Q1 **ARTICLE TYPE**

Quantification of *Shankhpushpi* using Phytochemical and Molecular Markers

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ABSTRACT

Shankhpushpi is a well-known brain tonic of *Ayurveda*. However, controversies associated with its authenticity due to several plant species such as *Convolvulus pluricaulis* Choisy., *Clitoria ternatea* L., *Evolvulus alsinoides* L., and *Tephrosia purpurea* (L.) Pers. reported in the literature. In the present study high-performance thin-layer chromatography (HPTLC) was used to know the concentration of phytochemical markers of all the collected species and commercial samples of *Shankhpushpi*. Random amplification of polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) profiling was done, and a phylogenetic tree was generated to check their genetic similarity with collected plant species. The results showed variation in phenolics (ferulic and caffeic acid) and terpenoid (β-sitosterol and lupeol) markers in all the *Shankhpushpi* samples. These variations were correlated with RAPD and ISSR profiling, which showed that *Shankhpushpi* samples of Delhi, Hisar, and Jaipur came in same cluster with *C. pluricaulis* in the dendrogram. Hence, the above commercial samples resembled *C. pluricaulis*. However, phytochemical markers, RAPD and ISSR profiling in other samples of *Shankhpushpi* were showed a mixture of two species. The Mumbai sample was unidentified. Phytochemical markers and molecular profiling are important tools for herbal drug authentication and applicable to assure the quality, efficacy, and batch to batch consistency in the pharmaceutical preparation of *Shankhpushpi*.

Keywords: Adulterants, HPTLC, ISSR, RAPD, Shankhpushpi, Substitutes.

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INTRODUCTION

Shankhpushpi is the conch or *shankh* shape flowers, which are considered to be auspicious. In most of the *Ayurvedic* text, *Convolvulus pluricaulis* Choisy. (CP) (Family, Convolvulaceae) which bears white, pink flowers is reported as *Shankhpushpi*.[1] However, due to similar flower structure in species like *Clitoria ternatea* L. (CT), *Evolvulus alsinoides* L.(EA) and *Tephrosia purpurea* (L.) Pers. (TP) are also called as *Shankhpushpi*.[2] *Clitoria ternatea* L. (Family, Fabaceae) as a climber has been accepted as *Shankhpushpi* by most of the South Indian *Vaidyas*.[2] Similar looking herb *Evolvulus alsinoides* L. (Family, Convolvulaceae) bearing blue flowers is commonly known as *Vishnukaranta*, believed to be a variety of *Shankhpushpi*.[3] Likewise, *Tephrosia purpurea* (L.) Pers. (Family, Fabaceae), a shrub with pink color flowers is also sold as *Shankhpushpi* in Maharashtra and South Indian markets.[4] On the basis of the above account, it is obvious that different plant species attributed to *Shankhpushpi* are present in the herbal drug market of India; therefore, correct identification and quality control of starting material is an essential prerequisite to ensure reproducibility and quality of this important *Ayurvedic* drug.

Shankhpushpi has several medicinal properties, *viz.*, intellect promoting augments memory, and rejuvenating to nervous tissue.[5] It is also used for alleviates abdominal pain, treatment of a cough, toxins, epilepsy, useful in urinary disorders, and hypertension.[4,5] Pharmacologically it has several activities like nootropic, anxiolytic, anticonvulsant, antistress, memory-enhancing activity, antidepressant, and antiepileptic activity.[6-9] Besides, anti-inflammatory, antipyretic, antimicrobial activity, antifungal properties, antidiabetic activity, analgesic activity, antitumor activity, and anti-ulcerogenic activity have also been reported.[10-12] There are several important *Ayurvedic* and *Unani* formulations of "*Shankhpushpi*" available in the herbal drug market, e.g.,

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Agastyaharitaki, BR-16 A Brahma Rasayana, Brahmi Ghrta, Brahmi Vati, Dimagheen, Gorocanadi Vati, Manasmitra Vataka, Shankhavali Churna, and *Shankhpushpi Syrup.*[1,13,14]

Nowadays, pharmacognosy has become more interdisciplinary because of subsequent advances in analytical chemistry and the application of genomics in herbal drug research. HPTLC is a widely used technique employed in the pharmaceutical industry for the identification and detection of adulterants in herbal products and quality control of herbs. In HPTLC, several samples can be run simultaneously by use of a smaller quantity of mobile phase; therefore, it has become one of the preferable chemoprofiling techniques and included in *Ayurvedic* Pharmacopeia for quality control of herbal drug.[1,15-17] Besides, molecular markers such as RAPD and ISSR are also being used to characterized and identify plants used in the herbal drug preparations.[18] The aim of the present study was to analyze the freshly collected and commercial samples of *Shankhpushpi* using HPTLC, RAPD, and ISSR methods for quality control in herbal drug preparations.

