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Original article

Molecular Authentication of Radix Behen Albi ("Bahman Sefid") Commercial Products Reveals Widespread Adulteration

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Abstract

Background and objectives: The roots of *Centaurea behen* L., (Asteraceae) known as Radix Behen Albi are used as an aphrodisiac, anti-lithiasis and general tonic. It is available as dried or powdered roots in the herbal markets of Iran. Confirming the identity of this medicinal root using conventional methods is challenging because of lack of the diagnostic characters and market samples are easy to misidentify or adulterate. **Methods:** This study aimed to authenticate 13 Radix Behen Albi samples purchased from different herbal markets in Iran and to identify the potential adulterants through DNA barcoding. Nuclear (nrITS) and plastid (*trn*L-F spacer, *mat*K and *rbc*L) DNA regions were used as barcoding markers. A reference database was compiled using sequences from herbarium voucher specimens and publicly available sequences. **Results:** Among used barcode regions nrITS was the best marker for species identification followed by *trn*L-F spacer. *Mat*K and *rbc*L were able to identify samples to the family level. This study showed that none of the market samples belonged to the authentic *Centaurea behen* L. Sixty-nine percent of samples were *Cousinia* spp. (Asteraceae), 23% *Korshinskya* spp. (Apiaceae) and 8% *Crambe* spp. (Brassicaceae). This substitution does not only hinder consumers obtain the desired medicinal effects of Radix Behen Albi but also raises concerns about the pharmacovigilance of this medicinal root sold in the markets. **Conclusion:** The present study shows the need for monitoring and authentication of crude herbal drugs in the markets of Iran, and that DNA barcoding is a suitable tool for this purpose.

Keywords: Asteraceae; *Centaurea behen*; DNA barcoding; herbal market; roots; traditional medicine

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Introduction

The resurgence of traditional medical systems has fueled global growth in traditional herbal products and the herbal dietary supplements market [1]. The demand for herbal medicine increases 6-10% annually and is projected to reach US$115 billion by 2020 [2]. However with the growth of the medicinal plant market, increasing reports of contamination and/or adulteration in herbal products has raised concerns about the quality and safety of these products [3,4]. Intentional substitution (adulteration) or accidental substitution

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(misidentification) of authentic medicinal species in raw materials as well as finished herbal products is a continuous problem in assuring authenticity of herbal medicine [4,5]. The World Health Organization has published guidelines for regulation and quality control of herbal medicines and emphasized the need for application of modern control techniques for quality control of herbal products [6,7].
Authentication and identification of raw and finished herbal products is challenging as they are mainly traded in the form of dried (roots, barks, leaves, etc.) or processed (as powders, capsules, etc.) plant parts, lacking important diagnostic characters [8]. Moreover, depending on the status of herbal products, taxonomic identification using macro- and micro-morphological, organoleptic and chemical methods can be time-consuming, error-prone and requires expertise and reliable references [9,10]. DNA barcoding provides an accurate and reliable tool to complement morphological and chemical profiling identification of herbal products, and is often applied when identification using above-mentioned methods is challenging [11]. This method is not affected by the plant's developmental stage, harvesting period, storage condition or processed stage, as long as DNA can be extracted [12-15].
Radix Behen Albi ("Bahman Sefid" /bæhmæn sefi̇d/ in Persian) is the dried root of *Centaurea behen* L. (Asteraceae), and has a long history of use in Arabic, Persian and Unani medicine [16,17]. The oldest ethnobotanical record of its use is connected to a voucher specimen of *C. behen* collected by Leonhard Rauwolf (1535-1596) from Lebanon in (1575 AD) [18], and mentions its use as a heart tonic. Nowadays this medicinal root is used mainly as aphrodisiac, anti-urolithiasis, sedative, cardiotonic and antiflatulent and to cure male infertility and jaundice, in the Middle East, Iran and India [19-21]. The dried roots or powdered roots of "Bahman Sefid" are sold in herbal shops in Iran and also exported to India [16,22]. Identification of these roots is significantly challenging, as they possess few or no diagnostic characters that can enable accurate morphological identification. Moreover, the monograph of Radix Behen Albi has not been mentioned in the Iranian Herbal Pharmacopoeia [23]. Therefore authentication and quality control assessment of marketed material is difficult due to the lack of morphological as well as chemical diagnostic characters.
This study has aimed to investigate the identity of Radix Behen Albi using DNA barcoding, and provide an accurate tool for authentication of herbal products from the markets of Iran. Our null hypothesis is that Radix Behen Albi products consist solely of roots of *Centaurea behen* [19,24]. To test this hypothesis, we pose the following research questions: 1) Can Radix Behen Albi products be authenticated using molecular identification?; 2) What are the potential adulterants of Radix Behen Albi in Iranian markets?; 3) What are the most suitable barcoding markers are most suitable for species level identification of Radix Behen Albi and its potential adulterants?; and 4) What species and barcoding markers are necessary to include in a sequence reference database for accurate species identification of Radix Behen Albi?

