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Identification of Shankhpushpi by morphological, chemical and molecular markers

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Abstract
Shankhpushpi", an important indigenous drug of Ayurveda, improves memory power and intellect. The different Ayurvedic preparations of this drug, either singly or in combination with other herbs, is meant for sleeplessness, epilepsy, hallucinations and anxiety. Survey of literature revealed that at least three different plants species viz., *Clitoria ternatea* L., *Convolvulus pluricaulis* Choisy and *Evolvulus alsinoides* L. are used as the source of this drug in the different parts of the country. Because of the adulteration in the Indian crude drug markets and increased demand and high price, shankhpushpi is often adulterated in the trade by other related/spurious species. Therefore, a reliable authentication method is needed to facilitate differentiation/ identification of the genuine material from its adulterants. The present review was aimed to collect morphological, chemical and molecular marker based data for the identification of *C. pluricaulis*, *E. alsinoides* and *C. ternatea*, to ascertain their authenticity. Morphological markers are subjective and identification of the medicinal plants in the dry and fragmented form is very difficult. Chemical markers though is superior over morphology, however, are tissue specific and age dependant. DNA-based markers are objective and not influenced by age, physiology and geographical conditions. The use of morphological chemical and molecular data is the ideal strategy to authenticate plant species rather than choosing a single marker; therefore we collected comparative data that will help identify authentic plant species of shankhpuspi.

Key words: Authentic sample, market sample, molecular marker, shankhpushpi.

Introduction
"Shankhapushpi" is an important drug of indigenous system of medicine. According to Ayurveda, Shankhpushpi is bitter, pungent, alexiteric, alternative, tonic, anti-helmintic, brightens intellect, useful in bronchitis, biliousness, epilepsy, leucoderma and teething troubles of infants etc. It contributes considerably to the improvement of the memory power and intellect (Nadkarni 1954). The whole plant is medicinal. It is astringent, hot, aphrodisiac and rejuvenating (Sharma *et al*. 1965; Chaturvedi *et al*. 1997; Mudgal *et al*. 1975; Singh and Mehta 1977; Shukla 1981). Investigators have reported barbiturate hypnosis potentiation effect in this drug. It improves strength, digestive power, and complexion. The herb is reported to be useful in fever and bronchitis (Kirtikar and Basu 1918). The drug also supposedly cures diseases due to evil spirits (Chuneker *et al*. 1969), the morbidity of tridosis and is useful in epilepsy insanity, insomnia, heart diseases and haemetemesis (Chuneker 1982). Many formulations containing Shankhpushpi as a single drug or in combination with other drugs are available in Indian market. Shankhpushpi is routinely advertised for memory enhancement. The important Ayurvedic formulations using the drug are "Abhrak bhasma", "Brahmi ghrita", "Brahmi vati", "Brahm rasayan", "Manasmrita gutika", Manjisthadi Kasayam, "Mukta vati", Memorex tablets, Stress guard capsules, "Medhya kashaya", and "Shankhapushpi panaka", Dimagheen (Dawakhana Tibiya College, Aligarh), Shankhpushpi syrup (Unjha), Shankhavali Churna (Narnaryan Pharmacy), BR-16A (Himalaya Drug. Co. Ltd.) etc.

Except for the term Shankhpushpi, which indicates the resemblance of its flower to a conch shell, the various Sanskrit synonyms given to this drug do not give any clue in identifying the source plant of the drug. There seems to be a lot of confusion in equating the Sanskrit terms Vishnukranta, Shankhpushpi, Aparijata, Girikarni etc. to their respective botanical source.

Therefore, the botanical identity of Shankhapushpi is highly controversial. While some authors equate it with *C. pluricaulis* of Convolvulaceae as the source plant (Singh and Chuneker 1972; Anonymous 1978; Dey 1980; Chuneker 1982), others consider *E. alsinoides* as the source plant of Shankhapushpi (Vaidya 1982). The former is extensively used as Shankhapushpi in North India. However, Kerala physicians do not discriminate between Aparajita and Shankhapushpi and use *C. ternatea* of Fabaceae. Regardless of the source, the drug is used for its therapeutic effects on Central Nervous System disorders like insanity, epilepsy, nervous debility and memory enhancement (Gupta *et al*. 2007).

Reason to authenticate Shankhpushpi

The Ayurvedic Pharmacopoeia (Anonymous 2001) mentions three plant species namely: *C. pluricaulis, E. alsinoides and C. ternatea* as the source of the drug Shankhpushpi. In addition to these three widely used plant species, further literature survey revealed that some unrelated plants-*Canscora decussata* Schult, of Gentianaceae (Vaidya 1936; Kapoor and Mitra 1979), *Lavandula bipinnata* Kuntz (Lamiaceae), *Goniogyne hirta* (Willd.) Ali (Fabaceae), *Tephrosia purpurea* Pers. (Fabaceae) (Singh and Viswanathan 2000) and *Cheilanthes farinosa*, a fern (Daniel 2004) have also been used as the source drug of Shankhpushpi. Because of the tremendous demand of shankhpushpi in markets, market samples of this drug are invariably adulterated and different unrelated drugs are being sold in crude drug markets of India in the name of Shankhpushpi (Singh and Viswanathan 2001). An estimate suggests that more than 60% phytochemical investigation carried out on market samples of Indian medicinal plants are based on wrong/adulterated samples procured from markets (Daniel 2004). Therefore, the present work was aimed to collect the comparative data regarding the authentication of plant species of shankhpushpi.

(A) Authentication by morphological study.

A comparison of morphological traits exhibited by *C. ternatea, C. pluricaulis* and *E. alsinoides* (Fig. 1) is given hereunder.

