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Variation in the marker content of five different *Dendrobium* species: Comparative evaluation using validated HPTLC technique

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ABSTRACT

Northeast India represents a rich floristic wealth of India. Orchids form a very noticeable feature of this vegetation. Amongst orchids, the genus *Dendrobium* forms a very important group of plants which are used by the locals for the treatment of various ailments. A through literature survey revealed that there is a paucity of data on the phytochemical evaluation of *Dendrobium* species using analytical methods. The chromatographic technique such as HPTLC is reported to be useful for standardization of plant materials in terms of phytochemical markers and evaluation of their quality. Therefore, in this study, five different *Dendrobium* species have been evaluated and compared in terms of their marker content using validated HPTLC methods. All the samples analyzed were found to be a good source of β-sitosterol with maximum content in *D. fimbriatum* stem and minimum content in *D. chrysanthum* roots. *D. nobile* (roots and stem) and *D. moschatum* (stem) were also found to be a source of ursolic acid and lupeol, respectively. Thus, this work will contribute in the identification, quality evaluation and standardization of different *Dendrobium* species. On the basis of maximum bioactive marker content, specific part of *Dendrobium* species can be selected which may be supported by its efficacy.

INTRODUCTION

The genus *Dendrobium* is one of the largest groups of family Orchidaceae (Gutierrez, 2010). Different species of these orchids are found in Northeast India, China and Japan and are recorded in the Chinese Pharmacopoeia. These species are not only valued for their ornamental beauty but also have important therapeutic properties (Gutierrez, 2010; Vij *et al*., 1997). Various *Dendrobium* species have been reported to possess secondary metabolites such as phenols, alkaloids, coumarins, terpenes, flavonoids as the major therapeutic agents (Meng *et al*., 2013). Even primary metabolites such as polysaccharides from different *Dendrobium* species have been shown to promote proliferation in macrophages and increase their phagocytic activity (Lan Zhen *et al*., 2013). *Dendrobium nobile* Lindl., is a precious herbal plant in Chinese traditional medicine and it is one of the *Dendrobium* species specified in Chinese Pharmacopoeia, 2005 (Luo *et al*., 2010). The stem of *D. nobile* has been used ethnomedicinally to nourish stomach, promote the production of body fluid and reducing fever. *D. nobile* has been a good source of compounds with anti-tumor activity (Singh *et al*., 2012). *D. nobile*, a highly prized orchid for its economic and medicinal importance is at the verge of extinction due to over-exploitation. Recently, the genetically stable regeneration protocol with increased phytochemical production and *in vitro* antioxidant activity of *D. nobile* has been reported (Bhattacharya *et al*., 2014). The efforts have also been made by researchers to propagate this endangered species through *in vitro* seed germination (Kumaria *et al*., 2005; Kumaria and Tandon, 2001).

Genetic characterization of this orchid, which is imperative for the prevention of elite germplasm erosion in breeding programs mainly during development of synthetic hybrids, has also been carried out using RAPD and SSR markers (Chattopadhyay *et al*., 2012; Zha *et al*., 2009). Phenanthrene and denbinobin isolated from aerial parts of *D. nobile* have been reported to possess cytotoxic activity against human lung and human ovary adenocarcinoma as well as antitumor activity (Gutierrez, 2010). The polysaccharides from different extracts of *D. nobile* possess antioxidant activity and activate macrophages (Lan Zhen *et al*., 2013; Luo *et al*., 2010).

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Amongst the other *Dendrobium* species, *Dendrobium fimbriatum* Hook. is one of the native species to India (Singh *et al.*, 2001). Infusion or decoction of its leaves is traditionally used to promote the production of body fluid and paste is applied on the fractured area to reset bones (Bi *et al*., 2003). *D. fimbriatum* is also used to treat liver disorders and nervous debility (Pant, 2013).