Material and Methods

Ethical considerations

This study was originated from the project with ethical code: IR.SBMU. RETECH. REC.1396.1330; approved by the ethical committee of Shahid Beheshti University of Medical Sciences.

Chemicals

GE Illustra GFX^{TM} PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK), Qiagen reaction buffer IV, $MgCl_2$, dNTP, Taq-polymerase (Qiagen NV, Venlo, Netherlands) were used in the experiment.

Plant material

Market samples including 12 roots and one powder sample of Radix Behen Albi (figure 1) were purchased from herbal shops in six different cities in Iran including Shiraz, Isfahan, Tehran, Mashhad, Jahrom, Hamedan from 2015 to 2017.
The specimens were registered at the herbal collection at the Herbarium of the Traditional Medicine and Materia Medica Research Center (HTMRC), Shahid Beheshti University of Medical Sciences. In addition, ten herbarium vouchers from HTMRC were sampled for the DNA library (table S1, supporting information).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from root and powder samples purchased from markets as well

as from herbarium voucher samples, using a CTAB protocol [25].

Extracted DNA was purified using a GE Illustra GFX™ PCR DNA and Gel Band Purification kit following the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Four barcode regions, nrITS (ITS1-5.8S-ITS2) as a nuclear marker, *trn*L-F spacer, *mat*K and *rbc*L as plastid markers were amplified using polymerase chain reaction (PCR) method. The primer pairs, their sequences and PCR conditions are given in table 1. PCR amplification was performed in 50 µL reactions

Figure 1. Market samples of Radix Behen Albi purchased from different herbal markets of Iran (refer to table 2 for sample details)

containing 5 µL Qiagen reaction buffer IV (NV, Venlo, Netherlands) (10x), 5 µL $MgCl_2$ (25mM), 1 µL dNTP (10 µM), 0.25 µL Taq-polymerase (Qiagen NV, Venlo, Netherlands) (5 U/ µL), 0.5 µl BSA, 1 µL of each primer (10 mM) and 1 µL of template DNA. Sequencing was performed by Macrogen Europe Inc. The same primers were used for sequencing reactions as in PCR amplification. Sequences were edited and assembled in Geneious 10.2.2 (Biomatters Ltd., USA). All sequences are available upon request from the authors.

Data analysis

The edited sequences were queried in GenBank and BOLD using Basic Local Alignment Search Tool (BLAST). Top corresponding species were extracted for including in reference database for phylogenetic analysis (table S1 supporting information). Top corresponding species were chosen based on the identity score (high identity: $i \geq 95\%$; medium identity: $90\% \leq i < 95\%$; low identity: $i < 90\%$) and the number of species within 1% deviation of the calculated similarity score [14]. High identity and one species within 1% deviation was assigned species-level confidence (in BLAST identification results); high identity and more than one species was assigned genus-level confidence; medium identity and one or more species within the same genus was assigned genus-level confidence; medium identity and species from more than one genus was assigned family-level confidence; and low identity was assigned family-level confidence [14].

