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ORIGINAL ARTICLE

DNA barcoding and NMR spectroscopy-based assessment of species adulteration in the raw herbal trade of *Saraca asoca* (Roxb.) Willd, an important medicinal plant

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Abstract *Saraca asoca* (Roxb.) Willd, commonly known as "Asoka" or "Ashoka," is one of the most important medicinal plants used in raw herbal trade in India. The bark extracts of the tree are used in the treatment of leucorrhea and other uterine disorders besides also having anti-inflammatory, anti-bacterial, anti-pyretic, anti-helminthic, and analgesic activity. The indiscriminate and rampant extraction of the wood to meet the ever-increasing market demand has led to a sharp decline in naturally occurring populations of the species in the country. Consequently, the species has recently been classified as "vulnerable" by the International Union for Conservation of Nature (IUCN). Increasing deforestation and increasing demand for this medicinal plant have resulted in a limited supply and suspected widespread adulteration of the species in the raw herbal trade market. Adulteration is a serious concern due to: (i) reduction in the efficacy of this traditional medicine, (ii) considerable health risk to consumers, and (iii) fraudulent product substitution that impacts the economy for the Natural Health Product (NHP) Industry and consumers. In this paper, we provide the first attempt to assess the extent of adulteration in the raw herbal trade of *S. asoca* using DNA barcoding validated by NMR spectroscopic techniques. Analyzing market samples drawn from 25 shops, mostly from peninsular India, we show that more than 80 % of the samples were spurious, representing plant material from at least 7 different families. This is the first comprehensive and large-scale study to demonstrate the widespread adulteration of market samples of *S. asoca* in India. These results pose grave implications for the use of raw herbal drugs, such as that of *S. asoca*, on consumer health and safety. Based on these findings, we argue for a strong and robust regulatory framework to be put in place, which would ensure the quality of raw herbal trade products and reassure consumer confidence in indigenous medicinal systems.

Keywords Herbal drugs · Species admixture · Ashoka · *Saraca asoca* · DNA barcoding · NMR spectroscopy

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Introduction

Saraca asoca (Roxb.) Willd (Caesalpiniaceae) commonly known as "Asoka" or "Ashoka" is one of the most important medicinal plants used in a variety of indigenous medicine

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systems, including Ayurvedic, Unani, and Siddha in India [1]. Its use has been documented in numerous classical Indian Ayurvedic texts such as Charaka Samhita dating to 1000 BC, Susruta dating to 500 BC, and Chakradatta dating to eleventh century AD [1]. Because of its overwhelming medicinal importance, the tree is revered in the Hindu tradition [2]. Almost all parts of the plant, including the bark, flowers, leaf, and seeds, have been attributed to have medicinal value [1]. One of the most prominent uses, known both from Ayurvedic texts and modern pharmacological studies, is the effect of *S. asoca* tissue extract in alleviating uterine and menstrual disorders [3]. It has been attributed to be useful in relieving uterine disorders such as menorrhiga, ammenorhea, endometrosis, etc. [1]. Besides, extracts of bark, leaves, roots, and dried flowers have been shown to have antibacterial [4, 5], anti-fungal [6, 7], anti-inflammatory [8], antioxidant [9], anti-pyretic [10], anti-helmintic [4], analgesic [11], anti-ulcer [8], chemo-preventive [12], and larvicidal activity [13]. Treatment with *S. asoca* increased the activity of lysosomal enzymes in both plasma and liver of adjuvant-induced arthritic rats [14]. Chemical analysis of the bark, roots, leaf, and flowers has indicated a wide range of compounds including glycosides, tannins, flavonoids, steroidal glycosides, saponins besides, carbohydrates, proteins, etc. [1, 12].

A large number of herbal formulations in the form of tablets, powders, and syrups of *S. asoca* are available commercially in local as well as national and international markets. With no documentation of plantations of the tree in the country, most of the collections must evidently come from natural populations of species [15]. In 1999–2000, the demand for bark of *S. asoca* in India was estimated to be 5332 metric ton and the demand is estimated to have risen to more than 15,000 metric ton in the year 2007–2011[16]. Distribution of the species is sparse, mainly in parts of the Western Ghats of Maharashtra, Goa and Karnataka and in parts of Tamilnadu, Kerala, Eastern Ghats of Odisha and Meghalaya [15]. Ved and Goraya believe that the huge demand is either met from some hitherto unknown source of *S. asoca* in the country or is simply unlikely to be *S. asoca* [17]. This suggests the very likely possibility that the demand for *S. asoca* is met by widespread adulteration with other plant materials. This claim is supported by several studies that have documented that market samples of Ashoka are often adulterated with bark of *Polyalthia* [15, 18] or with bark of *Trema orientalis* [19], *Shorea robusta* [20], and *Mallotus nudiflorus* [15].

Recently, a number of studies have shown that NHP market samples of raw herbal trade material are often not what they are claimed to be. Using DNA barcoding tools, Srirama et al. showed that 24 % of shops trading in *Phyllanthus amarus*, an important hepato-protective plant, were often mixed with six other *Phyllanthus* species [21]. Newmaster et al. reported as high as 63 % adulteration of herbal products sold in the North American herbal market [22]. Seethapathy et al. showed species admixtures ranging from 8 to 50 % in raw herbal trade of *Senna* species, known for their laxative property [23]. More recently, Santhosh et al. showed that over 80 % of the market samples of *Sida* species, known traditionally for improving vigor and vitality, contained species admixtures [24]. These studies confirm the widely held belief that there could be rampant species adulteration in raw herbal trade. An intrinsic problem associated with the adulteration of herbal products is the impact it may have on consumer health and safety [23, 25]. Unless regulated, such adulteration is bound to lower efficacy and subsequently the consumer confidence in plant medicine products, which will ultimately result in economic loss within raw herbal trade [24].

In this paper, we have attempted to assess the extent of adulteration in the raw herbal trade of *S. asoca* in India using DNA barcoding validated by NMR spectroscopy. DNA barcoding has been successfully used in detecting and quantifying adulteration in raw herbal trade of a variety of medicinal plants [21, 23, 24, 26, 27]. The success of this technique lies in its ability to develop species-specific or unique DNA sequence-based identifier for the species. A limitation of this technique, however, is the ability to extract good quality DNA from raw herbal material for PCR-amplification of the DNA barcode regions [24]. Often, the raw herbal trade material in the form of powder, billets, or even dried plant material is extremely recalcitrant to extraction [22, 24]. Under such situations, chemical fingerprinting, using techniques such as NMR spectroscopy could be used in conjunction with DNA barcoding to arrive at species identities and admixtures [28]. NMR spectroscopy is especially useful as it is non-destructive, quantitative, does not require sample pre-treatment such as column chromatography and derivatization, and can identify complex unidentified metabolites [29]. In fact, several recent studies have used 1H and 2H NMR combined with 2D diffusion ordered spectroscopy (DOSY) NMR experiments for the quantitative analysis of herbal medicines and for the identification of their geographical origin or supplier or adulteration [30, 31]. In this study, we use both DNA barcoding and NMR spectroscopic techniques to assess adulteration in medicinal products of *S. asoca* in India. More specifically, we have: (1) developed an authenticated Biological Reference Material (BRM) for *S. asoca* and (2) tested (DNA barcoding and NMR spectroscopic) market samples drawn from 25 shops, mostly from peninsular India. We discuss our results in the light of the possible consequences, NHP adulteration can have on consumer health and safety, which demand the need for imposing stringent regulations concerning NHP quality assurance.