Asymbiotic germination of *D. fimbriatum* seeds on six different media has also been reported (Kumaria and Tondon, 1991). *D. fimbriatum* has been reported to possess phytochemical constituents like fimbriatone, confusarin, crepidatin, physcion, rhein, ayapin, scopolin methyl ester and n-octacostyl ferulate (Bi *et al*., 2003).

A number of phytochemical constituents like alkaloids, bibenzyl derivatives, flavonoids etc., have been reported from *Dendrobium moschatum* (Buch. – Ham.) Sw. (Singh *et al*., 2012), but compared to *D. fimbriatum*, detail investigation on phytochemical profile and therapeutic potential of *D. moschatum* has been overlooked. So far, two phenanthrenes viz. rotundatin and moscatin from *D. moschatum* have been reported to be useful in the inhibition of aggregation of platelets induced by arachidonic acid and collagen (Chattopadhyay *et al*., 2012). Also, *in vitro* propagation of this epiphytic orchid through thidiazuron induced high-frequency shoot proliferation has been studied (Nayak *et al*., 1997).

Dendrobium chrysanthum Wall. ex Lindl. is widely distributed in South China. Dried and ground leaves of *D. chrysanthum* are traditionally used as antipyretic, eye-benefiting, immunomodulatory agents and in the treatment of skin diseases (Li *et al*., 2001). A phenanthrene dendrochrysanene from this orchid has also been reported to possess anti-inflammatory activity (Yang *et al*., 2006). The studies carried out on the stem of *D. chrysanthum* revealed erianin isolated from it possesses anticancer activity (Gong *et al*., 2004; Li *et al*., 2001).

Dendrobium chrysotoxum Lindl. is very abundant and mainly distributed in the Southwest of China, Malaysia, India and Myanmar (Jiangmiao *et al*., 2012). The plant has been studied for the genetic transformation mediated by *Agrobacterium tumefaciens* strain LBA4404 (Bunnag and Pilahome, 2012). Like *D. chrysanthum*, this orchid has also been reported to possess anti-angiogenic and anti-inflammatory activity which is attributed to the presence of bibenzyl derivative erianin and dendrochrysanene (Gutierrez, 2010, Yang *et al*., 2006, Gong *et al*., 2004). Two new fluorenones namely; 2, 4, 7-trihydroxy-5-methoxy-9-fluorenone and 2, 4, 7-trihydroxy-1, 5-dimethoxy-9-fluorenone have been isolated from *D. chrysotoxum* (Yang *et al*., 2006a).

The extensive literature survey revealed that though, these orchids are extensively used for their ethnomedicinal importance; there is a dearth of scientific data in terms of their phytochemical evaluation. Till date, there is no work reported on other *Dendrobium* species in search of some substitute to the endangered species *D. nobile* in terms of their phytochemical profile.

In line with these facts, in this research work five different *Dendrobium* species namely; *D. nobile*, *D. fimbriatum*, *D. moschatum*, *D. chrysanthum* and *D. chrysotoxum* were evaluated for their ursolic acid, β-sitosterol and lupeol (terpenoids) and gallic acid (a phenolic compound) content using validated HPTLC techniques. Based on the reported therapeutic activities of ursolic acid (Kaewthawee and Brimson, 2013), β-sitosterol (Saeidnia *et al*., 2014; Shailajan *et al*., 2013), lupeol (Gallo and Sarachine, 2009) and gallic acid (Harish *et al*., 2008) they were selected as markers for phytochemical characterization of various *Dendrobium* species.

MATERIALS AND METHODS

Plant materials

Five different species of *Dendrobium*, namely; *D. nobile*, *D. fimbriatum*, *D. moschatum*, *D. chrysanthum* and *D. chrysotoxum* were collected from Northeast India. The representative sample of all five species of *Dendrobium* was authenticated from Dr. Suman Kumaria, Department of Botany, North East Hill University, Shillong. Different plant parts (roots and stem of *D. nobile*; and leaves, roots and stem of *D. fimbriatum*, *D. moschatum*, *D. chrysanthum*, *D. chrysotoxum*) were separated, thoroughly washed, cleaned and shade dried for a week followed by oven drying at 37°C for four days. The materials were powdered and sieved through 85 mesh (BSS) and stored in airtight containers.