Evaluated characters	***C. ternatea***	***C. pluricaulis***	***E. alsinoides***
Habit	Perennial herb	Perennial herb	Perennial herb
Habitat	Widely cultivated as ornamental plant	Open grassy fields, rocky soil along road sides in northern and central India and Bihar	Forest edges, scrub jungles and Sandy localities throughout India
Root shape	Upright , cylindrical, tortuous branched	Cylindrical, ribbed, light yellow	Elongated, cylindrical with lateral branches
Root texture	Hairy	Hairy	Hairy
Stem	Twining, slender, sub-erect at base	Prostrate or ascending, branching basally, Slender, cylindrical	Prostrate or ascending, slender, pubescent
Leaves (stipule)	Stipulate	Ex-stipulate	Exstipulate
Stipule shape	Narrowly triangular	-	-
Petiole (present/absent)	Present	Sessile	Sub sessile
Leaf type	Compound	Simple	Simple
Shape of leaf/leaflet	Oblong or broadly ovate, slightly emarginate or obtuse	Linear to oblong, oblanceolate to lanceolate	Oblong, elliptic-oblong to lanceolate
Phyllotaxy of leaf	Opposite superposed	Alternate, distichous	Alternate, distichous
Venation	Reticulate unicostate	Reticulate unicostate	Reticulate unicostate
Inflorescence type	Solitary Axillary	1-3 flowers in axillary heads	Solitary Axillary
Flower bract	Linear	Linear to oblanceolate	Linear-subulate to linear – lanceolate
Bracteole	Suborbicular or obovate, membranous	No	No
Flower based on sex organs	Monoecious	Monoecious	Monoecious
Flower shape	Paplionaceous	Infundibuliform	Infundibuliform
Flower colour	Blue, white	White, light pink	Blue
Sepal shape	Infundibuliform	Infundibuliform	Infundibuliform
Sepals cohesion	Gamosepalous apex free	Polysepalous	Polysepalous
Sepals aestivation	Valvate	Quinquincial	Quinquincial
Corolla cohesion	Polypetalous	Gamopetalous	Gamopetalous
Corolla aestivation	Vexillary	Valvate	Valvate
Stamen number	10 (9+1) diadelphous	5	5
Filament colour	White	White	White
Anther colour	Light yellow	White	White
Anther position	Diadelphous	Polyandrous	Polyandrous
Stamen adhesion	Posterior stamen free, and filaments of nine stamens are fused to form sheath around the ovary	Epipetalous	Epipetalous
Anther fixation	Basifixed	Basifixed	Basifixed
Ovary position	Perigynous	Hypogynous	Hypogynous
Carpel number	01	02	02
Locule number	01	02	02

Style number	01	01	01 or 02
Stigma number	01	02	02 or 04
Fruit type	Legume (pod)	Capsule	Capsule
Fruit shape	Somewhat linear, flattened, beaked	Oblong globose	Spherical
Fruit surface	Smooth or sparsely hairy	Smooth	Smooth
Seed shape	Kidney shaped	Plano-convex	Ellipsoid
Seed surface	Smooth	Shining, faintly ridged	Ribbed