Materials and methods

Collection of authenticated Biological Reference Material

Bark samples of *S. asoca* (Roxb.) Willd., (Family: Caesalpiniaceae) were obtained from ten different geographic

locations in South India and from Odisha (Table 1) along with their respective leaf and flower (wherever available) samples. The taxonomic identity of the samples was established independently by two taxonomists after examining the leaf, bark, and flower samples. Each of the samples was then assigned a specific voucher identification number and this constituted the Biological Reference Material (BRM) of *S. asoca*. For each sample of the BRM, herbarium voucher specimens were prepared and deposited at the Herbaria at the Ashoka Trust for Research in Ecology and the Environment (ATREE), Bangalore, India. The BRM was used to develop species-specific DNA barcodes for two chloroplastic regions, namely *rbcL* and *psb*A-*trn*H, and NMR spectroscopic analysis. These DNA barcodes were later used to validate the species identities of the market samples of *S. asoca*.

Collection of trade samples of *S. asoca*

Vernacular names and trade names of *S. asoca* were obtained from "The Ayurvedic Pharmacopoeia of India" and ENVIS FRLHT, Bangalore (http://envis.frlht.org/). Based on these names, raw herbal trade samples of *S. asoca* were purchased from 25 shops across India (Fig. 1, Table 2). The shops typically stock collection of medicinal plants from collectors and contractors and supply them on retail or wholesale basis to customers, Vaidyas (practitioners of indigenous medicine; the Vaidyas use them to make the finished products or drugs) and herbal product industries. The purchases were done by one or more of the authors personally (Table 2) to ensure that there were no errors due to mis-communication between the purchaser and the supplier. About 100 g of the raw drug samples in triplicate was obtained from each shop. The products were in dry form either as bark or root samples or in powder form. The products bore no morphological resemblance to the species and hence could not be identified at the shop level. Each of the collected samples was given an Herbal Authentication Service (HAS) code with details of the date of collection, location of the shop, shop name, and collection number. To avoid mixup of samples, a chain of custody protocol was followed from the time of collection to DNA extraction, PCR amplification, and sequencing to the final data analysis. The obtained market samples were also deposited in the herbaria of ATREE, Bangalore (Table 2).

DNA barcoding

Genomic DNA extraction, PCR amplification, and sequencing

Total genomic DNA was isolated from each of the BRM samples of *S. asoca* ($n = 10$). Samples of each of the BRM were finely powdered, and 100 mg of the powder was extracted using Nucleospin Plant II Mini DNA extraction kit. The genomic DNA thus obtained was quantified using a nanophotometer and by visual inspection on 0.8 % agarose gel. Working concentration of genomic DNA was prepared by diluting the stock solution to a concentration of 25 ng/μL. Similar procedure was followed for extraction of DNA from trade samples.

Both the BRM of *S. asoca* and the trade samples were barcoded using two regions, namely *rbcL and psb*A-*trn*H. *psb*A-*trn*H has been one of the more preferred candidates

Table 1 Details of collection of Biological Reference Material (BRM) of *Saraca asoca* with their voucher number, collection locations, and GenBank accession numbers

Sl. No.	Biological Reference Material (BRM)	Voucher ID	Collection place	GenBank accession number	
				rbcL	*psb*A-*trn*H
1	BRM A1	UAS-SEC264	School of Ecology and Conservation, Bangalore, Karnataka	KU499908	KU375754
2	BRM A2	UAS-SEC265	University of Agricultural Sciences-GKVK, Bangalore, Karnataka	KU499909	KU375755
3	BRM A3	UAS-SEC266	Karwara, Uttara Kannada, Karnataka	KU499910	KU375756
4	BRM A4	UAS-SEC267	Indian Institute of Science, Bangalore, Karnataka	KU499911	–
5	BRM A5	UAS-SEC268	Sirsi, Uttara Kannada, Karnataka	KU499912	KU375757
6	BRM A6	UAS-SEC269	Similipal Biosphere reserve, Maturbhaij, Odisha	KU499913	KU375758
7	BRM A7	UAS-SEC270	Barbara, Balugaon range, Khordha forest division, Odisha	KU499914	KU375759
8	BRM A8	UAS-SEC271	Dhaunali, Khordha forest division, Odisha	KU499915	KU375760
9	BRM A9	UAS-SEC272	Kapilas, Dehenkanal forest division, Odisha	KU499916	KU375761
10	BRM A10	UAS-SEC273	RPBC, Bhubaneswar city forest division, Odihsa	–	–

– indicates poor quality sequence and hence not submitted to GenBank

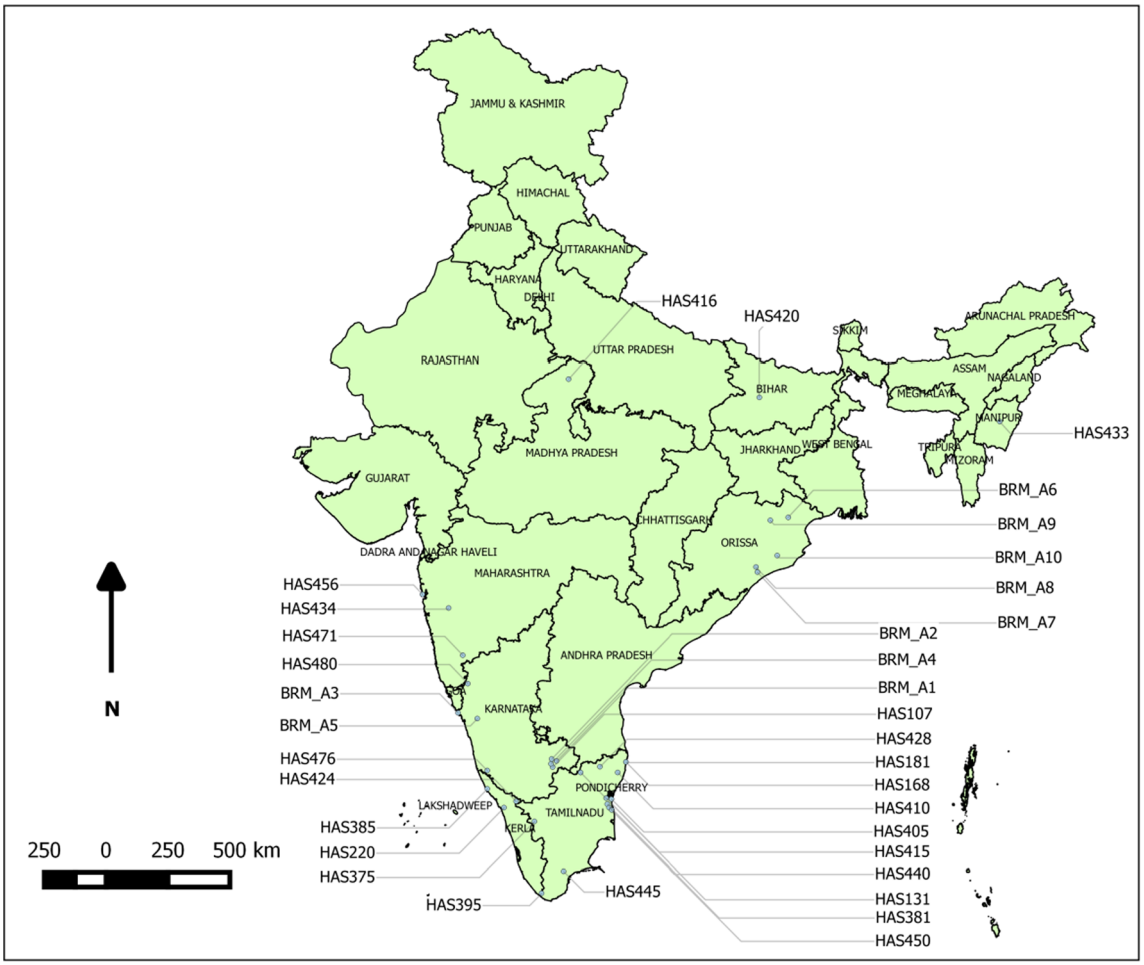

Fig. 1 Places of collection of Biological Reference Material (BRM) and trade samples of *Saraca asoca* (Ashoka) in India. The codes of the BRM and trade samples are represented in Table 1 and Table 2, respectively

for species identification [32, 33]. In a comparative study of seven loci, Pennisi found that the discriminatory power of *psb*A-*trn*H was the highest (69 %) among many other chloroplastic regions [34]. *rbcL* was evaluated as a possible region because of its universality, ease of amplification, ease of alignment, and evaluation. It has also been shown to differentiate a large percentage of congeneric plant species [35].