Chemicals and reagents

Ursolic acid, β-sitosterol, lupeol and gallic acid, (98 % purity each) were procured from Sigma-Aldrich Chemical Company, (Steinheim, Germany). Chemicals of analytical grade were purchased from Merck Specialties Private Limited, Mumbai.

Chromatographic characterization

Extraction of phytochemical constituents from different samples of Dendrobium species

In order to extract terpenoids namely ursolic acid (**Figure 1A**), β-sitosterol (**Figure 1B**) and lupeol (**Figure 1C**) from the complex matrix of leaves, roots and stem of *D. nobile*, *D. fimbriatum*, *D. moschatum*, *D. chrysanthum* and *D. chrysotoxum*; each powdered sample (1.0 g) was extracted with 10.0 mL petroleum ether (Shailajan and Gurjar, 2015; Shailajan and Gurjar, 2014; Shailajan *et al*., 2012), vortex mixed for a minute and then sonicated for 20 min followed by filtration through Whatman filter paper no. 1.

The filtrate was evaporated to dryness under vacuum using rotary evaporator at 40°C, reconstituted in an equal volume of methanol and subjected to HPTLC analysis for the separation of these terpenoids. In order to extract the phenolic compound gallic acid (Figure 1D), the powdered sample (1.0 g) was extracted with methanol (10.0 mL), vortex mixed for a minute and sonicated for 20 min followed by filtration through Whatman filter paper no. 1. The filtrate was subjected to HPTLC analysis for the separation of gallic acid.

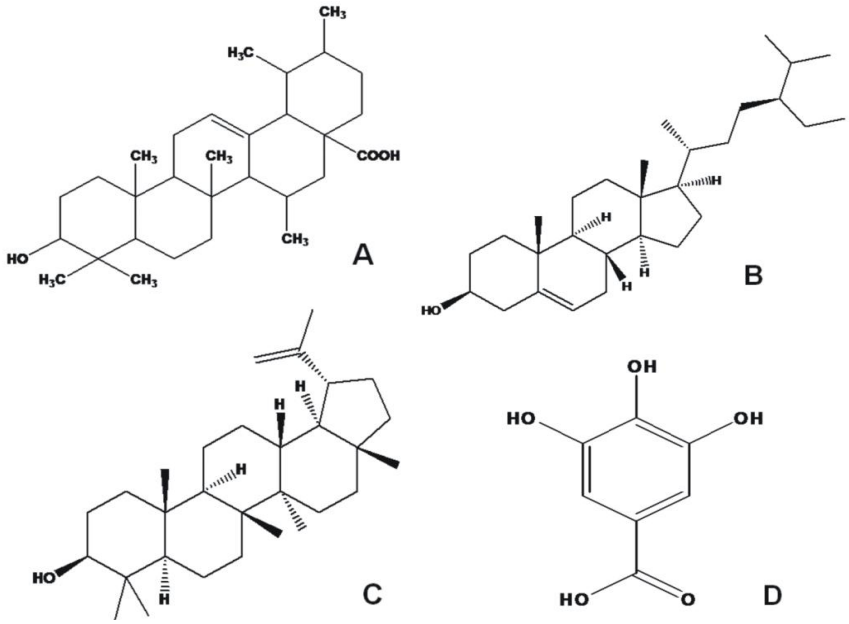

Fig. 1: Structure of (A) Ursolic acid (B) β-sitosterol (C) Lupeol and (D) Gallic acid.

Preparation of standard stock solutions

The standard stock solution of ursolic acid, β-sitosterol, lupeol and gallic acid (1000.0 µg/mL each) was prepared in methanol. Serial dilution of the stock solution in methanol was carried out in order to prepare calibrant/ quality control samples.