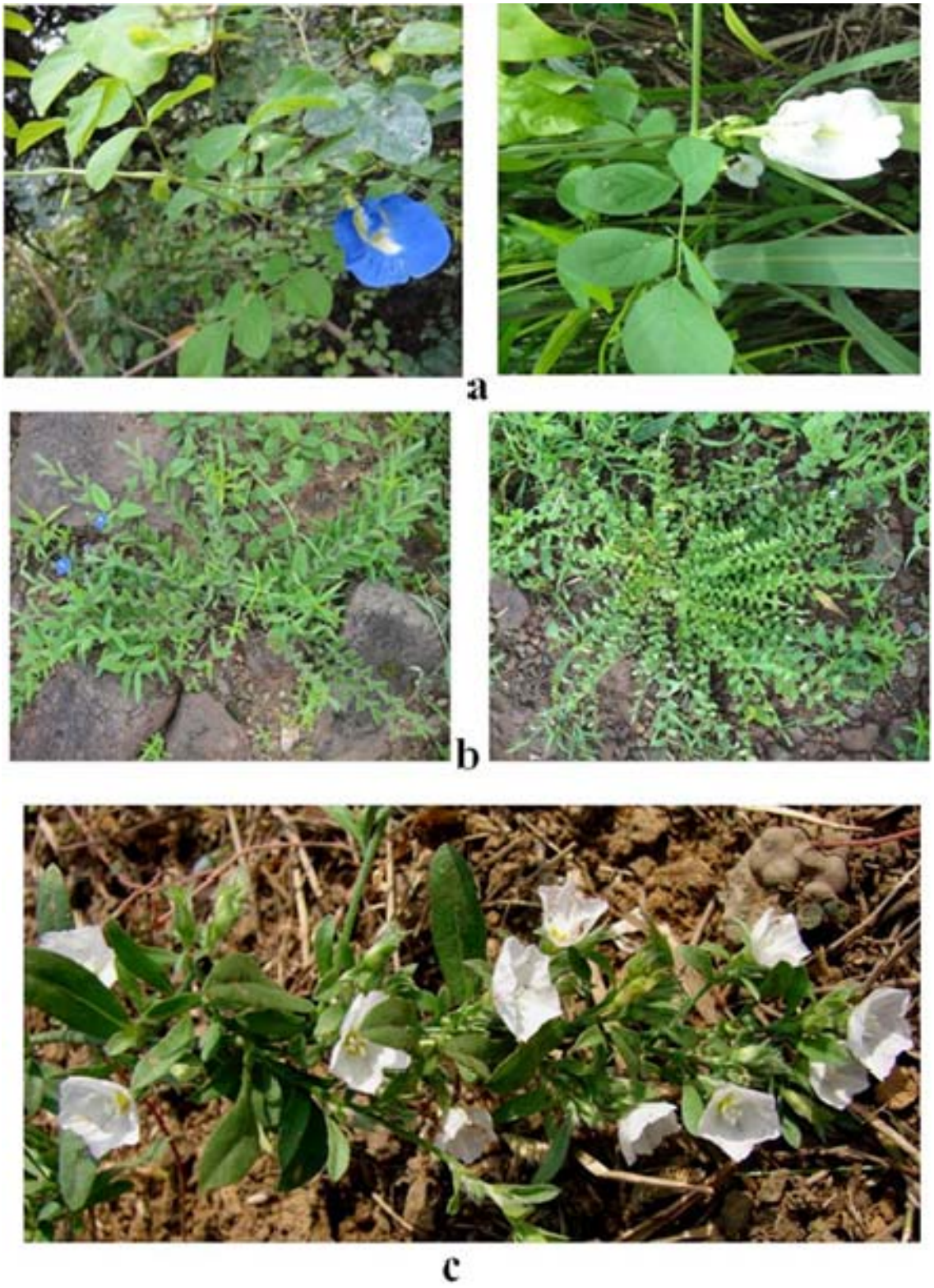

Fig. 1: Plant species equated with the traditional herbal drug Shankhpushpi (a) *C. ternatea* (b) *E. alsinoides* (c) *C. pluricaulis*

The detailed phenotypic evaluation of plant and plant material provide means of standardization of an herb that can be used as drug or as raw material. Morphologically all the three plant species are distinct in their appearance and can be easily identified. *C. ternatea* (Fabaceae) is quite distinct in relation to habit, leaf shape, phyllotaxy, inflorescence, flower shape, fruit and seed structure from *C. pluricaulis* and *E. alsinoides*. The striking feature about this plant are its vivid deep blue flowers; solitary, with light yellow markings. There are some varieties that have white flowers. The other species i.e. *C. pluricaulis* and *E. alsinoides,* share a number of features as both belong to the same family Convolvulaceae. Both are prostrate, spreading, perennial herbs with woody rootstock but can be easily distinguished on the basis of their flower colour, leaf shape and other morphological characters as delineated in the above table. Phenotypic evaluation is simple and direct but its accuracy depends heavily on the researcher's experience and judgement, which is subjective and error-prone. It is also difficult to identify processed herbal material when they are in powder form or in shredded pieces. Therefore, in addition to morphological characterization, medicinal plants need to be characterized through chemical and molecular markers.

(B) Authentication through chemical markers

The medicinal effectiveness of the plant species is related to the quantity of that marker compound. Plant species, strain and geographical origin can be distinguished using chemical fingerprinting. The chromatographic analysis employing High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) as well as Mass Spectroscopy (MS) and Nuclear Magnetic Resonance (NMR) are capable techniques to create chemical profile of active constituents of herbal material. Kaempferol, a natural flavonol is reported to be present in all three plant species of shankhpushpi (Austin 2008; Mukherjee *et al*. 2008; Andrade *et al*. 2012) and, therefore, the said metabolite was selected as a chemical marker and quantified it in different samples through HPLC (Ali *et al*. 2013; Ganie *et al*. 2014; Ganie and Sharma 2014). The kaempferol content found by Ali *et al*. (2014) in different accessions of *C. ternatea* was in the range of 9.311±0.0277-20.018±0.006; and when quantified in accessions of *C. pluricaulis* (Ganie *et al*. 2014) and *E. alsinoides* (Ganie and Sharma 2014) it ranged from 0.076±0.002-0.495±0.014 and 0.026±0.007-0.120±0.018 respectively (Table 1). The kaempferol content was higher in *C. ternatea* followed by *C. pluricaulis* and *E. alsinoides*. Sharma *et al*. (2008) did almost similar study in *Andrographis paniculata* in which andrographolide content was quantified in different accessions using HPLC. As the plants were collected from the same locality, the variation, they found in andrographolide content was due to intra-specific differences in nucleotide sequences only. As the plant species of the drug shankhpushpi collected at different geographical zones, variations observed in the kaempferol content in different accessions may probably be due to both environmental and genetic factors. Therefore, the variation analysis could be correlated by the Sabu *et al*. (2001) equation: VK = VG + VE, where VK = is the variation in kaempferol content, VG = genetic variance and VE = environmental variance. The present study revealed that blue flowered variety of *C. ternatea* collected from Haryana (Kurukshetra) is the highest accumulator of kaempferol. This variety may potentially be multiplied and used on a large scale for commercial cultivation.

The composition and relative amount of chemical in a plant species may also have been developed with the growing conditions, harvesting periods, post harvest periods and storage. The variation of chemical composition may hinder the authentication, and in some cases, may be misleading if the samples are adulterated. Moreover, it is difficult to distinguish closely related species due to similar chemical compounds. Therefore, it is necessary to develop a more effective, reliable and sensitive technology for the authentication of herbal drugs.

(c) Authentication by DNA markers

Molecular or DNA-based markers are now becoming a popular means for the identification of medicinal plants (Yip *et al*. 2007). Molecular markers have the advantage over chemical markers as the genetic composition is unique for each individual and is least affected by age (Kumble 2003), environmental factors and physiological conditions (Macbeath and Schreiber 2000), harvest, storage and processing of the samples (Scheitzer *et al*. 2003). DNA extracted from leaves, stems or roots of an herb all carry the same genetic information. In general, extracted DNA is stable and can be stored at -20ºC for a reasonable period of time, thus eliminating the time constraint in performing the analysis. A small amount of sample is sufficient for analysis and the physical form of the sample does not restrict the detection. Molecular markers are not stage and tissue specific and thus can be detected at any stage of development. This is particularly true for similar looking herbal materials that can often vary greatly in their medicinal properties and market value. DNA based technology can provide an efficient and accurate means of testing the authenticity of hundreds of samples simultaneously while conventional chemical methodologies usually take several days for verification. DNA based authentication of medicinal plants can be useful as a tool for quality control and safety monitoring of herbal pharmaceuticals and neutraceuticals and will significantly add to the medical potential and commercial profitability of herbal products.