The PCR amplification was performed in a 20-μL reaction mixture that contained 2.5 μL of genomic DNA, 2.5 μL of 10 × *Pfu* buffer with $MgSO_4$ (Fermentas®), 2.5 μL of 2 mM dNTPs (Fermentas), 0.5 μL each of forward and reverse primers (10 pM), and 0.2 μL of 2.5 U *Pfu* DNA polymerase (Fermentas). The primers and the reaction conditions were as those published for *rbcL* [36] and *psb*A-*trn*H [32]. The PCR products obtained from these reactions were subjected to Big Dye (version 3.1) sequencing reactions that had a total volume of 10 μL and included 10 pMol of each primer. The sequencing amplification protocol consisted of 1 cycle of 1 min at 96 °C, followed by 30 cycles of 10 s at 96 °C, 50 s at 55 °C, and 4 min at 60 °C. The amplicons were bidirectionally sequenced using ABI 377 sequencer (Applied Biosystems). The chromatographic traces were aligned and codon read in the Codon code Aligner ver. 3.0 (CodonCode, Dedham, MA, USA) and contigs were generated. Sequences were deposited in NCBI GenBank.

Data analysis and validation of raw herbal drugs

The sequence results obtained for the BRM samples were edited manually using BioEdit (Version 5.0.6). The species sequences were then queried in a BLASTn search to ascertain if the two barcode regions, *rbcL* and *psb*A-*trn*H, distinctly qualify as unique descriptors of all the BRM samples. Our results indicated that all the 10 BRM samples, independent of their sources of collection, could be uniquely described by their *rbcL* and *psb*A-*trn*H sequence. Hence, these were considered as the DNA barcode descriptors of *S. asoca*. The sequences obtained from the trade samples were then queried and compared with those of the BRM barcode library with a minimum BLAST cutoff of 97 % identity for a top match [37].

Table 2 Details of collection of market samples of *Saraca asoca*

SL. No.	HAS Code	Longitude	Latitude	Place of collection	Collector
1	HAS107	77.5770° E	12.9679° N	Mamulpet, Bangalore, Karnataka	SK
2	HAS131	78.614511° E	12.682564° N	Vaniyambadi, Tamil Nadu	SE
3	HAS168	80.2787° E	13.086628° N	Park town, Tamil Nadu	SE
4	HAS181	80.2787° E	13.086628° N	Park town, Tamil Nadu	SE
5	HAS220	75.8300° E	11.4500° N	Balusherry, Kerala	SK
6	HAS375	76.932625° E	10.943178° N	Kovaipudur, Tamil Nodu	SE
7	HAS381	78.888597° E	10.257953° N	Periya kadai street, Tamil Nodu	SE
8	HAS385	75.208217° E	12.106803° N	Court Road, Payyanur, Kerala	SK
9	HAS395	76.947778° E	8.494431° N	Puthenchanthai-Pullimood, Kerala	RK
10	HAS405	79.748083° E	11.746675° N	Thirupapuliyur, Tamil Nadu	SE
11	HAS410	79.491761° E	11.940247° N	KK Nagar, Old Bus Strand, Tamil Nadu	SE
12	HAS415	79.553147° E	11.769394° N	Gandhi Road, Panruti, Tamil Nadu	SE
13	HAS416	78.197078° E	26.211464° N	Bara-Dari, Gwalior, Madhya Pradesh	RK
14	HAS420	85.154417° E	25.616797° N	Bakarganj, Bihar	RK
15	HAS424	76.256719° E	11.664694° N	Sulthan Bathery, Kerala	SK
16	HAS428	79.326542° E	12.905044° N	Kalavai road, Arcot, Tamil Nadu	SE
17	HAS433	93.940811° E	24.804128° N	Imphal, Manipur	US
18	HAS434	73.790181° E	18.482464° N	Warje, Maharastra	US
19	HAS440	78.167736° E	11.656556° N	Chinna kadai street, Tamil Nadu	SE
20	HAS445	79.141253° E	10.791069° N	Keelavasal, Tamil Nadu	SE
21	HAS450	79.692581° E	11.400078° N	Chidambaram, Cuddalore	RK
22	HAS456	72.837811° E	19.023597° N	Hanuman Mandir, Dadar (W), Maharashtra	US
23	HAS471	74.314092° E	16.837822° N	Peth Vadgaon, Maharastra	US
24	HAS476	75.205975° E	12.768097° N	Puttur, Karnataka	SK
25	HAS480	74.507872° E	15.858233° N	Belgaum, Karnataka	SK

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A UPGMA phylogenetic tree was constructed using both the trade and the BRM samples using MEGA 6.06 [38].

NMR spectroscopic analysis

Sample preparation

Representative samples of BRM ($n = 5$) and the market samples ($n = 25$) that were used for DNA barcoding were dried in a hot air oven (42 to 45 °C) and ground to a fine powder using liquid nitrogen. One gram of the fine powder was taken and dissolved in methanol. The methanolic extracts were vacuum dried, and the residues were taken for the NMR analysis. The evaporated methanol extracts were re-dissolved in deuterated methanol to make the total volume to 600 μl per sample and were later transferred to a 5-mm NMR tube.

NMR spectroscopy

NMR spectra were recorded on a BrukerBiospin 600-MHz Avance-III spectrometer operating at a 1H NMR frequency of 600.13 MHz at 298 K, equipped with a 5-mm BBI probe. Gradient shimming was performed prior to signal acquisition. Deuterated methanol was used to provide an internal lock. Water suppression of the residual water signal was achieved with a presaturation sequence with low-power selective irradiation at 4.9 ppm during the recycle delay. The proton spectra were collected with a 90° pulse width of 6.97 μs, a recycle delay of 2 s, 16 scans, 2K data points, and a spectral width of 12 ppm. Data were zero-filled by a factor of 2, and the FIDs were multiplied by an exponential weighting function equivalent to a line broadening of 1 Hz prior to Fourier transformation. The spectra were phase- and baseline-corrected and referenced to the solvent peak of methanol-D4 at 3.31 ppm.

For resonance assignment and metabolite identification, two-dimensional NMR spectra were recorded, including 1H-1H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and 1H-13C heteronuclear coherence spectroscopy (HSQC, HMQC). 2D 1H-13C heteronuclear spectra were obtained with a spectral width of 12 and 220 ppm in the proton and carbon dimensions, respectively, 1 K data points, 16 scans, 256 t1 increments, and a recycle delay of 2 s. The COSY and TOCSY spectra were acquired with a spectral width of 12 ppm in both dimensions, 1 K data points, 16 scans, and 256 t1 increments. For 2D DOSY 1H NMR, stimulated echo bipolar gradient pulse experiments were used with a pulse delay of 1 ms after each gradient and a diffusion delay of 100 ms. Forty experiments were recorded with gradient intensity linearly sampled from 5 to 95 %. The gradient system had been calibrated to 46.25 G cm^{-1} at maximum intensity. All data were processed using the DOSY Toolbox software. DOSY spectra are presented with chemical shifts on the horizontal axis and diffusion coefficients on the vertical axis (expressed in m^2 s^{-1}) (S1 Fig. 1).