Optimized chromatographic conditions for quantitation of markers

High Performance Thin Layer Chromatography

The HPTLC system used consisted of CAMAG TLC Scanner 4 supported by winCATS software version 1.4.7 equipped with CAMAG Linomat 5 sample spotter and CAMAG Reprostar 3 system for photo-documentation. Chromatographic separation of the phytochemical constituents was achieved on TLC plates (E. Merck) pre-coated with silica gel 60 F_{254} (0.2 mm thickness) on aluminium sheet support.

For simultaneous separation of ursolic acid, β-sitosterol and lupeol from leaves, roots and stem of *D. nobile*, *D. fimbriatum*, *D. moschatum*, *D. chrysanthum* and *D. chrysotoxum*, 10.0 µL of samples along with the standards-ursolic acid (10.0 µg/mL), β-sitosterol (50.0 µg/mL) and lupeol (10.0 µg/mL) of 10.0 µL each were spotted on TLC plates as bands of 8.0 mm and at a distance of 15.0 mm from the edges. Plates were developed up to a distance of 85.0 mm in CAMAG twin trough glass chamber pre-saturated with mobile phase toluene: methanol (8:1, v/v) for 15 min and derivatized using freshly prepared 10% methanolic sulphuric acid and dried in an oven preset at 110°C for 10 min. For densitometric scanning, the source of radiation was a mercury lamp (366 nm). All measurements were performed at 22 ± 1°C. Plates were photo-documented at 366 nm. To separate gallic acid from leaves, roots and stem of *D. nobile*, *D. fimbriatum*, *D. moschatum*, *D. chrysanthum* and *D. chrysotoxum*, the sample (10.0 µL) and gallic acid standard (100.0 µg/mL, 10.0 µL) were Gedsvfcrrdeswa spotted on TLC plate as bands of 8.0 mm and at a distance of 15.0 mm from the edges under mentioned instrumental conditions. The plate was developed up to a distance of 85.0 mm in CAMAG twin trough glass chamber pre-saturated with mobile phase toluene: ethyl acetate: formic acid (2:7:1, v/v/v) for 15 min. The plate was scanned and photo documented at 254 nm.

Method validation

The HPTLC methods were validated as per ICH guidelines for parameters like sensitivity, linearity, precision, selectivity, recovery and ruggedness (International Conference on Harmonization, 1997).

Statistical analysis

Microsoft Excel-2007 was used to determine mean, standard deviation (SD), relative standard deviation (RSD) and mean difference during the analysis.

RESULTS AND DISCUSSION

Quality evaluation of herbal medicine is an important factor and basic requirement for the herbal drug industry. The analysis and quality control of herbal medicines are moving a step ahead towards an integrative and comprehensive direction, in order to tackle the complex nature of herbal medicines. High-performance thin layer chromatography (HPTLC) is one of the sophisticated instrumental techniques for standardization of the herbs and herbal drugs in terms of phytochemical constituents (Shivatare *et al.*, 2013). *Dendrobium* species could be useful in the selection of some promising alternative to an endangered species like *D. nobile*. Therefore, in this study five *Dendrobium* species were comparatively evaluated for the presence of terpenoids (ursolic acid, β-sitosterol, and lupeol) and a phenolic compound

(gallic acid). HPTLC analysis of different *Dendrobium* species was carried out using our recently published report on the estimation of ursolic acid, β-sitosterol, lupeol and gallic acid (Shailajan and Gurjar, 2015). Use of mobile phase composition toluene: methanol (8:1, v/v) showed a good resolution of terpenoids (ursolic acid at R_f = 0.28 ± 0.02, β-sitosterol at R_f = 0.48 ± 0.02 and lupeol at R_f = 0.58 ± 0.02) during the HPTLC experiment. Separation of β-sitosterol was easily achieved from the phytochemical matrix of different *Dendrobium* species. Ursolic acid was detected in both the parts (roots and stem) of *D. nobile*, whereas lupeol was detected only in the stem of *D. moschatum*. The presence of terpenoids in the samples was confirmed by overlay and color of the characteristic band with that of the standard. The plate photo, overlay along with the chromatograms of standards and different parts of five *Dendrobium* species are represented in Figure 2 (*D. nobile*), Figure 3 (*D. fimbriatum*), Figure 4 (*D. moschatum*), Figure 5 (*D. chrysanthum*) and Figure 6 (*D. chrysotoxum*), respectively.