Plant species of Shankhpushpi have been characterized through RAPD markers (Ali *et al*. 2013, Ganie *et al*. 2014; Ganie and Sharma 2014). Seven different RAPD markers revealed 45% polymorphism across different geographical locations. Of the seven primers, OPN-02 could be used as molecular ID for *C. ternatea* as all the different accessions generated similar genetic profile with the said primer (Fig. 2a). In addition, population specific bands were identified with primers OPN-01 and OPN-06 (Table 2). Dendrogram, splitted the different accessions according to the geographical regions (Fig. 3a). In *C. Pluricaulis*, 5 primers amplified 37 amplicons with percentage polymorphism of 59.45. Primer OPN-09 (Fig. 2b) generated maximum monomorphism, therefore, is the source to identify *C. pluricaulis*. Besides, several unique bands amplified by different primers help identify geographically different Populations (Table 2). Differentiation of accessions by UPGMA was not in accordance with locality of the plants (Fig. 3b). In *E. alsinoides*, of the 47 RAPD fragments, 39 were polymorphic, with 80% polymorphism. Like *C.*

Pluricaulis none of the primers generated absolute monomorphism; however, a unique band of primer OPN-05 with size 2.2 kb (Fig. 2c) being present in all accessions is the identification of *E. alsinoides*. Region specific bands have also been obtained using other primers (Table 2). Dendrogram generated on similarity coefficient separated the accessions that represent lack of any defined population structure in this species (Fig. 3c).

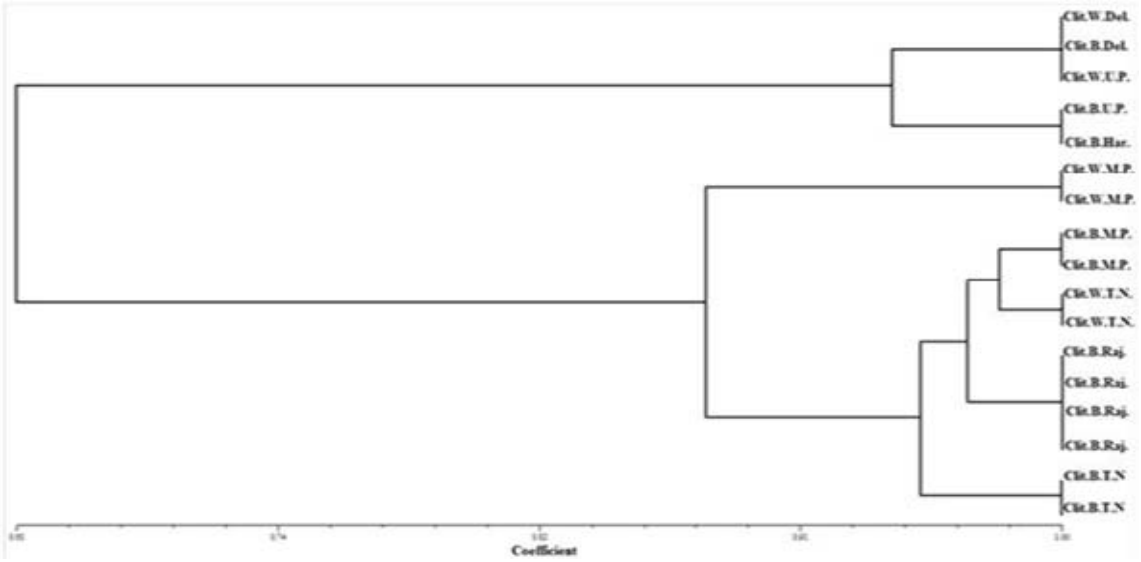

Fig: (2a**)** Dendrogram based on RAPD analysis for the estimation of genetic diversity in different accessions of *C. ternatea* collected from different locations. Clit. W. Del. = *C. ternatea* white Delhi- Hamdard Campus (1 accession), Clit. B. Del. = *C. ternatea* blue Delhi- Hamdard Campus (1 accession), Clit. W. U. P. = *C. ternatea* white Uttar Pradesh- Lucknow (1 accession), Clit. B. U. P. = *C. ternatea* blue Uttar Pradesh- Lucknow (1 accession), Clit. B. Har. = *C. ternatea* Blue Haryana- Kurukshetra (1 accession), Clit. W. M. P. = *C. ternatea* white Madhya Pradesh- Bhopal (2 accessions), Clit. B. M. P. = *C. ternatea* blue Madhya Pradesh- Bhopal (2 accessions), Clit. W. T. N. = *C. ternatea* white Tamil Nadu- Coimbatore (2 accessions), Clit. B. T. N. = *C. ternatea* blue Tamil Nadu- Coimbatore (2 accessions) Clit. B. Raj. = *C. ternatea* blue Rajasthan- Jaipur and Udaipur (4 accessions). (Ali *et al*. 2013

