCAR primer of *S. catechu* (lanes 3-4).

Discussion

The incessant adulteration of food and herbal ingredients renders it important for the standardization and quality assurance of the raw materials and the finished products. Due to similar morphological characteristics and lack of botanical identification, adulteration of the raw materials, whether deliberate or unintentional, remains an indispensable problem in domestic and export markets (Patwardhan *et al*., 2005; Saad *et al*., 2006). This adulteration tremendously decreases the value and efficacy of the medicinal plants whether used in raw form or in prepared polyherbal medicines. According to WHO, correct identification is the first step towards assuring quality, safety and efficacy of traditional herbal medicines (Anon., 2000). Purity assessments based on physical, chemical or organoleptic markers are not always reliable as they are affected by environmental conditions and require higher resources and time (Marieschi *et al*., 2012).

Molecular marker technology is increasingly becoming popular as a potent tool for detection of adulteration as they are not affected by plant age, physical form or agroclimatic sources (Devaiah *et al*., 2011). In recent years, DNA markers such as RFLP, AFLP and RAPD have been extensively used for identification purposes. The study of genomic polymorphism using RAPDs has been widely used for DNA fingerprinting, varietal identification, classification, and population genetics (Shinwari *et al*., 1994, 2011; Kathidi *et al*., 2003; Jan *et al*., 2011; Jabeen *et al*., 2012). In our RAPD analysis, *C. sativus* and *A. barbadensis*, and their adulterants *C. tinctorius* and *S. catechu* revealed significant polymorphism and unique, reproducible amplicons were observed. The primers in our study clearly discriminated the genuine as well as the adulterant samples through generation of high intensity unique amplicons. The RAPD technique has been reported to differentiate a large number of species from their close relatives or adulterant (Sasikumar *et al*., 2004; Na *et al*., 2004; Qi *et al*., 2008; Irshad *et al*., 2009; Hussain *et al*., 2009; Khan *et al*., 2010). High degree of polymorphism is revealed by RAPD markers, but due to their low reproducibility and lower annealing temperature (Theerakulpisut *et al*., 2008), these RAPD amplicons are converted to a more stable and reproducible SCAR markers (Devaiah *et al*., 2011).

SCAR markers have many advantages over RAPD markers as the conditions for annealing are stringent and only a single locus is detected (Kiran *et al*., 2010; Khan *et al*., 2010). They are more specific as only one species-specific DNA fragment is amplified in PCR amplification, which can be visualized either by agarose gel electrophoresis or by measuring the DNA concentration in the solution of PCR product using an ELISA reader or by reading absorbance at 260nm (Weeden, 1994). The SCAR technique was first applied to the identification of the downy mildew resistance genes in lettuce (Paran and Michelmore, 1993). SCAR markers have been used for authentication of large number of medicinal plants such as *Artemisia* (Lee *et al*., 2006), *Phyllanthus* (Theerakulpisut *et al*., 2008), *Anthricus sylvestris* (Choo *et al*., 2009), *Piper longum* (Manoj *et al*., 2005), *Jatropha curcas* (Basha *et al*., 2009), *Aconitum heterophyllum* and *Cyperus rotundus* (Seethapathy *et al*., 2014). In our study, unique RAPD amplicons of herbal constituents and their adulterants were sequenced and internal sets of primers (SCAR primers) were designed. These SCAR primers generate a product only in the presence of a DNA from a given species increasing the specificity of SCAR markers. Thus, these SCAR primers employed herein can be used to authenticate the genuine herbal constituents from their adulterants in the raw material source as well as in polyherbal medicine.

The use of species-specific primers in PCR is a rapid means of sample identification and it may also be used to screen species in complex mixture of DNAs of finished products (Seethapathy *et al*., 2014). This enlightens the fact that the SCAR markers can be used in samples where DNA might be partially degraded due to treatment and storage conditions (Dhanya *et al*., 2011). In this study, the validation of commercially procured polyherbal medicine, Habb-e-Mudir having *C. sativus* and *A. barbadensis* as its constituents through SCAR markers has revealed that *C. sativus* may not have been used in the preparation of the drug whereas its more readily available and cheaper adulterant *C. tinctorius* has been used. The validation by amplification of genomic DNA of the health product with SCAR primers showed that only *C. tinctorius* is amplified for its SCAR marker of 800 bp and not *C. sativus* (900 bp). Further, the amplification of 550 bp product corresponding to *A. barbadensis* from complex mixture of the DNA molecules suggests that authentic *A. barbadensis* has been used in the health product. No amplification was detected with SCAR primers of its adulterant, *S. catechu*.

Conclusion

Adulteration, substitution and product mislabelling is a common practice, whether intentional or accidental in the traditional medicines. DNA-based authentication offers powerful tools aimed at quality control and quality assurance of medicinal plants as well as their finished polyherbal medicines. Our analysis strengthens the effectiveness of these DNA-based molecular marker techniques as an authentication tool with widespread applicability. Our work further demonstrates a fast, stringent and low cost method based on SCAR markers which enabled the unequivocal detection of adulterants. The method meets the need of the present market and can play a key role in developing a more foolproof protocol for their regulation.

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