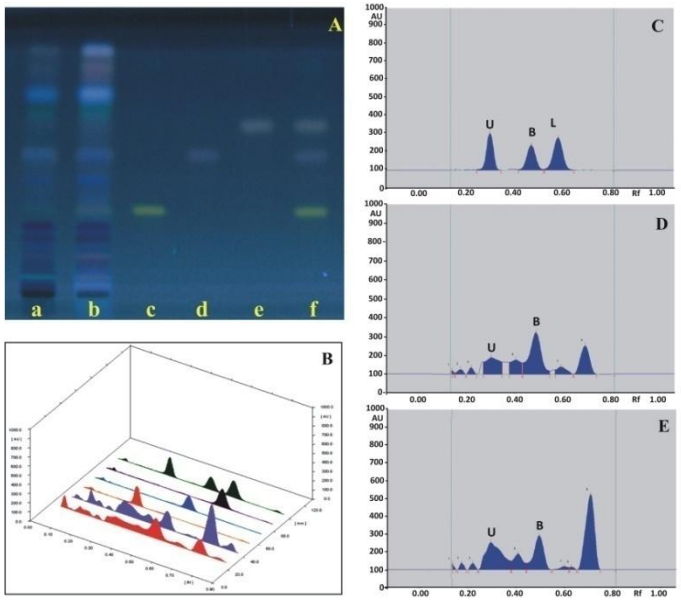

Fig. 2: TLC plate photo (A) and overlay of the chromatograms (B) showing presence of terpenoids - ursolic acid and β-sitosterol at 366 nm in *Dendrobium nobile* roots and stem.
Track details for plate A: a) *Dendrobium nobile* roots, b) *Dendrobium nobile* stem, c) ursolic acid (10 µg/mL), d) β-sitosterol (50 µg/mL), e) lupeol (10 µg/mL), f) mixture of ursolic acid, β-sitosterol and lupeol (10 µg/mL, 50 µg/mL and 10 µg/mL, respectively). C represents the chromatogram of three terpenoids - ursolic acid, β-sitosterol and lupeol. D represents the chromatogram showing ursolic acid and β-sitosterol in *Dendrobium nobile* roots. E represents the chromatogram showing ursolic acid and β-sitosterol in *Dendrobium nobile* stem.

The HPTLC method was validated as per ICH guidelines and found rapid, specific, precise, accurate and rugged (Table 1). The regression analysis of calibrant samples for each marker (ursolic acid, β-sitosterol, and lupeol) resulted in the form of regression equation which was further used to estimate their content in samples. Along with the bands of selected markers, the same mobile phase also showed separation of few more bands as follows: 6 and 7 bands in *D. nobile* roots and stem, respectively; 7, 5 and 8 bands in *D. fimbriatum* leaves, roots and stem, respectively; 9, 6 and 8 bands in *D. moschatum* leaves, roots and stem, respectively; 7, 2 and 6 bands in *D. chrysanthum* leaves, roots and stem, respectively and 9, 6 and 8 bands in *D. chrysotoxum* leaves, roots and stem, respectively.