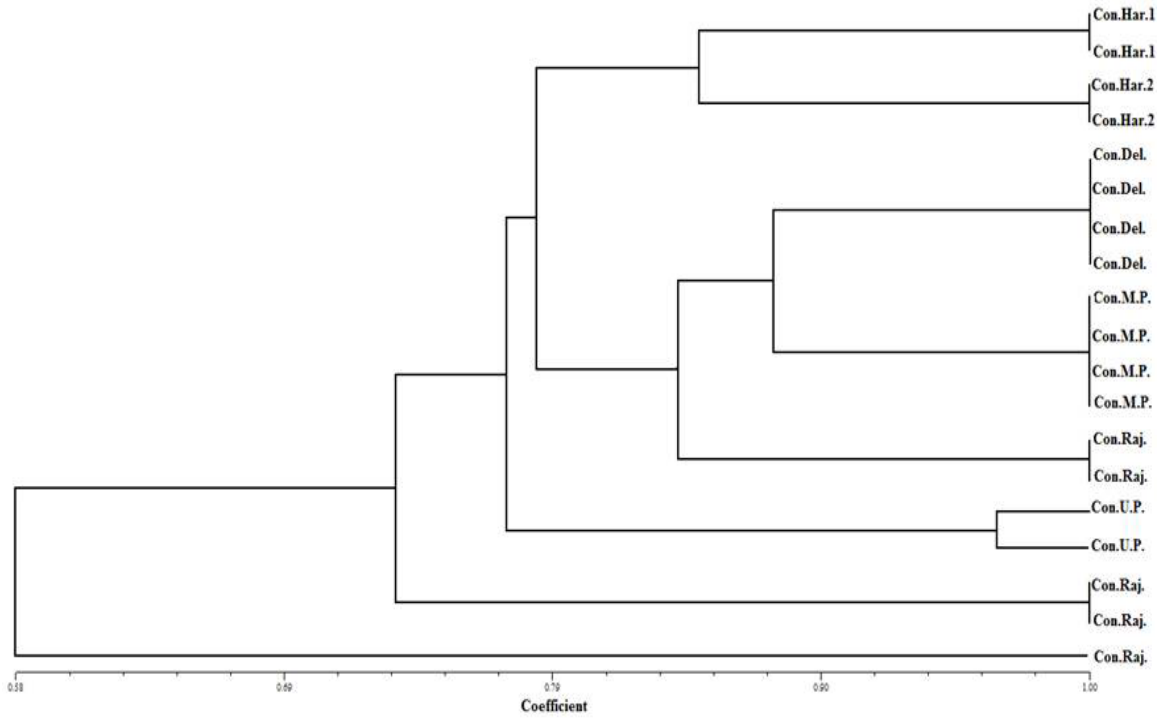

Fig: (2b) Dendrogram based on RAPD analysis for the estimation of genetic diversity in different populations of *C. pluricaulis* collected from different locations. Har. 1 = (Haryana, Kurukshetra Campus), Har. 2 = (Haryana, Arjun Herbal Park, Kurukshetra), Del = (Delhi, Hamdard Campus), M. P. = (Madhya Pradesh, Bhopal), Raj. = (Rajasthan- Udaipur, Jaipur and Jodhpur), U. P. = (Uttar pradesh, Lucknow). (Ganie *et al*. 2014

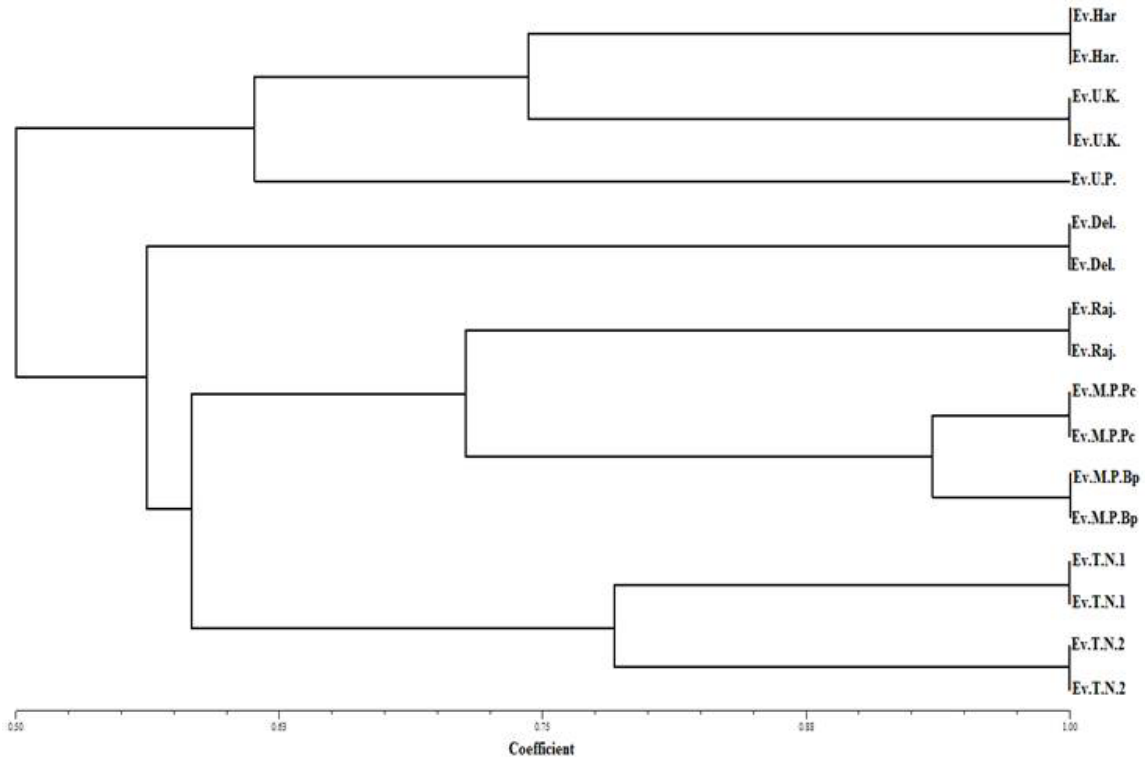

Fig: (2c) Dendrogram based on RAPD analysis for the estimation of genetic diversity in different populations of *E. alsinoides* collected from different locations. Ev. = *Evolvulus*, Har. = Haryana, U. K. = Uttarakhand, U. P. = Uttar Pradesh, Raj. = Rajasthan, M. P. Pc. = (Madhya Pradesh- Pachmarhi), M. P. Bp. = (Madhya Pradesh- Bhopal), T. N.1 = (Tamil Nadu- Botanical Survey of India- Coimbatore), T. N.2 = (Tamil Nadu- Mother Cry Temple- Coimbatore). (Ganie and Sharma 2014