MATERIAL AND METHODS

Collection and Procurement of Plant Materials

The plant species of drug attributed to *Shankhpushpi* were collected from their natural habitats of different cities of India (Table 1), and their herbarium specimens were prepared and identified as per standard herbarium procedure[19] and deposited in the herbarium of CSIR-National Botanical Research Institute, Lucknow, for accession number LWG series (Table 1). The commercial samples of *Shankhpushpi* were procured from crude drug markets of Lucknow, Delhi, Varanasi, Hisar, Jalandhar, Dehradun, Mumbai, and Jaipur (SM1, SM2, SM3, SM4, SM5, SM6, SM7, and SM8) in India.

Processing of Plant Material

All the samples of *Shankhpushpi* grounded into a coarse powder and placed in appropriately sized volumetric flasks, and 100 mL methanol was added to 10 grams of powder of each plant and then shaken on a shaker for 5–6 hours, kept at rest overnight and filtered after that. The procedure was repeated thrice with methanol (100 mL) at room temperature (25 ± 2°C). The methanolic extracts were filtered through Whatman No. 1 filter paper and combined. The combined extracts were concentrated under reduced pressure using Rotavapor R-114 (Buchi) at a temperature of 45°C and freeze-dried in the freeze-dry system/Freezone 4.5 (Labconco). Dissolved the accurately weighed 10 mg of the extract in 1 mL methanol and filtered through a 0.45 µm filter membrane, the filtrate was used as a sample solution.

High-Performance Thin Layer Chromatography (HPTLC) and Estimation of Phytochemical Marker

The HPTLC has performed on 20 × 10 cm silica TLC plates, coated with 0.2 mm layers of nanosilica G containing ultraviolet (UV) 254 fluorescent indicator (Aluchrosep nanosilica G/ UV254, S.D. Fine Chem. Ltd., Mumbai, India). All the samples (20 µL) were applied using 100 µL hamilton syringes with Linomat 5 applicator (Camag, Muttenz, Switzerland) under a flow of N2 gas. Extract solutions were applied as bands onto the TLC plate. The plates were developed using a standardized solvent system toluene-ethyl acetate-formic acid (8.5:1.5:0.1 v/v/v) in a Camag glass twin-trough chamber (20 × 10 cm), by applying the following experimental conditions: temperature, 25 ± 2°C, and relative humidity, 40% (a pre-equilibrate twin-trough chamber with the mobile phase for 30 minutes before analysis). Scanning was performed using a TLC Scanner 3 (Camag). The plate developing distance was 8 cm from the lower edge of the plate. The plate was dried in a stream of warm air for 5 minutes. The densitometry scanning was done at absorbance mode at 500 nm using Camag TLC scanner III with winCATS 3.2.1 software for ferulic acid (FA), caffeic acid (CA), β-sitosterol (BS), and lupeol (LUP). The source of light was the deuterium and tungsten beam. Method of quantification followed according to the standard protocol.[15,16,20]

Genomic DNA Extraction

Total genomic DNA was isolated from different genotypes of *Shankhpushpi* following the cetyl trimethyl ammonium bromide (CTAB) method.[21] Quantitation of purified DNA was carried out by UV spectrophotometry using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA).

RAPD and ISSR Amplification

Initially, 20 RAPD and 16 ISSR primers were screened with two templates DNA as, and only those primers were considered, which produced consistent and reproducible profiles. Finally,

Table 1: List of plant materials used in the present study

S. No.	*Plant name*	*Sample code*	*Source*	*Accession No.*
1	*Convolvulus pluricaulis*	CP1	Lucknow	LWG-0001
2	*Convolvulus pluricaulis*	CP2	Haryana	LWG-34
3	*Convolvulus pluricaulis*	CP3	Chitrakoot	LWG-35
4	*Clitoria ternatea* (white)	CT1	Lucknow	LWG-0002
5	*Clitoria ternatea* (white)	CT2	Faizabad	LWG-0003
6	*Clitoria ternatea* (white)	CT3	Trivandrum	LWG-32
7	*Evolvulus alsinoides*	EA1	Chhattisgarh	LWG-31
8	*Evolvulus alsinoides*	EA2	Chitrakoot	LWG-30
9	*Tephrosia purpurea*	TP	Lucknow	LWG-41

6 RAPD and 5 ISSR primers were considered in the present study. The RAPD and ISSR PCR reactions were carried out according to Singh N *et al.*[22] The PCR products were resolved on 1.5% agarose gel in 1X TBE buffer stained with ethidium bromide at 100 V, and the fragments were visualized and documented in UV Tech gel documentation system (UK). The gel profiles were photographed and stored as digital pictures in the gel documentation system.