Metabolite databases and software

Metabolite fingerprinting of NMR spectra for identifying metabolites in *S. asoca* was done by checking identified metabolite peaks with standard NMR data recorded in databases such as Biological Magnetic Resonance Data Bank (BMRB) (http://www.bmrb.wisc.edu) and the Madison Metabolomics Consortium Database (MMCD) (http://mmcd.nmrfam.wisc.edu).

Multivariate statistical analysis

1D NMR spectra of both plant and the trade samples were processed using MestReNova (Mestrelab Research, Spain). For statistical analysis, NMR data was binned into integrated regions of equal width (0.06 ppm) corresponding to the region of 0.0–9.0 ppm. Data were normalized to a total integral of 100 to compensate for possible differences in signal-to-noise ratios between spectra. The region corresponding to the signal from the solvent peak was removed from analysis. Univariate and multivariate statistical analysis was done using the Metaboanalyst software [39]. PCA with Pareto scaling and PLS-DA with mean-centering were performed. The binned data was first analyzed by PCA, which enabled detection and removal of any outliers (located outside the 95 % confidence region of the model). Outliers were hence removed prior to PLS-DA analysis. The predictive power of the PLS-DA model was evaluated from the values of the parameters Q^2 and R^2Y, representing the explained variance and the predictive capability respectively. The statistical significance of these parameters was estimated by performing a permutation test where the *Y* matrix was randomly permuted 1000 times, keeping the *X* matrix as fixed, with a threshold *p* value of <0.01, indicating that none of the results are better than the original one. Significant metabolites contributing to the differences between groups were identified from the PLS-DA loadings plot. Significant metabolites were ranked according to their variable influence on the projection (VIP) score, which is a weighted sum of squares of the PLS weights, accounting for the Y variance in each dimension. VIP analysis displays the metabolites ordered according to their influence on group separation, with metabolites arranged according to their VIP values, and the *y*-axis denoting their relative intensities. The significance of metabolites contributing to differences between clusters in the PLS-DA score plots was analyzed using *t* tests and ANOVA, setting $p < 0.01$ as the level of statistical significance.

Results and discussion

DNA barcoding of BRM of *S. asoca* and market samples

Two DNA barcode regions, both of chloroplastic origin, namely *rbcL* and *psb*A-*trn*H, were used to develop species-specific barcode signatures for the BRM of *S. asoca*. These regions are among the half-a-dozen regions that have been commonly used in barcoding plant taxa [33]. All the ten BRM samples yielded good amplification of these regions, with sequence lengths corresponding to 568 bp for *rbcL* and 515 bp for *psb*A-*trn*H. No major length variations were found among the BRM samples (Table 3). The sequences obtained from the BRM were queried with existing sequences using BLAST. Sequences of *rbcL* matched with *S. asoca* (GenBank accession JQ673549.1) as well as also *Saraca declinata* (GenBank accession JX856761.1). There were no records of any sequence of *psb*A-*trn*H for *S. asoca* in the database. In summary, the BRM of *S. asoca* were clustered in a single group with 63 % bootstrap support for *rbcL* and 99 % bootstrap support for *psb*A-*trn*H. Thus, irrespective of the geographical regions of their collection, all the samples in BRM could serve as reference for validating the market samples.

Table 3 Evaluation of the two DNA barcode regions used in this study

Variables	*rbcL*	*psb*A-*trn*H
PCR success, BRM/Market samples (%)	100/68	100/44
Amplified product length (bp) of BRM	545–568	480–515
Amplified product length (bp) of raw drugs	271–380	385–538
Aligned sequence length (bp)	278	388
Mean distance ± SD (Within BRM)	0.000	0.000
Mean distance ± SD (BRM vs. raw drugs)	0.076 ± 0.056	0.416 ± 0.235

BRM biological reference material

Analysis of the market samples for the DNA barcode regions was however not as successful as that of the BRM material. For many of the market samples, either because of the extended period of storage or semi-processing such as drying, we could not recover good quality DNA. Consequently, the amplification resulting from the market samples was limited to only 68 % (17/25) and 44 % (11/25) of market samples that yielded good quality sequences for *rbcL* and *psb*A-*trn*H regions, respectively (Table 3). These sequences were queried against those of the BRM generated in the study using BLASTn and also queried in a BLASTn search against the GenBank database (S1 Table 1).

For the DNA barcode region, *rbcL*, 14 of the 17 market samples did not match those of *S. asoca*, while for *psb*A-*trn*H region, 10 of 11 samples did not match. The market samples that matched *S. asoca* using the *rbcL* region were HAS 220 (from Kerala), HAS 107 (from Bangalore), and HAS 424 (from Kerala). On the other hand, only HAS 220 (from Kerala) matched with *S. asoca* using the *psb*A-*trn*H region. HAS 107 (from Bangalore) did not amplify the *psb*A-*trn*H region and hence was omitted from the analysis. It is not immediately clear why HAS 424, which matched *S. asoca* in the *rbcL* region, did not match at the *psb*A-*trn*H region. The inconsistency may have arisen because of the poor amplification success that was obtained for the two barcodes. For example, in HAS 424, the sequence length obtained was only 271 bp against the expected 545 bp. On the other hand, the sequence length obtained for *psb*A-*trn*H was good (330 bp) against an expected 480 bp.

This aside, the DNA barcoding analysis of the market samples clearly showed that there is a widespread adulteration or species admixture in trade of *S. asoca* in the country. The majority of market samples instead seem to align with many other species belonging to the families: Fabaceae, Combreatace, Caricaceae, Moringaceae, Meliaceae, Rhamnaceae and Putranjivaceae. In summary, and as evident from the UPGMA phylogenetic tree, the market samples, save a few, largely sit outside the group representing the authentic *S. asoca* samples (Figs. 2 and 3).

NMR spectroscopy of BRM of *S. asoca* and market samples

Metabolite identification using 1D and 2D NMR

NMR spectra of BRM of *S. asoca* bark samples showed the presence of a wide variety of metabolites. The metabolites identified using 1D and 2D NMR experiments have been summarized in S1 Table 2 and include peaks for metabolites such as amino acids, lipids, and phytosterols in the high-field region from 0.8 to 3.5 ppm, carbohydrates in the mid-field region from 3.5 to 5.5 ppm, and aromatic compounds in the low-field region from 6.0 to 9.0 ppm. Further, 1D and 2D NMR spectra were recorded for the market samples obtained from different sources. All the market samples showed the presence of a mixture of molecules, spanning the whole range of NMR spectra from 0.7 to 9 ppm. Figure 4, for example, shows an illustrative 1D 1H NMR spectra of *S. asoca* bark sample against those obtained from the market samples HAS 450 and HAS 318, recorded on 600 MHz NMR machine. Clearly, over the entire span, the spectra of the market samples do not match those of the BRM sample of *S. asoca*.

Aromatic region of *S. asoca* samples showed peaks for many pharmacologically significant phenolic metabolites such as rutin, hesperidin, naringenin, and caffeic acid. Such medicinally significant metabolites varied in their presence/absence in different market samples (Fig. 5). To further identify the different metabolites present in samples, 2D 1H DOSY NMR was used, which provides comprehensive information of the samples by virtual separation of the sample components where one dimension accounts for chemical shift values and other for diffusion coefficients. Diffusion NMR spectra for *S. asoca* and market sample HAS 181, showing differences in metabolites separated in both the cases are presented in S1 Fig. 1.