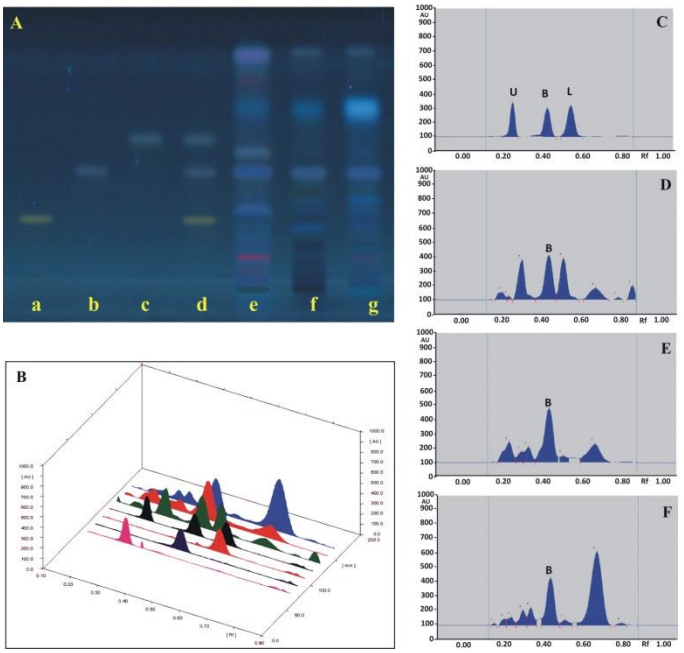

Fig. 3: TLC plate photo (A) and overlay of the chromatograms (B) showing the presence of a terpenoid - β-sitosterol at 366 nm in *Dendrobium fimbriatum* leaves, roots and stem.
Track details for plate A: a) ursolic acid (10 µg/mL), b) β-sitosterol (50 µg/mL), c) lupeol (10 µg/mL), d) mixture of ursolic acid, β-sitosterol and lupeol (10 µg/mL, 50 µg/mL and 10 µg/mL, respectively), e) *Dendrobium fimbriatum* leaves, f) *Dendrobium fimbriatum* roots and g) *Dendrobium fimbriatum* stem. C represents the chromatogram of three terpenoids - ursolic acid, β-sitosterol and lupeol. D represents the chromatogram showing β-sitosterol in *Dendrobium fimbriatum* leaves. E represents the chromatogram showing β-sitosterol in *Dendrobium fimbriatum* roots. F represents the chromatogram showing β-sitosterol in *Dendrobium fimbriatum* stem.

The mobile phase toluene: ethyl acetate: formic acid (2:7:1, v/v/v) showed good resolution of a phenolic compound (gallic acid at R_f = 0.53). But it was found absent in all the five *Dendrobium* species during HPTLC experiment (data not shown). This may be due to the presence of gallic acid in traces or presence of some other phenolic compounds in the plant samples. The content of phytochemical markers in different parts of five *Dendrobium* species is summarized in Table 2.

Amongst the five different species of *Dendrobium*, the maximum content of β-sitosterol was observed in stem of *D. fimbriatum* followed by leaves of *D. chrysanthum* and roots of *D. nobile* whereas ursolic acid was detected only in *D. nobile* and found maximum in its stem compared to the roots. Lupeol was detected only in the stem of *D. moschatum* (Table 2). This suggests the need of some other sensitive method to detect and quantitate markers such as ursolic acid and lupeol from these samples if present in traces.