Data Analysis

All the experiments were carried out in triplicate, and results represented as average with ± SD. Data analysis for RAPD and ISSR was carried out only for those accessions that resulted in consistent and reproducible profiles. Data were scored as presence (1) or absence (0) of a band, and only distinct and well-separated bands were included in the final analysis. The polymorphic information content (PIC) was calculated[23] for each primer. In order to determine the utility of each of the marker systems, the diversity index (DI), marker index (MI), and resolving power (RP) were calculated as well as the percentage of polymorphism (% P) were estimated.[24] A pairwise matrix of similarity between genotypes was determined for the band data using Jaccard's similarity coefficient for the UPGMA method, and the dendrogram was constructed in the Free Tree program (ver. 0.9.1.5).[25]

RESULTS

Phytochemical Marker Quantification

The HPTLC analysis showed considerable variation in phenolics and terpenoid (ferulic acid, caffeic acid, lupeol, and β-sitosterol) content in different genotypes investigated. The results are presented in Table 2. *C. pluricaulis* can be considered as the actual sources of *Shankhpushpi* and *C. pluricaulis* contains the average concentration (9.223 ± 0.013, 11.15 ± 0.041, 24.65 ± 0.032, and 46.61 ± 0.051 mg/100g) of ferulic acid, caffeic acid, lupeol, and β-sitosterol, respectively. Chitrakoot population of *C. pluricaulis* was found to be the highest accumulator of phenolics, and terpenoid content, followed by *C. ternatea* and *E. alsinoides*, and least phenolics was observed in *T. purpurea* in which cafferic acid was absent. The calibration curve for each estimated phytochemical marker was obtained by linear least square regression of peak areas of the developed spots *vs.* concentrations (Figure 1). The linear regression equation for FA (y = 106.332 + 0.427 × x, r = 0.9822, SD = 5.38%), CA (y = 16.674 + 0.370 × x, r = 0.999, SD = 1.64%), BS (y = 56.130 + 0.080 × x, r = 0.9894, SD = 2.44%), and LUP (y = 251.157 + 0.366 × x, r = 0.96868, SD = 4.32%). The concentration of the above four quantified phytochemical markers in all the samples of *Shankhpushpi* were quantified and are given in Table 2.

Molecular Markers

Twenty RAPD and sixteen ISSR primers were used, of which only six RAPD and five ISSR primers generated clear and reproducible banding patterns. In RAPD analysis, a total of 52 bands were produced with an average of 8.33 bands per primer, among which 49 bands were polymorphic, showing 95% polymorphism. Polymorphic information content, resolving power, and marker index values for RAPD markers were found 0.4, 10.07, and 15.7, respectively. However, a total of 45 ISSR bands were produced with five ISSR primers, of which 43 bands were polymorphic, showing 96% polymorphism. Polymorphic information content, resolving power, and marker index values for ISSR markers were found 0.4, 9.06, and 17.7, respectively (Table 3).

Table 2: Phenolics and terpenoid markers concentration in collected as well as market samples of *Shankhpushpi*

		Phytochemical markers mg/100g			
S. No.	*Samples*	*Ferulic acid*	*Caffeic acid*	*β-sitosterol*	*Lupeol*
1	CT1	6.78 ± 0.02	1.25 ± 0.031	49.63 ± 0.002	20.24 ± 0.013
2	CT2	7.24 ± 0.013	1.44 ± 0.006	50.81 ± 0.012	22.41 ± 0.015
3	CT3	1.68 ± 0.002	0.38 ± 0.005	14.76 ± 0.007	20.22 ± 0.93
4	CP1	9.71 ± 0.021	9.42 ± 0.023	8.21 ± 0.008	25.33 ± 0.005
5	CP2	1.75 ± 0.013	1.09 ± 0.014	23.21 ± 0.017	13.10 ± 0.007
6	CP3	16.21 ± 0.006	23.02 ± 0.003	108.50 ± 0.002	37.40 ± 0.42
7	EA1	0.52 ± 0.011	0.148 ± 0.022	13.12 ± 0.012	9.01 ± 0.002
8	EA2	8.41 ± 0.012	0.38 ± 0.006	23.8 ± 0.014	11.9 ± 0.005
9	TP	2.07 ± 0.024	–	105.4 ± 0.012	17.44 ± 0.032
10	SM1	18.35 ± 0.02	30.37 ± 0.05	6.47 ± 0.03	2.40 ± 0.31
11	SM2	15.0 ± 0.41	61.95 ± 0.23	2.08 ± 0.21	1.21 ± 0.04
12	SM3	23.2 ± 0.05	30.59 ± 0.13	5.25 ± 0.04	3.05 ± 0.01
13	SM4	6.36 ± 0.02	45.31 ± 0.12	2.52 ± 0.21	3.63 ± 0.04
14	SM5	18.5 ± 0.014	55.03 ± 0.06	9.07 ± 0.06	5.85 ± 0.13
15	SM6	0.32 ± 0.06	4.152 ± 0.02	7.01 ± 0.04	9.40 ± 0.18
16	SM7	13.44 ± 0.15	26.4 ± 0.16	6.36 ± 0.12	17.68 ± 0.09
17	SM8	19.53 ± 0.06	36.57 ± 0.05	2.83 ± 0.15	2.95 ± 0.23