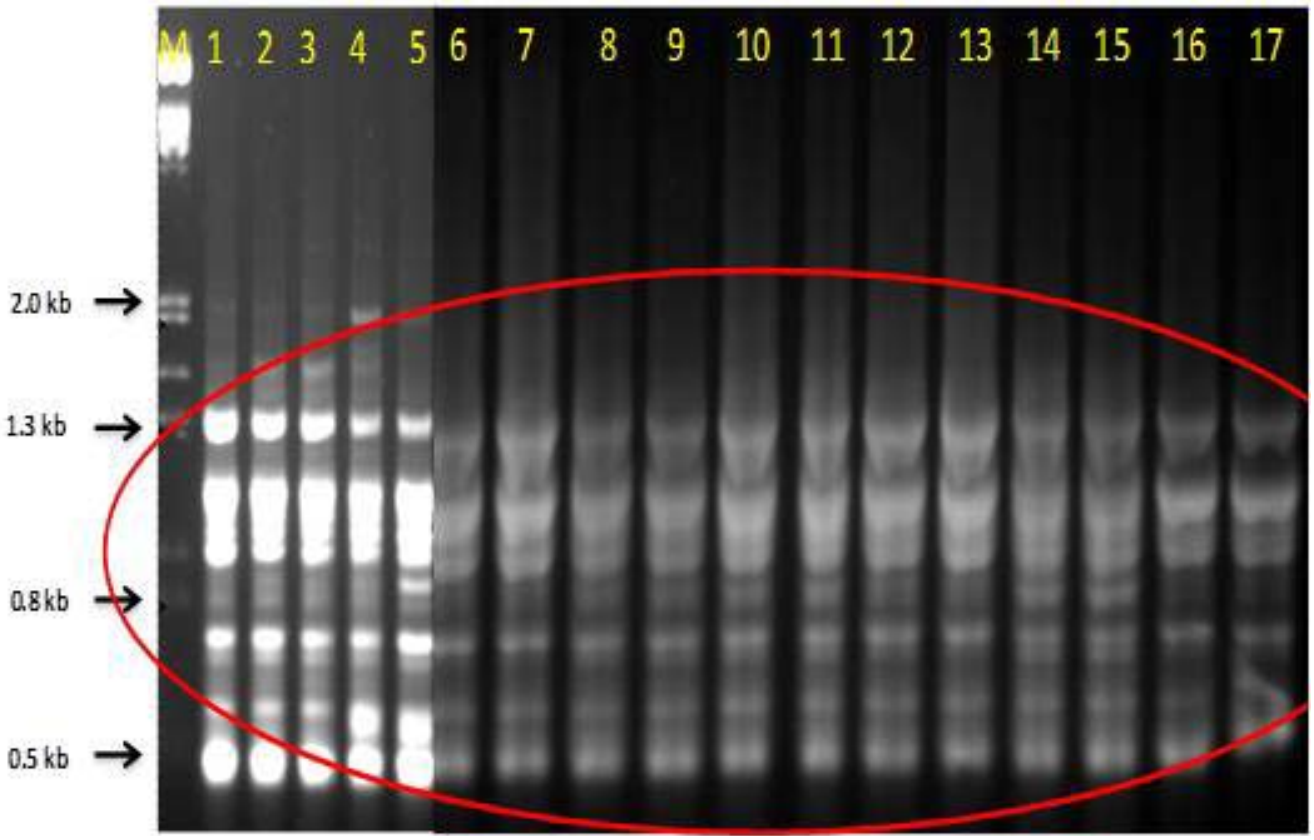

Fig (3a) RAPD fingerprint obtained with OPN-02 primers in different accessions of *C. ternatea:* M = Marker (λ DNA digested with *Hind* III and *Eco*R I), 1- *Clitoria* white Delhi, 2- *Clitoria* blue Delhi, 3- *Clitoria* white U. P., 4- *Clitoria* blue U. P., 5- *Clitoria* blue Haryana, 6, 7- *Clitoria* white M. P., 8, 9- *Clitoria* blue M. P., 10, 11- *Clitoria* white Tamil Nadu, 12, 13- *Clitoria* blue Tamil Nadu, 14, 15- *Clitoria* blue Udaipur, (Rajasthan), 16, 17- *Clitoria* blue Jodhpur (Rajasthan). Circle represents monomorphism. (Ali *et al*. 20