Sequence matrices per marker were aligned using ClustalW as implemented in MEGA 7 using default settings [31]. The resulting alignments were manually adjusted. Maximum likelihood method based on kimura 2-parameter model and gamma distribution and 1000 bootstraping replicates were used to construct phylogenetic trees in MEGA 7 [31]. If the unknown market sample was found within a cluster with one species, it was accepted as a member of that species. If it was clustered with different species of the same genus it was accepted as member of that genus and if it was clustered with different genera of the same family, then it was accepted as a member of that family.

Table 1. Primer pairs used for amplification and sequencing and PCR conditions used in this study(For each primer pair the upper one is forward and the lower one is reverse sequence)

Locus	Primer	Primer sequence 5' - 3'	Reaction conditions	Reference
nrITS	17SE	ATGGTCCGGTGAAGTGTTC	95 °C 3 min (95 °C 20 s, 60 °C 1 min, 72 °C 2 min) × 35 cycles; 72 °C, 8 min	[26]
	26SE	CCCGGTTCGCTCGCCGTTAC		
*mat*K	matK-xf	TAATTTACGATCAATTCATTC	95 °C 3 min (95 °C 20 s, 54 °C 1 min, 72 °C 3 min) × 35 cycles; 72 °C, 10 min	[27]
	matK-MALP	ACAAGAAAGTCGAAGTAT		
*trn*L_F	trnL_c2	GGATAGGTGCAGAGACTCAAT	95 °C 3 min (95 °C 20 s, 59 °C 1 min, 72 °C 3 min) × 35 cycles; 72 °C, 8 min	[28]
	trnL_f	ATTTGAACTGGTGACACGAG		
*rbc*L	rbcLa_F	ATGTCACCACAAACAGAGACTAAAGC	95 °C 3 min (95 °C 20 s, 55 °C 1 min, 72 °C 3 min) × 35 cycles; 72 °C, 10 min	[29]
	rbcLa_R	GTAAAATCAAGTCCACCRCG		[30]

Final consensus identification was made based on the results from all markers [14]. Species level identification was assigned if at least two markers with species level identifications yielded the same species identification. Genus level identification was assigned if identifications resulted in multiple species of the same genus**.**

Results and Discussion

The sequencing success of market samples for nrITS and *rbc*L was 100% (13 samples), for *trn*L-F 69% (9 samples) and for *mat*K 61% (8 samples). BLAST querying of nrITS sequences resulted in 69% (9 samples) species level identifications, 23% (3 samples) genus level and 8% (1 sample) family level identifications. For *trn*L-F it resulted in 11% (1 sample out of 9) species level identifications, 44% (4 samples out of 9) genus level and 44% (4 samples out of 9) family level identifications. *Mat*K and *rbc*L yielded only family level identifications. Details of BLAST query identifications are given in table 2.

Phylogenetic analysis using a maximum likelihood framework of nrITS was able to identify five samples (38%) to species level and eight samples (62%) to genus level. For the *trn*L-F spacer sequences, it resulted in identification of one sample (11%) to species level, five samples (56%) to genus level and three samples (33%) to family level. Analysis of *mat*K and *rbc*L yielded only family level identifications. Table 2 shows details of the tree-based phylogenetic identifications for each sample and marker. Phylogenetic trees of all markers are available upon request from the authors.

Consensus identification results revealed that 69% (9 samples) belonged to the genus *Cousinia* Cass. (Asteraceae), 23% (3 samples) to the genus *Korshinskya* Lipsky (Apiaceae) and 8% (1 sample) to the genus *Crambe* L. (Brassicaceae). None of the samples could be identified to species level when the consensus results of all markers were considered. Importantly, none of the tested samples belonged to the authentic species *Centaurea behen*, and none even to the genus *Centaurea* L. Potential adulterants of Radix Behen Albi in the markets of Iran are mainly species of *Cousinia*, *Korshinskya* and *Crambe*. The root samples from Tehran markets (2 sample), Hamedan (1 samples) and Jahrom (1 samples) belonged to unrelated families than the authentic species.