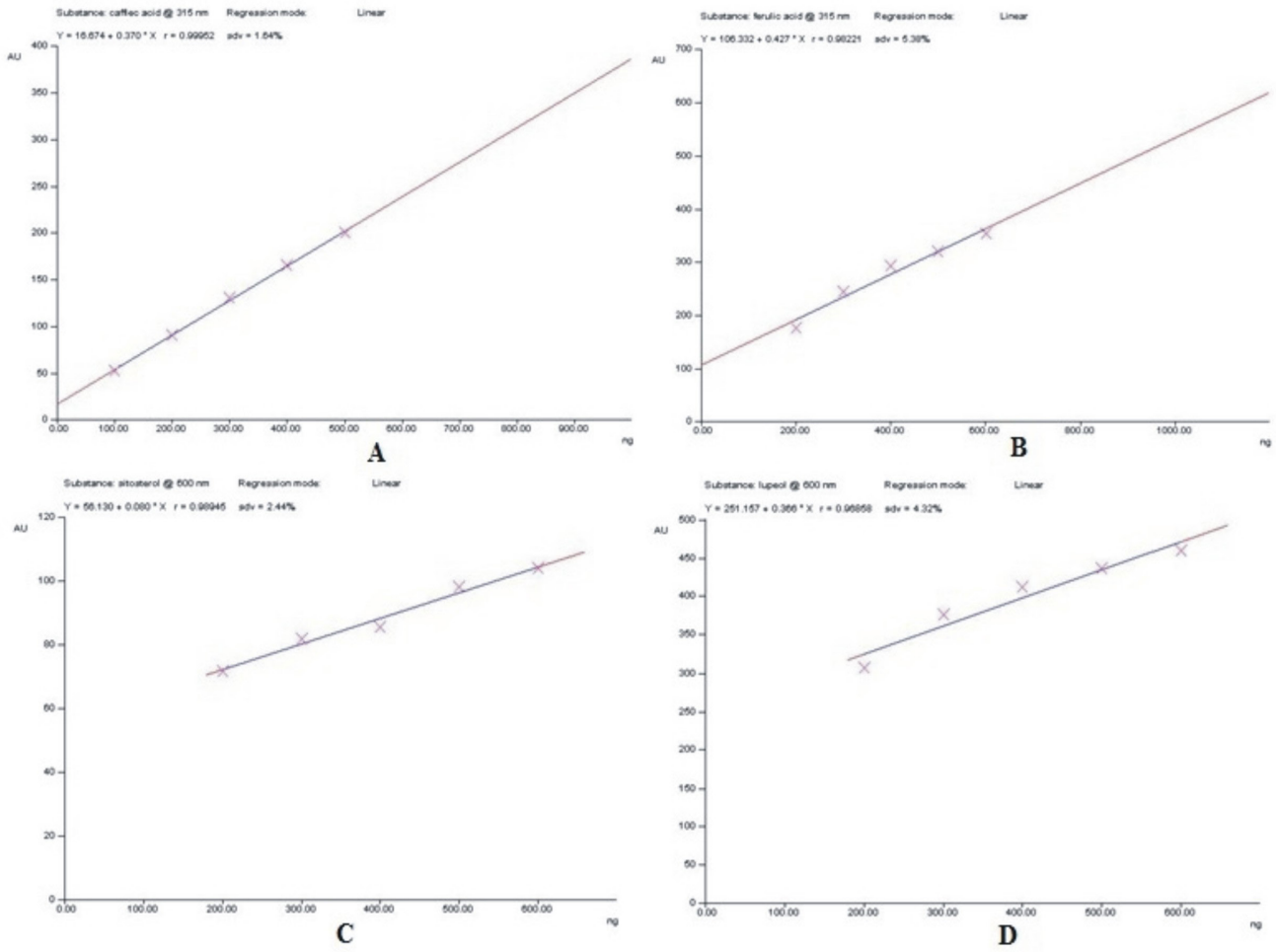

Figure 1: Linear regression graph for four phytochemical markers quantified in *Shankhpushpi*; A = caffeic acid; B = ferulic acid; C = beta-sitosterol; D = lupeol

Table 3: RAPD and ISSR primers used for amplification

RAPD	*Sequence (5-3')*	*TB*	*PB*	*PPB*	*PIC*	*RP*	*DI*	*MI*
OPG-05	CTGAGACGGA	13	11	85	0.4	14.47	3.1	15.7
OPG-06	GTGCCTAACC	9	8	89	0.3	8.24		
OPG-10	AGGGCCGTCT	7	7	100	0.4	7.76		
OPG-13	CTCTCCGCCA	9	9	100	0.4	10		
OPG-14	GGATGAGACC	8	8	100	0.3	8.24		
OPG-16	AGCGTCCTCC	6	6	100	0.4	5.65		
	Total	52	49	95	0.4	10.07		
**ISSR*		*TB*	*PB*	*PPB*	*PIC*	*RP*	*DI*	*MI*
UBC-809	AGAGAGAGAGAGAGAGG	8	6	75	0.3	11.88	3.4	17.7
UBC-836	AGAGAGAGAGAGAGAGYA	7	7	100	0.4	7.76		
UBC-841	GAGAGAGAGAGAGAGAYC	9	9	100	0.4	9.06		
UBC-861	ACCACCACCACCACCACC	9	9	100	0.4	9.18		
UBC-886	CTCCTCCTCCTCCTCCTC	12	12	100	0.4	12.47		
	Total	45	43	96	0.4	9.06		
	Cumulative*	97	92	95	0.4	9.56	3.3	16.7