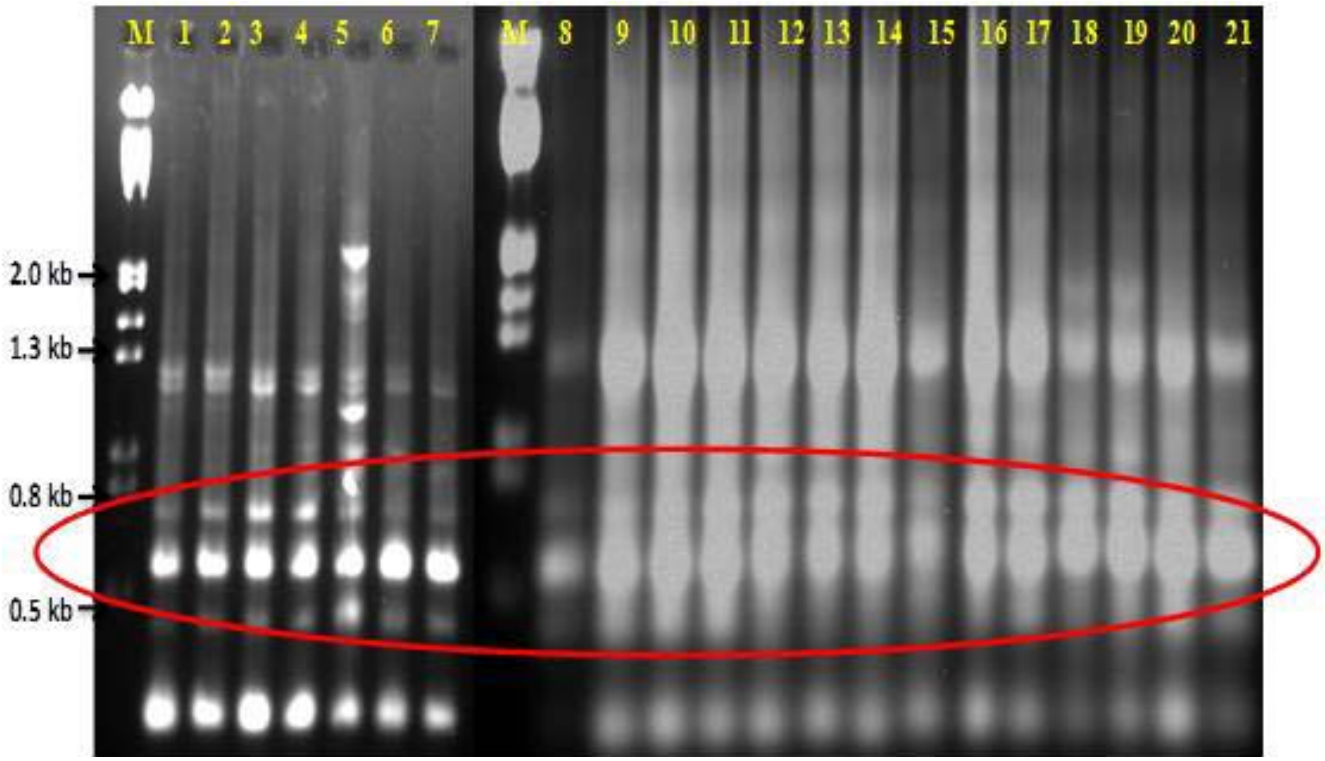

Fig. (3b) RAPD fingerprint obtained with OPN-09 primer in different accessions of *C. pluricaulis:* M = Marker (λ DNA digested with *Hind* III and *Eco*R I), 1, 2 *C. pluricaulis* from, Haryana (Kurukshetra Campus), 3, 4 *C. pluricaulis* from Haryana (Arjun Herbal Park Kurukshetra), 5 *C. pluricaulis* from Jodhpur (Rajasthan), 6, 7 *C. pluricaulis* from U. P., 8-11 *C. pluricaulis* from Delhi, 12-15 *C. pluricaulis* from M. P., 16-18 *C. pluricaulis* from Udaipur (Rajasthan), 19-21 *C. pluricaulis* from Jaipur (Rajasthan). Circles represent monomorphic bands. (Ganie *et al*. 2014)

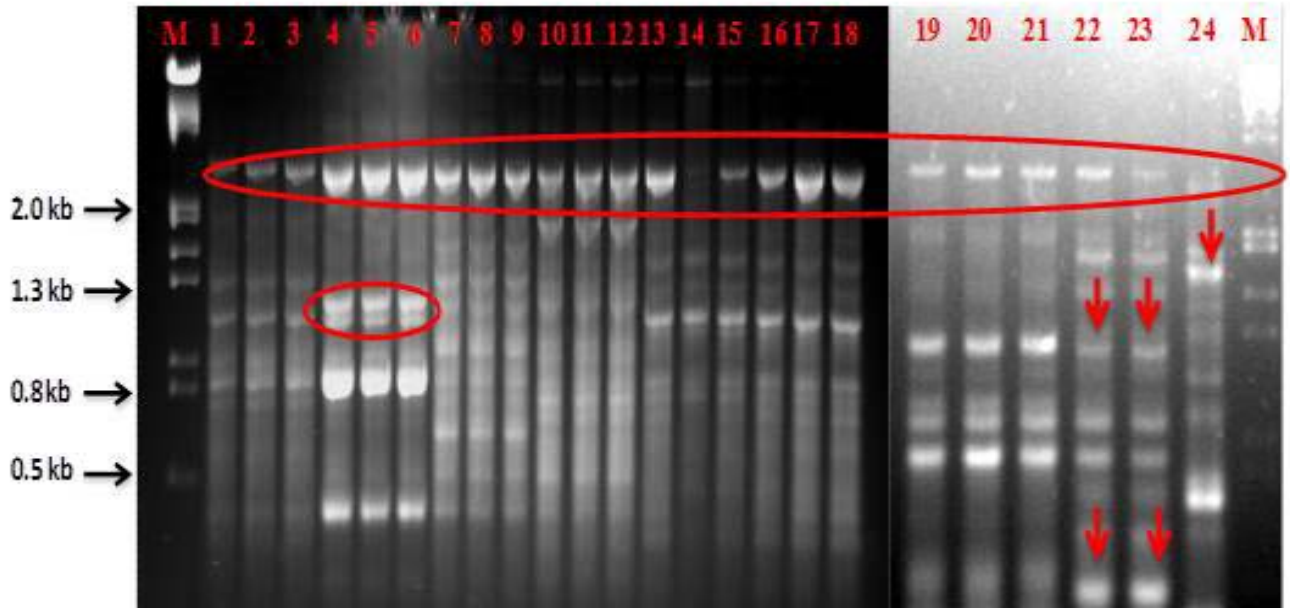

Fig: (3c) RAPD fingerprint obtained with OPN-05 primer in different accessions of *E. alsinoides*: M = Marker (λ DNA digested with *Hind* III and *Eco*R I), 1-3 *E. alsinoides* from Delhi, 4-6 *E. alsinoides* from Rajasthan, 7-9 *E. alsinoides* from Tamil Nadu (BSI), 10-12 *E. alsinoides* from Tamil Nadu (Mother Cry Temple), 13-15 *E. alsinoides* from, Pachmarhi (M. P.), 16-18 *E. alsinoides* from Bhopal (M. P.), 19-21 *E. alsinoides* from Haryana, 22, 23 *E. alsinoides* from Uttarakhand, 24 *E. alsinoides* from U. P. Top circle represent monomorphic band. (Ganie and Sharma 2014