Multivariate statistical analysis

Chemical classification of metabolites responsible for differentiation between BRM of *S. asoca* and different market samples was performed using multivariate methods. Following detection of outliers using PCA, the binned data for the authentic plant extract replicates and a total of 25 market samples was first subjected to PLS-DA analysis to examine how much each market sample differentiates from the authentic plant samples on the visible score plot (S1 Fig. 2). Since each market sample varied individually from each other and from the BRM of *S. asoca*, to simplify and make the analysis more meaningful, market samples were divided into two main groups prior to further analysis based on separation of market sample by component 2 on scores plot. Market samples were divided such that group A consisted of 9 samples below 0 of component 2 and group B consisted of 14 samples above 0 of component 2. Market samples in each group were further compared separately with the authentic plant extract samples.

Figure 6 shows the dendrogram (which summarizes all the variation present in the dataset) prepared using hierarchical cluster analysis (HCA). Comparative PLS-DA analysis of individual trade samples in group A and authentic plant extract replicates, with the PLS-DA score plot (Fig. 7), shows a clear separation between the individual trade samples of the group and plant extract replicates. Taking sample HAS 450 as an example, Fig. 7b shows the variables (in bins) responsible for the separation in the score plot, in the corresponding loadings plot. Metabolites marked above 0.0 in the loadings plot are present in higher quantities in HAS 450 sample where as metabolites marked below 0.0 in the loadings plot are present

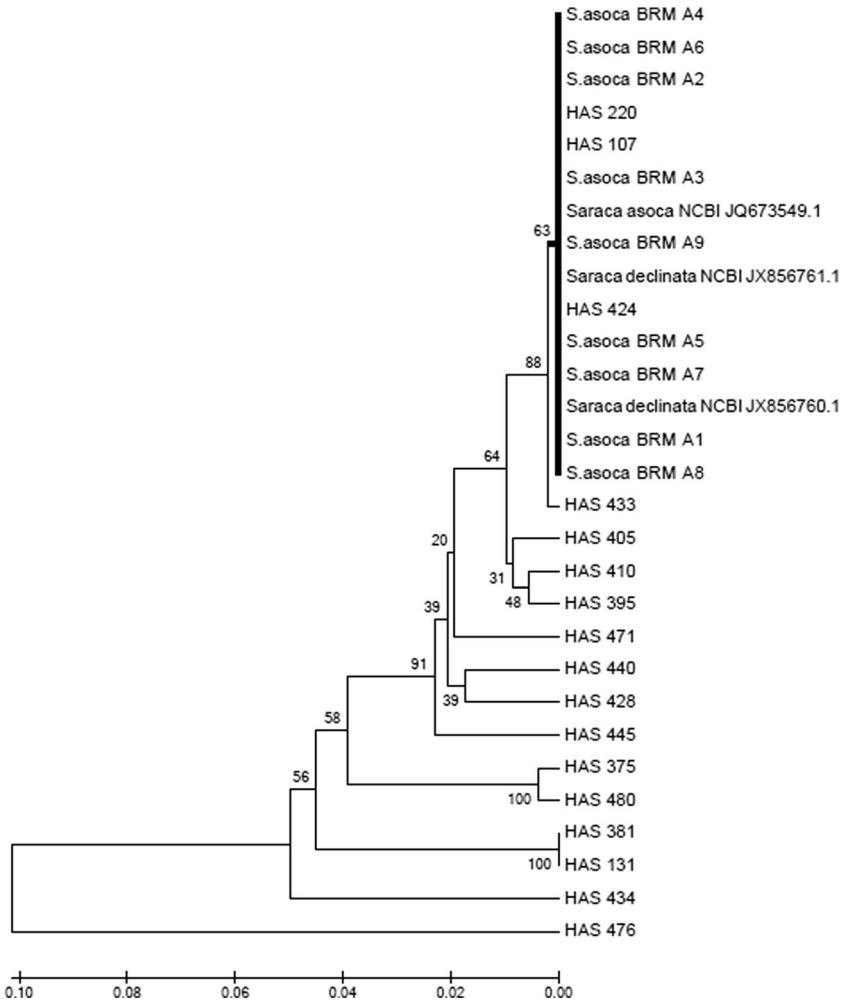

Fig. 2 *UPGMA* tree of Biological Reference Material (BRM) of *Saraca asoca* and raw drug trade samples using *rbcL* region. Codes for the BRM and trade samples are as presented in Table 1 and Table 2. The numbers at the nodes refer to the bootstrap values

in higher quantities in authentic plant extract samples. VIP scores parameter used to further confirm the variables identified to be responsible for group separations (Fig. 7c). A similar result was obtained for analysis of trade samples in group B (Fig. 8).

For each group, the results were further tested for statistical significance using ANOVA ($p < 0.01$) to identify which of the metabolites significantly contributed to differentiation between the BRM of *S. asoca* and trade sample followed by post hoc analysis (Fisher's LSD) to identify which samples were responsible for such differentiation. Based on this, 10 metabolites were identified for group A and 13 metabolites were identified for group B to be contributing most significantly towards differentiation (S1 Tables 3–4).

As can be seen, market samples, HAS 220, 405, and 375 were most similar to the authentic plant extracts as compared to other market samples. In other words, based on the NMR spectroscopic analysis, only 3 of the 25 market samples matched the authentic *S. asoca* samples. These results independently confirm those obtained from the DNA barcoding analysis that there is widespread species adulteration in the trade of *S. asoca*. However, only HAS 220 (from Kerala) was found to match with the authentic plant material, based on both DNA barcoding and NMR spectroscopic analysis. HAS 405 and 375 did not show a match with *S. asoca* based on DNA barcoding analysis (for HAS 405 based on *rbcL* and for HAS 375 based on both *rbcL* and *psb*A-*trn*H). This discordance can be best explained by the fact that NMR spectroscopic technique, unlike DNA barcoding, only provides for species chemical signatures and by itself cannot be used as species identifier, for two or more different species may share the same metabolite pool. Thus, though HAS 405 and 375 did not match *S. asoca* genetically, it is likely that they share similar chemical profiles. Similarly, though HAS 107 and HAS 424 had similar genetic makeup as authentic plant samples as shown by DNA bar coding, they did not have same chemical profiles as authentic plant material though their metabolite profiles were closely related to those of plant materials, as can be seen from dendrogram (Fig. 6). This difference in profiles can be attributed to several factors such as degradation of certain metabolites on storage or drying, different environmental conditions of samples, etc.