ISSR = primers: Y = (C, T); Cumulative = Combined data of RAPD and ISSR; TB = total band; PB = polymorphic band; PPB = percentage polymorphic band; PIC = polymorphic information content; RP = resolving power; DI = diversity index; MI = marker index

Cumulative (RAPD + ISSR) marker analysis showed 97 amplified bands, of which 92 bands were polymorphic, showing 95% polymorphism. Polymorphic information content, resolving power, and marker index values for cumulative markers were found 0.4, 9.56, and 16.7, respectively (Table 3).

Species-specific bands generated through different RAPD and ISSR primers represented the identification markers for various species. The unique bands of 310 bp (approximately) amplified by primer OPG16 for *C. pluricaulis* and the unique band of 208 bp (approximately) amplified by primer OPG14 for *E. alsinoides*, a similarly unique band of 334 bp (approximately) amplified by primer OPG06 for *C. ternatea* and the unique band of 144 bp (approximately) amplified by primer ISSR-836 for *T. purpurea* (Figure 2).

The UPGMA method used to generate a tree for cluster analysis using the similarity coefficients. The resulting dendrogram differentiated all genotypes in two major clusters. Cluster 1 represented 12 genotypes consisting of all *C. pluricaulis* and *E. alsinoides* species with seven market samples SM1 to SM8 except SM7, which was not grouped with any cluster. Cluster 2 represented all genotypes of *C. ternatea* and *T. purpurea* species (Figure 3).

DISCUSSION

Chemical and molecular analysis is the most accepted and appropriate method for the authentication of plant-based drug/formulation.[15,18,20,26-29] As plants are an important source of potentially bioactive chemicals, phytochemical analysis helps in determining the quality, purity, and efficacy; however, the molecular aspect identifies the true genetic source, especially in the powder form.[15,17,18,30] In recent years, TLC/HPTLC is considered a significant phytochemical analytical technique