Table 1: Kaempferol concentration in different accessions of plant species of shankhpushpi (Ali *et al*. 2013; Ganie *et al*. 2014; Ganie and Sharma 2014)

S.No	Plant samples	Amount of kaempferol (mg/g dry wt) ± SE
1	*Clitoria ternatea* (Blue flowered), New Delhi (Hamdard Campus)	10.115±0.003
2	*C. ternatea* (White flowered), New Delhi (Hamdard Campus)	10.921±0.005
3	*C. ternatea* (Blue Flowered), U. P. (Lucknow)	11.653±0.032
4	*C. ternatea* (White Flowered), U. P. (Lucknow)	10.149±0.290
5	*C. ternatea* (Blue Flowered), M. P. (Bhopal)	16.67±0.017
6	*C. ternatea* (White Flowered), M. P. (Bhopal)	9.311±0.0277
7	*C. ternatea* (Blue Flowered), Tamil Nadu (Coimbatore)	9.876±0.362
8	*C. ternatea* (White Flowered), Tamil Nadu (Coimbatore)	11.448±0.052
9	*C. ternatea* (Blue Flowered), Haryana (Kurukshetra)	20.018±0.006
10	*Convolvulus pluricaulis*, M. P. (Bhopal)	0.076±0.002
11	*C. pluricaulis*, Haryana (Kurukshetra)	0.375±0.002
12	*C. pluricaulis*, U. P. (Lucknow)	0.184±0.007
13	*C. pluricaulis*, Rajasthan (Jodhpur)	0.279±0.013
14	*C. pluricaulis*, New Delhi (Hamdard Campus)	0.495±0.014
15	*Evolvulus alsinoides*, New Delhi (Hamdard Campus)	0.120±0.018
16	*E. alsinoides*, Haryana (Sohna)	0.051±0.002
17	*E. alsinoides*, Uttarakhand (Srinagar)	0.033±0.004
18	*E. alsinoides*, U. P. (Lucknow)	0.074±0.002
19	*E. alsinoides*, M. P. (Pachmarhi)	0.035±0.002
20	*E. alsinoides*, M. P. (Bhopal)	0.026±0.007
21	*E. alsinoides*, Tamil Nadu (Coimbatore)	0.028±0.007

Table 2: Unique bands of different accessions of shankhpushpi amplified by different primers (Ali *et al*. 2013; Ganie *et al*. 2014; Ganie and Sharma 2014)

Name of plant specie	Place of the collected accession	Primer code	Sequence (5′-3′)	Unique bands amplified	Size of the unique bands (kb)
C. ternatea	Delhi	OPN-06	TGAGACGCACA	1	1.0
	Madhya Pradesh	-	-	1	0.8
	Tamil Nadu	-	-	1	1.0
C. pluricaulis	Rajasthan	OPN-01	CCTCAGCTTGG	2	0.6, 0.8
	Rajasthan	0PN-02	AACCAGGGGCA	1	0.35
	Rajasthan	OPN-09	TTGCCGGCTTG	2	1.0, 2.1
E. alsinoides	Delhi	OPN-01	CCTCAGCTTGG	4	1.4, 1.6, 1.8, 1.9
	Uttrakhand	-	-	1	1.1
	Delhi	OPN-02	AACCAGGGGCA	3	1.3, 1.9. 2.0
	Uttrakhand	-	-	2	0.6, 2.2
	Rajasthan	OPN-06	TGAGACGCACA	1	2.2
	Tamil Nadu	-	-	2	1.1, 1.2
	Uttar Pradesh	-	-	1	1.5
	Delhi	OPN-05	TACTGAACGCC	1	1.2
	Uttrakhand	-	-	2	0.3, 1.0
	Uttar Pradesh	-	-	1	1.5

Comparison of molecular characterization of *C. ternatea*, *C. pluricaulis* and *E. alsinoides*:

Molecular analysis revealed higher polymorphism in *E. alsinoides* followed by *C. pluricaulis* and *C. ternatea*. The *E. alsinoides* is widely distributed throughout India under varied habitats and environmental conditions, which is probably responsible for its higher genetic diversity. Additionally, high polymorphism could be due to the xenogamic nature of *E. alsinoides* (Singh *et al*. 2010). The average polymorphism of *C. pluricaulis* might be due to its restricted distribution (being found only in some parts of north India), non effective gene flow, low fecundity, low pollen flow, local selection procedure (environment and struggle for existence), inbreeding systems, biotic factors like human interference, habitat destruction and commercial exploitation (Ganie *et al*. 2014). The low genetic diversity in *C. ternatea* can be explained because the plant is cultivated throughout India in herbal gardens and nurseries that ensures less impact of changing environmental conditions (Ali *et al*. 2013); and the self pollination.

Conclusion

The morphological analysis, chemical and genetic profile of the plant species of Shankhpushpi will find applications in the quality control of the drugs obtained from these species not only by the pharmaceutical industries, but also Govt, agencies responsible for monitoring their quality.

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