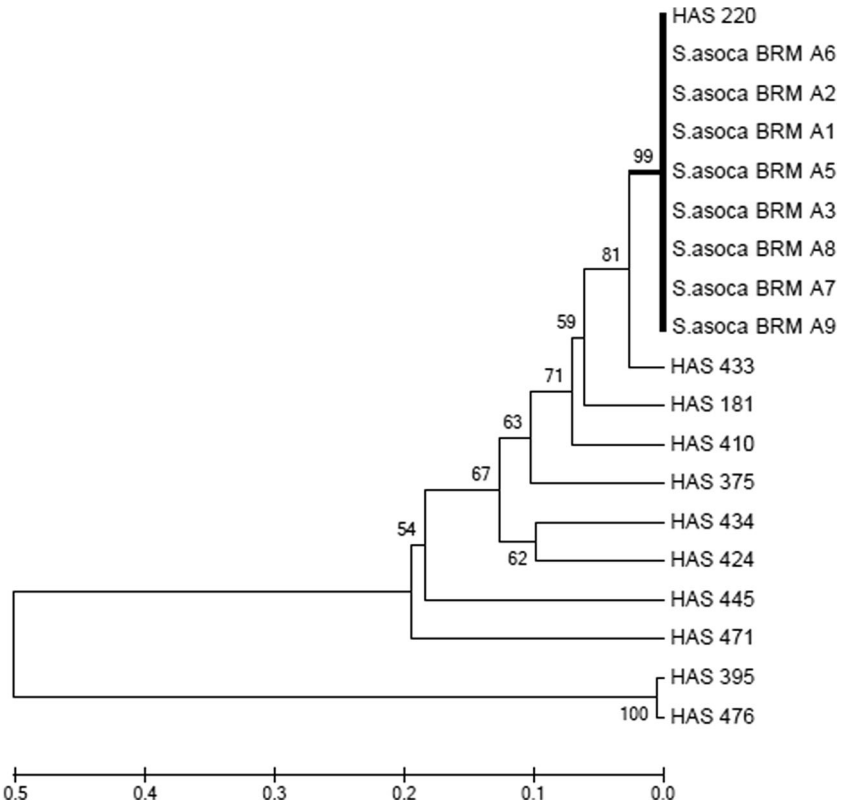

Fig. 3 *UPGMA* tree of Biological Reference Material (BRM) of *Saraca asoca* and raw drug trade samples using *psb*A-*trn*H region. Codes for the BRM and trade samples are as presented in Table 1 and Table 2. The numbers at the nodes refer to the bootstrap values

Since HAS 220 was found to match the most with authentic plant samples, we tried to identify the metabolites that still contributed to its slight differentiation from plant materials using the multivariate analysis of OPLS-DA, with one predictive and one orthogonal components (S1 Fig. 3) [40]. HAS 220 was found to be highly similar to *S. asoca* plant materials with very few metabolites differing in their concentrations to be responsible for separation. S1 Table 5 provides the list of metabolites contributing to differences between HAS 220 and authentic plant samples. This further confirmed the authenticity of HAS 220. Another sample HAS 476 was found to be highly different from authentic plant materials, both by DNA barcoding and NMR multivariate analysis, as can be seen in Figs. 2, 3, and 6. We thus tried to identify the metabolites responsible for such a differentiation using the same method of OPLS-DA (S1 Fig. 4). S1 Table 6 provides the list of metabolites responsible for separation. Fig S1 5 shows comparative NMR spectra for authentic plant material, HAS 220 and

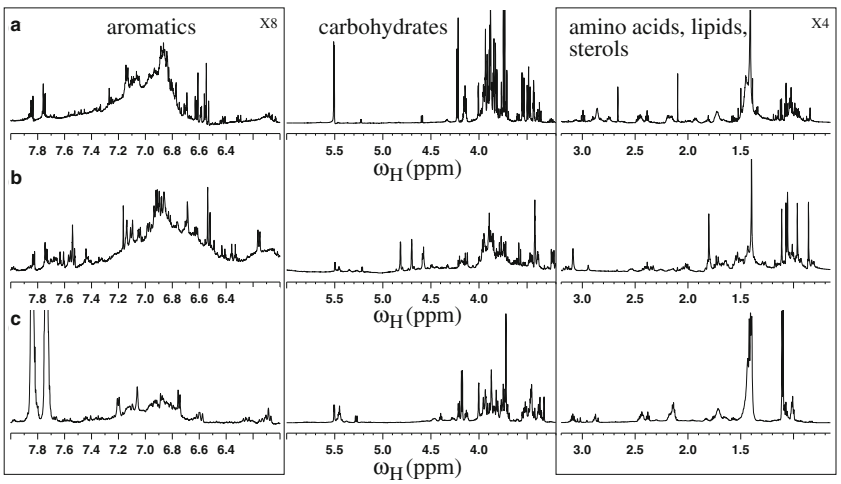

Fig. 4 1D ^{1}H NMR spectrum recorded at 600 MHz of **a** *Saraca asoca* (BRM A4) bark, **b** drug HAS 450 from group A, and **c** drug HAS 318 from group B, showing NMR resonances of amino acids, lipids, sterols, carbohydrates, and aromatic groups. Codes of the BRM and HAS are given in Table 1 and Table 2

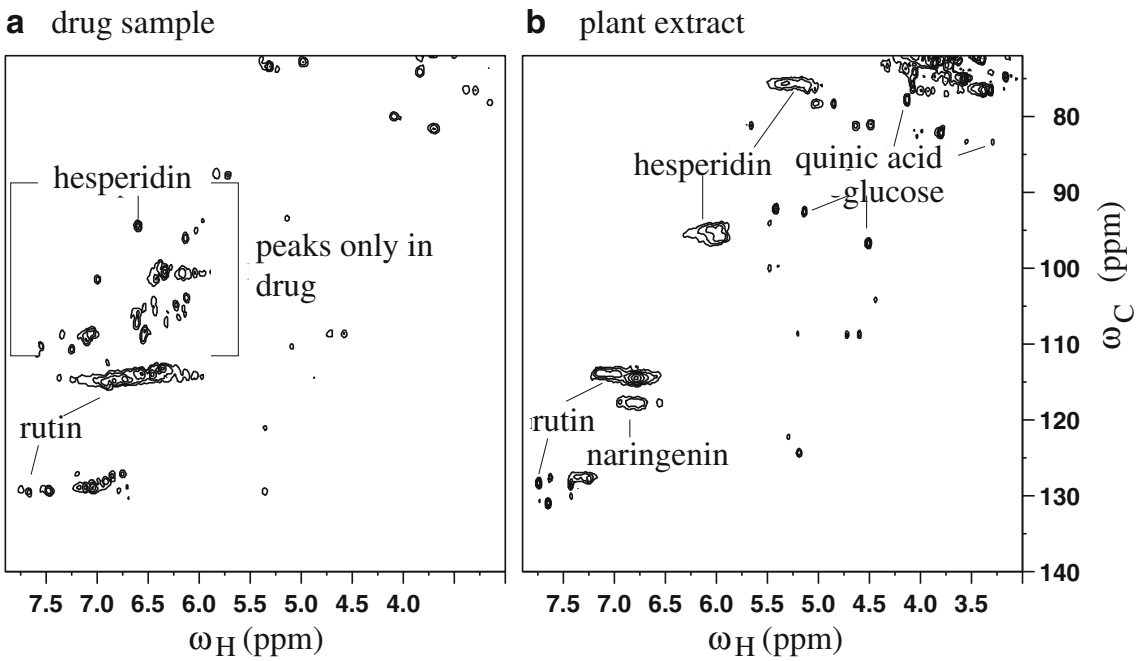

Fig. 5 2D HSQC NMR spectrum of **a** representative raw drug sample (HAS 318) and **b** *Saraca asoca* (BRM A4) bark recorded at 600 MHz showing different metabolites identified in bark samples and additional peaks observed in drug samples in the aromatic region. Codes of the BRM and HAS are given in Table 1 and Table 2