Our results show that among the standard plant DNA barcoding markers, nrITS is the most suitable for molecular authentication of Radix Behen Albi market samples. Other studies have also shown that nrITS is a good marker for authentication of herbal products [32-34]. Furthermore, our study showed that *rbc*L and *mat*K are not suitable for species level identification of Radix Behen Albi and its adulterants. Previous studies have also shown that *rbc*L and *mat*K are mainly suitable for identification to family and genus level [35-38]. Using consensus identification criteria, we were able to identify the samples only up to the genus. In general, DNA barcoding is a useful technique for molecular authentication of Radix Behen Albi; however, a more comprehensive DNA reference library that includes all *Centaurea* and *Cousinia* species in Iran might help in detailed identification of adulterants of Radix Behen Albi market products to species level [39,40].

Radix Behen Albi material in the market originates mainly from wild resources in Iran. Widespread substitution of *Centaurea behen* with species of *Cousinia* might be due to morphological similarities and subsequent misidentification of plants by collectors. *Cousinia* is a very common and diverse genus with more than 200 species in Iran [41]. The distribution range of *Centaurea behen* [42] overlaps with the distribution range of *Cousinia* [43] (figure 2) and because of morphological similarities and lack of knowledge of collectors in identifying authentic species, it maybe misidentified with *Cousinia*.

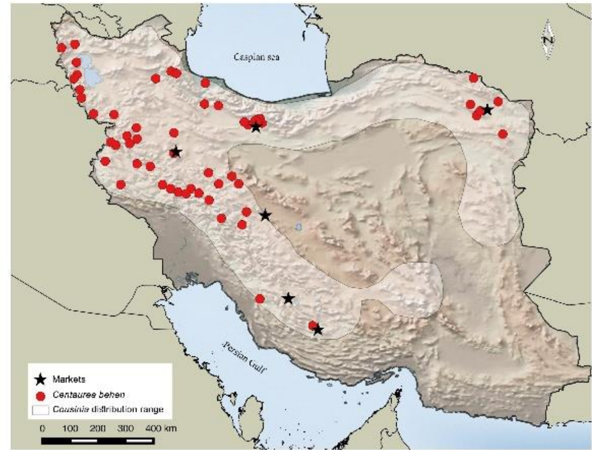

Figure 2. Location of markets where root samples were purchased; general distribution of *Centaurea behen* [42] in Iran and general distribution range of *Cousinia* [43] in Iran

Table 2. Detailed results of identification success of Radix Behen Albi by each marker and BLAST match and phylogenetic analysis