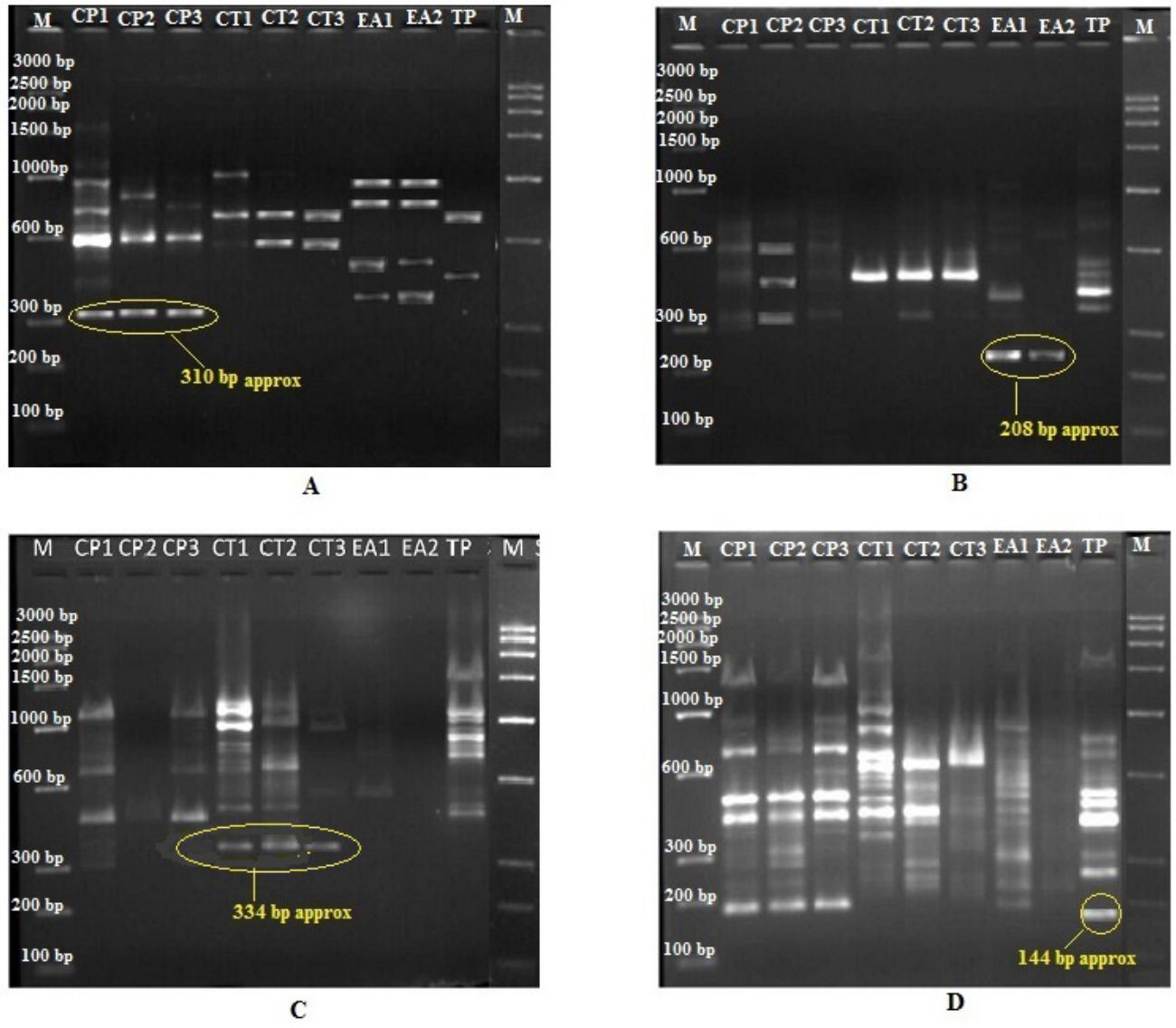

Figure 2: RAPD/ISSR profile showing species-specific band using primer A-OPG 16; B-OPG 14; C-OPG 6; D-UBC 836; Track showed from left low range DNA ruler (M); *Convolvulus pluricaulis*-Lucknow (CP1), Haryana (CP2), and Chitrakoot (CP3); *Clitoria ternatea*-Lucknow (CT1), Trivandrum (CT2), and Faizabad (CT3); *Evolvulus alsinoides*-Durg (EA1) and Chitrakoot (EA2); *Tephrosia purpurea*-Lucknow (TP)

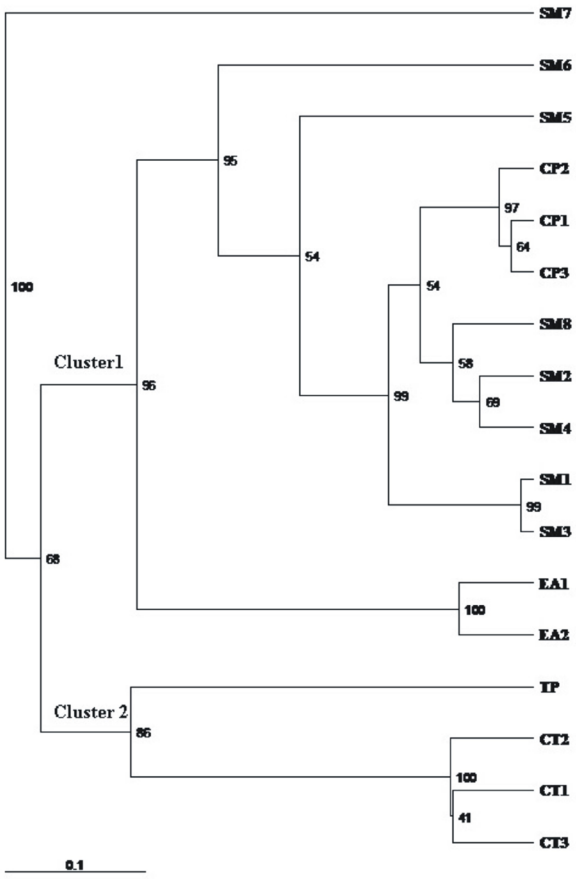

Figure 3: Dendrogram of all the *Shankhpushpi* samples showed *Convolvulus pluricaulis*-Lucknow (CP1), Haryana (CP2), and Chitrakoot (CP3); *Evolvulus alsinoides*-Durg (EA1) and Chitrakoot (EA2); *E. nummularius*-Lucknow (EN); *Clitoria ternatea*-Lucknow (CT1), Trivandrum (CT2), and Faizabad (CT3); *Tephrosia purpurea*-Lucknow (TP); market samples-Lucknow (SM1), Delhi (SM2), Varanasi (SM3), Hisar (SM4), Jalandhar (SM5), Dehradun (SM6), Mumbai (SM7), and Jaipur (SM8)

and extensively being used in the quality evaluation of plant materials. It includes fingerprint profiling for the assessment of chemical constituents, identification of adulterants/substitutes, and quantitative estimation of bio-markers in plant drugs to maintain batch to batch consistency of the products.[16,20] However, we have already published a TLC profile based authentication report in the case of *Shankhpushpi*,[31] therefore, in the present study, the quantity of two phenolics (ferulic acid and caffeic acid) and two triterpenoids (β-sitosterol and lupeol) markers were estimated. The data showed (Table 2) that *C. pluricaulis* contains the average concentration (9.223 ± 0.013, 11.15 ± 0.041, 24.65 ± 0.032, and 46.61 ± 0.051 mg/100 g) of ferulic acid, caffeic acid, lupeol, and β-sitosterol, respectively. Likewise, SM2, SM4, and SM8 samples contained FA, CA, BS, and LUP (Table 2) similar to the range of markers present in CP whole plant, therefore, the above samples may be the whole plant of *C. pluricaulis.* While the other studied plant *C. ternatea* and *E. alsinoides* have the variable range of all the four markers, but CA was not found in *T. purpurea* (Table 2). SM1, SM3, and SM6 sample seem to be a mixture of CP and EA. The SM1 sample might contain more amounts of CP than SM3 and SM6. Similarly, the SM5 sample consists of three markers FA, BS, and LUP lower than the average value of net concentration CP and TP, and also CA in the range of CP; therefore, it may be the mixture of CP and TP (Table 2). Lastly, the SM7 sample was unidentified, as shown in Table 2.