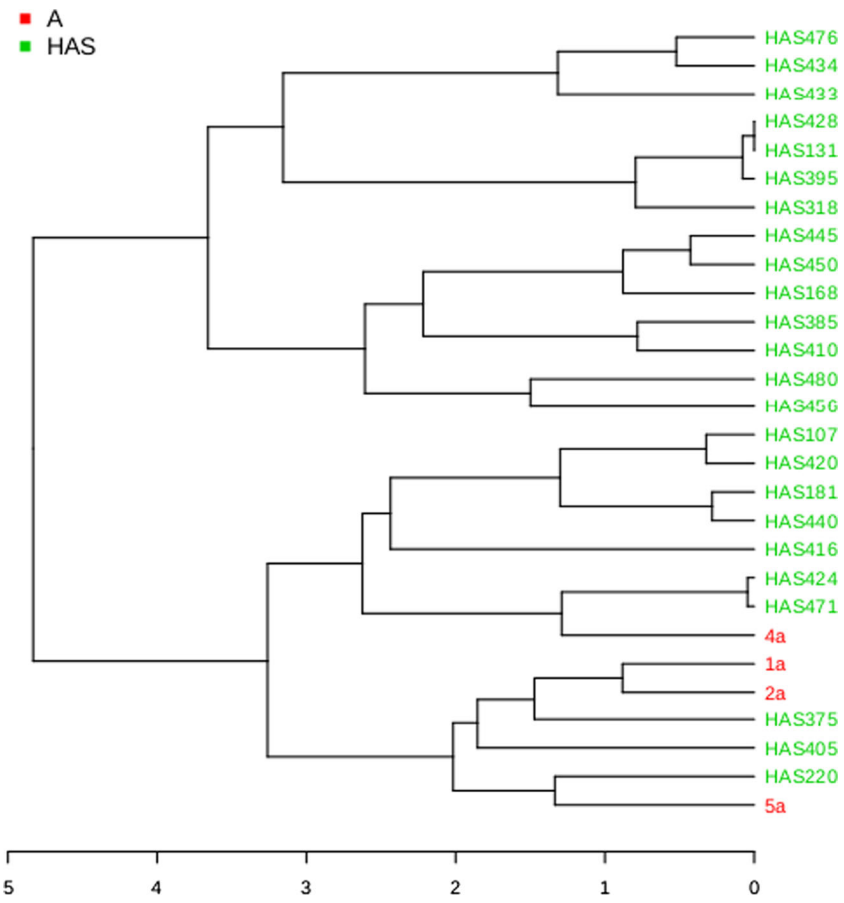

Fig. 6 Clustering result shown as dendrogram. *1a*, *2a*, *4a*, and *5a* represent *Saraca asoca* BRM bark samples BRM A1, BRM A2, BRM A4, and BRM A5, respectively, while HAS are drug samples. Codes of the BRM and HAS are given in Table 1 and Table 2

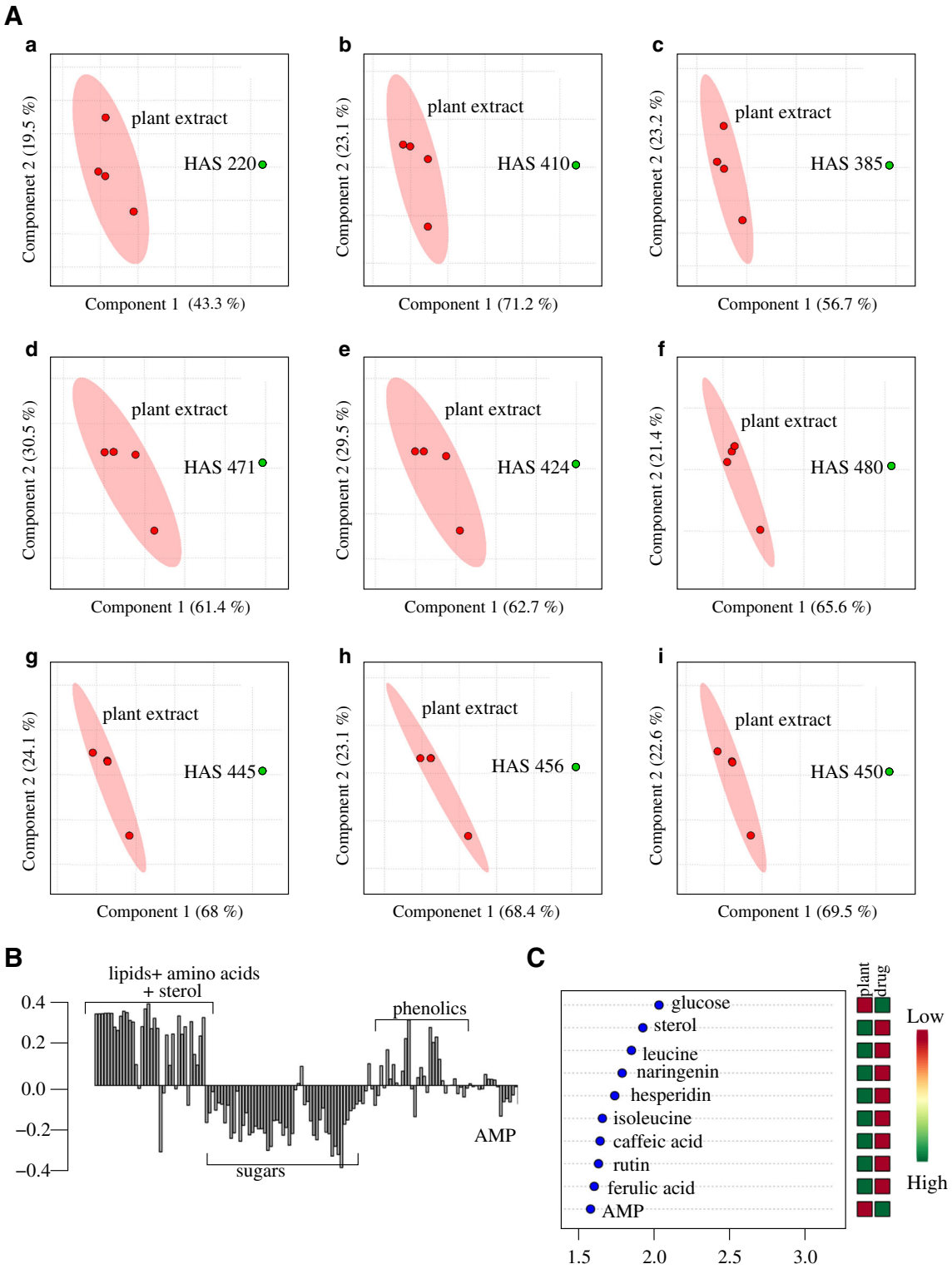

Fig. 7 **a** PLS-DA score plots (Component 1 vs Component 2) for comparing *Saraca asoca* (BRM A4) bark samples with (*a*) drug HAS 220, (*b*) drug HAS 410, (*c*) drug HAS 385, (*d*) drug HAS 471, (*e*) drug HAS 424, (*f*) drug HAS 480, (*g*) drug HAS 445, (*h*) drug HAS 456, and (*i*) drug HAS 450. **b** Loadings plot showing metabolites present in higher quantities in drug samples (HAS 450), marked above 0.0 and metabolites present in higher quantities in plant bark samples, marked below 0.0 and **c** VIP scores with the corresponding expression heat map. *Red and green* indicate decreased and increased metabolite levels, respectively