Sample information			BLAST Results (Identifications)				Tree based identification				Consensus
Samples variety	**Sample origin**	**Voucher**	**ITS_BLAST**	**trnL_BLAST**	**rbcL_BLAST**	**matK_BLAST**	**ITS_Tree**	**trnL_Tree**	**rbcL_Tree**	**matK_Tree**	**Identification**
Bahman-Sefid	Shiraz	R_363	*Cousinia microcarpa* Boiss.	*Cousinia* sp.	Asteraceae	Asteraceae	*Cousinia* sp.	*Cousinia* sp.	Asteraceae	Asteraceae	*Cousinia* sp.
Bahman-Sefid	Isfahan	R_364	*Cousinia microcarpa* Boiss.	*Cousinia* sp.	Asteraceae	Asteraceae	*Cousinia* sp.	*Cousinia* sp.	Asteraceae	Asteraceae	*Cousinia* sp.
Bahman-Sefid	Tehran	R_367	*Cousinia microcarpa* Boiss.	*Cousinia* sp.	Asteraceae	Asteraceae	*Cousinia* sp.	*Cousinia* sp.	Asteraceae	Asteraceae	*Cousinia* sp.
Bahman-Sefid	Tehran	R_369	*Cousinia* sp.	Asteraceae	Asteraceae	Asteraceae	*Cousinia microcarpa* Boiss.	*Cousinia* sp.	Asteraceae	Asteraceae	*Cousinia* sp.
Bahman-Sefid	Tehran	R_370	*Korshinskya olgae* Lipsky	Apiaceae	Apiaceae	Apiaceae	*Korshinskya kopetdaghensis* Pimenov & Kljuykov	Apiaceae	Apiaceae	Apiaceae	*Korshinskya* sp.
Bahman-Sefid	Hamedan	R_486	*Cousinia* sp.	*Cousinia* sp.	Asteraceae	NA	*Cousinia* sp.	*Cousinia* sp.	Asteraceae	NA	*Cousinia* sp.
Bahman Sefid_powder	Hamedan	R_487	*Brassica* sp.	*Crambe cordifolia* subsp. *kotschyana* (Boiss.) Jafri	Brassicaceae	NA	*Crambe* sp.	*Crambe cordifolia* subsp. *kotschyana* (Boiss.) Jafri	Brassicaceae	NA	*Crambe* sp.
Bahman-Sefid	Mashhad	R_385	*Cousinia microcarpa* Boiss.	NA	Asteraceae	Asteraceae	*Cousinia* sp.	NA	Asteraceae	Asteraceae	*Cousinia* sp.
Bahman-Sefid	Mashhad	R_386	*Cousinia microcarpa* Boiss.	NA	Asteraceae	NA	*Cousinia* sp.	NA	Asteraceae	NA	*Cousinia* sp.
Bahman-Sefid	Jahrom	R_437	Apiaceae	Apiaceae	Apiaceae	Apiaceae	*Korshinskya kopetdaghensis* Pimenov & Kljuykov	Apiaceae	Apiaceae	Apiaceae	*Korshinskya* sp.
Bahman-Sefid	Shiraz	R_440	*Cousinia microcarpa* Boiss.	NA	Asteraceae	NA	*Cousinia* sp.	NA	Asteraceae	NA	*Cousinia* sp.
Bahman-Sefid	Tehran	R_442	*Cousinia microcarpa* Boiss.	NA	Asteraceae	Asteraceae	*Cousinia microcarpa* Boiss.	NA	Asteraceae	Asteraceae	*Cousinia* sp.
Bahman-Sefid	Tehran	R_444	*Korshinskya olgae* Lipsky	Apiaceae	Apiaceae	No sequence	*Korshinskya kopetdaghensis* Pimenov & Kljuykov	Apiaceae	Apiaceae	NA	*Korshinskya* sp.

NA: not applicable

Morphological identification of root products is difficult and as soon as the root material is dried and enters the market supply chain, authentication by traders and middlemen becomes impossible. However, substitution might also be due to intentional exchange for roots of other species by collectors, middlemen or retailers [13,44,45]. Ouarghidi et al. [46] found 54.3% substitution among 33 common medicinal roots in the markets of Morocco. They concluded that substitution is due to scarcity, high demand and high price of the authentic product [46]. Other studies also found scarcity of plant species in the wild and continuous market demand as underlying reasons and motivations for substitution [44,47-49]. However, in case of *Centaurea behen* scarcity does not seem to be the main reason for substitution. Further studies are necessary to understand the exact reasons underlying the pervasive substitution of Radix Behen Albi in the markets of Iran.

DNA barcoding of commercial samples of Radix Behen Albi in Iran revealed that none of the tested samples belonged to the authentic species of *Centaurea behen*. It showed that Radix Behen Albi is widely substituted or misidentified with roots of other species of both related (*Cousinia*) and unrelated (*Crambe* and *Korshinskya*) genera. This finding highlights the necessity of proper authentication and regular monitoring of commercialized medicinal roots in the herbal markets of Iran. We suggest that DNA barcoding of Radix Behen Albi be included in the herbal pharmacopoeia monograph of this plant along with other methods of authentication.

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Author contributions

Abdolbaset Ghorbani, Somayeh Esmaeili and Mahmoud Mosaddegh conceived and designed the study; Somayeh Esmaeili and Mahmoud Mosaddegh performed fieldwork; Abdolbaset Ghorbani performed experiments; Abdolbaset Ghorbani and Hugo De Boer analyzed the data; Abdolbaset Ghorbani, Somayeh Esmaeili and Hugo De Boer wrote the first draft of the manuscript. All authors have read and approved the final manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

HTMRC: Herbarium of the Traditional Medicine and Materia Medica Research Center; BLAST: Basic Local Alignment Search Tool; PCR: Polymerase Chain Reaction