Several studies have been done in the last decade to distinguish the relationship between DNA markers with phytochemical composition among closely related species.[18,32,33] In the present paper, RAPD and ISSR profile of all the plants showed maximum numbers of bands amplified through OPG-5 and UBC-886 that are 13 and 12, respectively. According to the molecular characterization data, all the studied samples have higher polymorphism with a maximum of 100% with most of the OPG and UBC primers that indicate the high genetic variability in all the plants attributed to *Shankhpushpi*. The results of the study showed that OPG-16, UBC-861, and UBC-886 marker gave maximum information of polymorphism while OPG-5 and UBC-886 showed the maximum resolving power (RP) (Table 3), which is the ability to detect the level of variation between individual.[22,34] Based on RAPD and ISSR profile, the dendrogram was generated according to the similarity matrices between all the samples (Figure 3). It clearly divided into two clusters (clusters 1 and 2). Cluster 1 contained CP1, CP2, CP3, EA1, and EA2 along with the samples SM1, SM2, SM3, SM4, SM5, SM6, and SM8. However, cluster 2 contained CT1, CT2, CT3, and TP (Figure 3). The results clearly demonstrated that out of eight SM samples, seven were genetically related to the *C. pluricaulis*, *E. alsinoides*, and *T. purpurea*. However, one sample from Mumbai, i.e., SM7, showed a totally different genotype. Hence, the dendrogram showed that CP, SM2, SM4, and SM8 clustered together, therefore genetically similar and justified the phytochemical inferences.

The above data showed that there was significant correlation between phytochemical (TLC profiling and marker concentration) and molecular (RAPD and ISSR profiles) studies which up to some extent authenticates our results and species-specific bands for *C. pluricaulis, E. alsinoides*, *C. ternatea*, and *T. purpurea* generated through different RAPD and ISSR primers will be used for identification of different species of *Shankhpushpi.*

CONCLUSION

Phytochemical quantification and molecular analysis have been concluded that the market sample of Delhi, Hisar, and Jaipur were pure *C. pluricaulis.* However, other market samples of *Shankhpushpi* were the mixture of two species, while the Mumbai market sample did not match any collected plant species and unidentified. The studied techniques are an important tool for quality control and identification of adulterant/substituent of herbal drug and give significant identification marker at phytochemical and DNA level to maintain batch to batch consistency of herbal drug and ascertain the drug quality.

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REFERENCES

Q5
1. Anonymous, The Ayurvedic pharmacopoeia of India (API). Part I, Vol II, Government of India, Ministry of Health and Family Welfare, New Delhi, India, 2000.
2. Vaidya B, Some controversial drugs in India. Chowkhamba orientalia, Delhi, India, 1982.
3. Iyer RS, Indian Medicinal plants: compendium of 500 species. Orient longman private limited, Chenni, 1994.