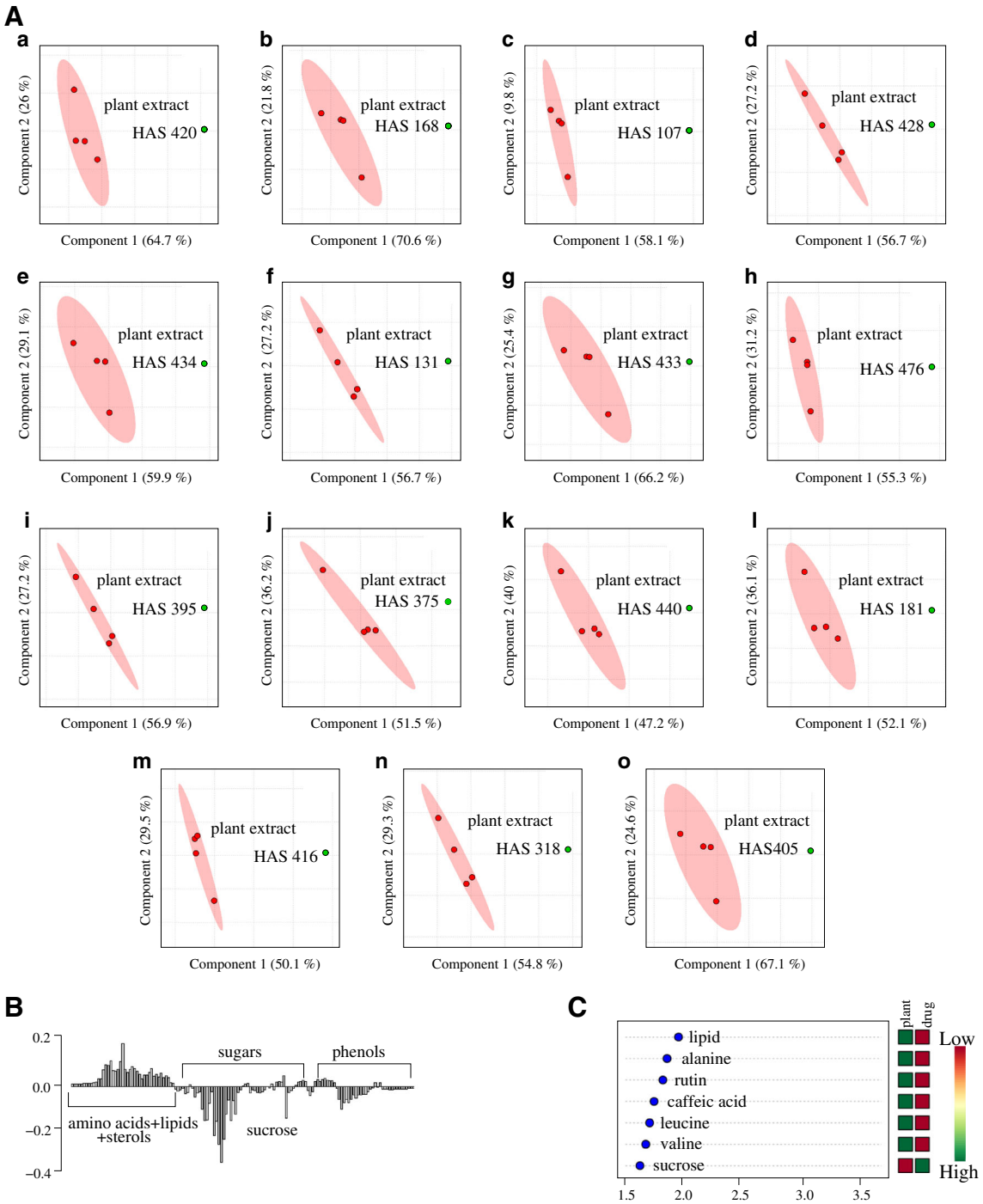

Fig. 8 **a** PLS-DA score plots (Component 1 vs Component 2) for comparing *Saraca asoca* (BRM A4) bark samples with (*a*) drug HAS 420, (*b*) drug HAS 168, (*c*) drug HAS 107, (*d*) drug HAS 428, (*e*) drug HAS 434, (*f*) drug HAS 131, (*g*) drug HAS 433, (*h*) drug HAS 476, (*i*) drug HAS 395, (*j*) drug HAS 375, (*k*) drug HAS 440, (*l*) drug HAS 181, (*m*) drug HAS 416, (*n*) drug HAS 318, and (*o*) drug HAS 405. **b** Loadings plot showing metabolites present in higher quantities in drug samples (HAS 318), marked above 0.0 and metabolites present in higher quantities in plant bark samples, marked below 0.0 and (C) VIP scores with the corresponding expression heat map. *Red and green* indicate decreased and increased metabolite levels, respectively

HAS 476 in the aromatic region where most pharmacologically significant metabolites are identified. As can be seen, NMR spectra of plant material and HAS 220 are highly similar, whereas NMR spectra of HAS 476 differ a lot from both authentic plant material and HAS 220, thereby showing its adulterated nature.

In summary, our study provides the first comprehensive analysis to demonstrate the widespread adulteration in the

market samples of the highly traded medicinal plant, *S. asoca* in India. To the extent that we have been able to determine, the adulterants comprise of several plant species spread over 7 different families. The consequences of these species on the effectiveness and safety of consumers remain to be addressed. Srirama et al. (2010) addressing the primary drivers of adulteration in herbal trade argued that adulteration in many cases could simply be due to the naivety of the collectors who may not be able to taxonomically discriminate between co-occurring conspecific species (such as between *P. amarus* and other species of *Phyllanthus* occurring in the same area) [21]. However, another reason could be because of the fact that two or more species may share the same vernacular name and thus lead to confusion in the minds of naive collectors [23]. The latter seems to be partially true with adulterants recovered from *S. asoca* market samples by earlier workers and this study. For example, two of the adulterants recovered, namely *S. declinata* and *Polyalthia longifolia*, share the same vernacular name, Ashoka or Asoka with *S. asoca* [41]. However, beyond these species, our study revealed a number of other adulterants that neither are phylogenetically related to *S. asoca* nor share a common vernacular name with *S. asoca*. Such adulterations seem to be primarily motivated towards profit and to keep pace with the increasing market demand for *S. asoca*.

Irrespective of the immediate drivers leading to such widespread adulteration is the consequence of such adulteration on the health and safety of unsuspecting consumers. Only recently have studies begun to address these consequences [42]. For example, a study by Wallace et al. showed that products of *Echinaceae* that had admixtures of walnut lead to severe nut allergies in consumers [37]. In a more alarming case, where roots of *Stephania tetrandra* (for anti-inflammatory property) were contaminated with roots of a toxic herb *Aristolochia fangchi*, perhaps due to them sharing the same vernacular name, led to more than 100 women suffering kidney failures [42]. To date, there are no published studies describing the consequences of adulterants in *S. asoca* products on consumer health.

Raw herbal trade markets in India, as perhaps in many other parts of the world, have been largely unregulated [43]. The markets were operated in a close-knit comprising the collectors, local healers or herbal physicians, and the consumers. Essentially, the entire market was local and rarely even regional. However, with opening up of trade across countries and demand for natural products as opposed to conventional allopathic remedies, the demand and consequently the trade of raw herbal trade grew by leaps and bounds. For example, trade of medicinal plant products, a decade after globalization in India, grew by several folds compared to a decade before globalization [43]. Dependent as it is still on natural populations of the species, the increased demand has led to the severe decimation of populations of a large number of species [43]. Faced with dwindling resource bases but with increased demand probably arose the drive to adulterate raw herbal trade products. Unfortunately, not only is this counter-productive but also has the ability in the long term to discredit the indigenous systems of medicine leading to loss of consumer confidence and lowering of trade of herbal products [44, 45]. Today, the large-scale international trade in herbal products has heightened the concern of the safety and efficacy of herbal products [22, 46, 47].

Conclusion

The challenges of meeting the increased demand for raw herbal drugs must be met eventually by domesticating and cultivating medicinal plants that are highly traded. However, meanwhile, it is important that stringent regulations be imposed to ensure the quality of herbal products by authenticating them at various levels of the production. Srirama et al. (unpublished) proposed the establishment of an Herbal Authentication System that not only establishes a national level Biological Reference Material (BRM) library of all plants in trade but also develops DNA barcodes of the material. All herbal material can then be authenticated and certified before use and transformation into products by herbal industries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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