Q6
4. Upadhya AS, Kumbhojkar MS. Studies on ayurvedic drug Shankhpushpi from western Maharashtra medicobotanical reported aspects. Bulletin of Medicine and Ethnobotanical Research. 1993;14:64-69.
5. Chunekar KC, Bhavprakash nighantu. Chukhamba vidhya bhavan, Varanasi, 1969.
6. Asuntha G, Prasannaraju Y, Sujatha D, Prasad KVSR. Assessment of effect of ethanolic extract of *Tephrosia purpurea* (L.) Pers., Fabaceae, activity on lithium-pilocarpine induced status epilepticus and oxidative stress in Wistar rats. Revista brasileira Farmacognosia 2010; 20: 767-772.
7. Nahata A, Patil UK, Dixit VK. Anxiolytic activity of *Evolvulus alsinoides* and *Convulvulus pluricaulis* in rodents. Pharmaceutical Biology 2009; 47: 444-451.
8. Ratha KK, Mishra SS. Anticonvulsant activity of Shankhpushpi (*Convolvulus pluricaulis*) on strychnine induced seizure in experimental animals. International Journal of Ayurvedic Medicine 2012; 3: 82-87.
9. Malik J, Karan M, Vasisht K. Nootropic, anxiolitic and CNS depressant studies on different plant sources of Shankhpushpi. Pharmaceutical Biology 2011; 49: 1234-1242.
10. Sharath SS, Preethy J, Kumar SG, Revannaswami A, Muralidhar P, Rajshekhar ST, Prabhawati, Rohini KR, Screening for antiulcer activity of *Convolvulus pluricaulis* using Puloric ligation method in wistar rats. International Journal of Pharmaceutical Science and Research 2015; 6: 89-99.
11. Sethiya NK, Thakor SG, Mishra SH. Comparative evaluation on commercial sources of indigenous medicine Shankhpushpi for antistress potential a preliminary study. Pharmacology online 2009; 2: 460-67.
12. Das N, Chatterjee P. Evaluation of brine shrimp cytotoxicity of 50% aquous ethanolic leaf extract of *Clitoria ternatea* L. Asian Journal of Pharmaceutical and Clinical Research 2014; 7: 118-120.
13. Kumar A, Kulkarni SK. Effect of BR16A (Mentat), a polyherbal formulation on drug- induced catalepsy is mice. Indian Journal of Experimental Biology 2006; 44: 45-48.
14. Mitra S. Comparative pharmaceutical studies of different formulations of Shankhpushpi and microbial analysis using ganga jal and tap water. Journal of Ayurvedic and Holistic Medicine 2015; 3: 6-10.
15. Khatoon S, Singh N, Kumar S, Srivastava N, Rathi A & Mehrotra S, Authentication and Quality Evaluation of an Important Ayurvedic Drug – 'Ashoka Bark'. Journal of Scientific and Industrial Research 2009; 68: 393-400.
16. Khatoon S, Singh H, Goel AK. Use of HPTLC to establish the chemotype of a parasitic plant *Dendrophthoe fulcata* (Linn. F.) Etting. (Lorenthaceae), growing on different substrates. Journal of Planar Chromatography 2011; 24: 60-65.
17. Khatoon S. Macro-microscopy and planer chromatography-important tool for quality control and identification of adulterant/substitute of Unani drugs. International Journal of Advances in Pharmacy Medicine and Bioallied Science 2015; 3: 58-64.
18. Kalpana J, Preeti C, Dnyaneshwar W, Bhushan P. Molecular markers in herbal drug technology. Current Science 2004; 87: 159-165.
19. Jain SK, Rao RR. A hand Book of field and herbarium methods. Today and Tomarrow pinters and publications, New Delhi, 1977.
20. Khatoon S, Singh N, Kumar S, Srivastava N, Rathi A, Mehrotra S. Authentication and quality evaluation of an important Ayurvedic drug- Ashoka bark. Journal of Scientific and Industrial Research 2010; 68: 393-400.
21. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus 1990; 12: 13-15.
22. Singh N, Pal AK, Roy RK, Tewari SK, Tamta S, Rana TS. Assessment of genetic variation and population structure in indigenous *Gladiolus* cultivars inferred from molecular markers. Nucleus 2016; 59: 235-244.
23. Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics 1980; 32: 314-331.
24. Powell WW, Koput KW, Doerr LS. Interorganizational Collaboration and the Locus of Innovation: Networks of Learning in Biotechnology. Administrative Science Quarterly 1996; 41: 116-145.
25. Pavlicek A, Hrda S, Flegr J. FreeTree – Freeware program for construction of phylogenetic trees on the basis of distance data and bootstrapping /jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia*. Folia Biologica 1999; 45: 97-99.
26. Lazarowych NJ, Pekos P. The use of fingerprint and marker compounds for identification and standardization of botanical drugs. Journal of Drug Information 1998; 32: 497-512.
27. Irshad S, Singh J, Kakkar P, Mehrotra S. Molecular characterization of Desmodium species- an important ingredient of 'Dashmoola' by RAPD analysis. Fitoterapia 2009; 80: 115–118.
28. Shikha S, Mishra N. Genetic markers - a cutting-edge technology in herbal drug research. Journal of Chemical and Pharmaceutical Research 2009; 1: 1-18.
29. Heubl G. New aspects of DNA-based authentication of Chinese medicinal plants by molecular biological techniques. Planta Medica 2010; 76: 1963-1974.
30. Evans WC. In: *Trease and Evans Pharmacognosy*. 15th ed., Saunders, London, 2002.
31. Irshad S, Misra PK, Rawat AKS, Khatoon S. Authentication of commercial samples of Shankhpushpi through physico-phytochemical analysis and TLC fingerprinting. Indian Journal of Traditional Knowledge 2016; 15: 646-653.
32. Ganie SH, Srivastava PS, Narula A, Ali Z. Authentication of shankhpushpi by RAPD markers. EurAsian Journal of

Biosciences 2012; 6: 39–46.
33. Rasheed NMA, Nagaiah K, Goud PR, Sharma VUM. Chemical marker compounds and their essential role in quality control of herbal medicines. Annals of Phytomedicine 2012; 1: 1-8.
34. Lamare A, Rao SR. Efficacy of RAPD, ISSR and DAMD markers in assessment of genetic variability and population structure of wild *Musa accuminata* colla. Physiology and Molecular Biology of Plants 2015; 21: 349-358.

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