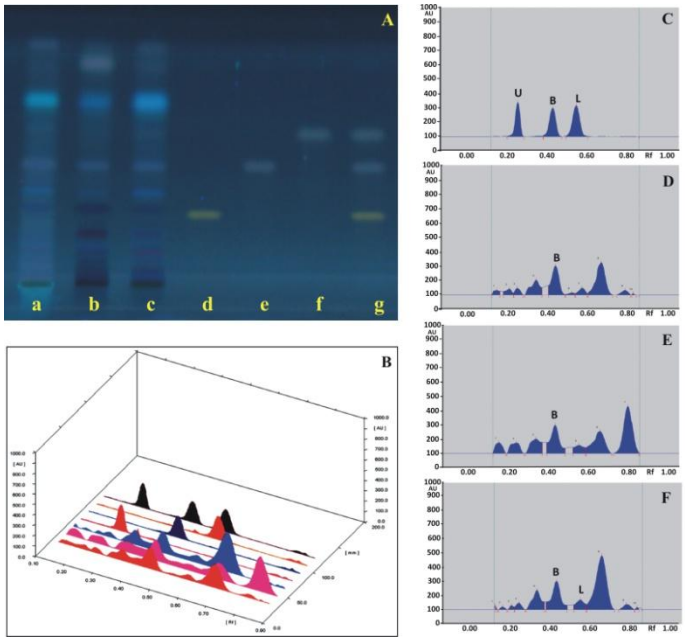

Fig. 4: TLC plate photo (A) and overlay of the chromatograms (B) showing the presence of terpenoids - β-sitosterol and lupeol at 366 nm in *Dendrobium moschatum* leaves, roots and stem.
Track details for plate A: a) *Dendrobium moschatum* leaves, b) *Dendrobium moschatum* roots, c) *Dendrobium moschatum* stem, d) ursolic acid (10 µg/mL), e) β-sitosterol (50 µg/mL), f) lupeol (10 µg/mL), and g) mixture of ursolic acid, β-sitosterol and lupeol (10 µg/mL, 50µg/mL and 10 µg/mL, respectively). C represents the chromatogram of three terpenoids - ursolic acid, β-sitosterol and lupeol. D represents the chromatogram showing β-sitosterol in *Dendrobium moschatum* leaves. E represents the chromatogram showing β-sitosterol in *Dendrobium moschatum* roots. F represents the chromatogram showing β-sitosterol and lupeol in *Dendrobium moschatum* stem.

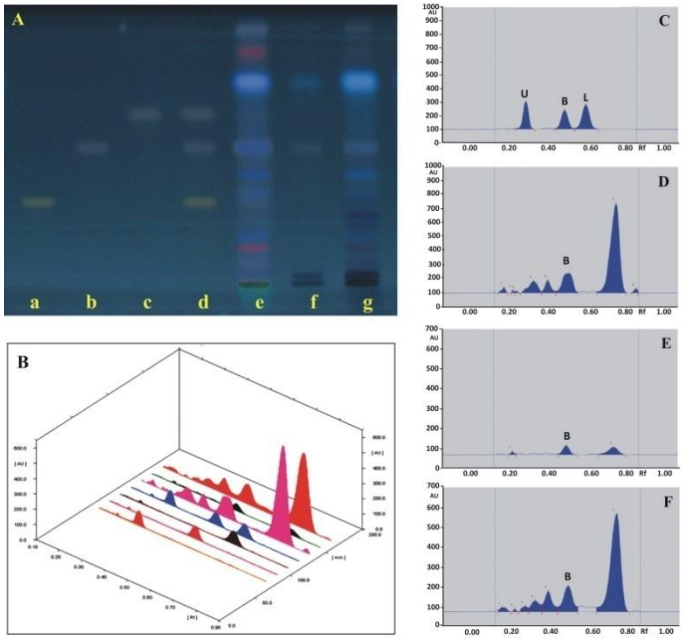

Fig. 5: TLC plate photo (A) and overlay of the chromatograms (B) showing the presence of a terpenoid - β-sitosterol at 366 nm in *Dendrobium chrysanthum* leaves, roots and stem.
Track details for plate A: a) ursolic acid (10 µg/mL), b) β-sitosterol (50 µg/mL), c) lupeol (10 µg/mL), d) mixture of ursolic acid, β-sitosterol and lupeol (10 µg/mL, 50 µg/mL and 10 µg/mL, respectively), e) *Dendrobium chrysanthum* leaves, f) *Dendrobium chrysanthum* roots and g) *Dendrobium chrysanthum* stem. C represents the chromatogram of three terpenoids - ursolic acid, β-sitosterol and lupeol. D represents the chromatogram showing β-sitosterol in *Dendrobium chrysanthum* leaves. E represents the chromatogram showing β-sitosterol in *Dendrobium chrysanthum* roots. F represents the chromatogram showing β-sitosterol in *Dendrobium chrysanthum* stem.

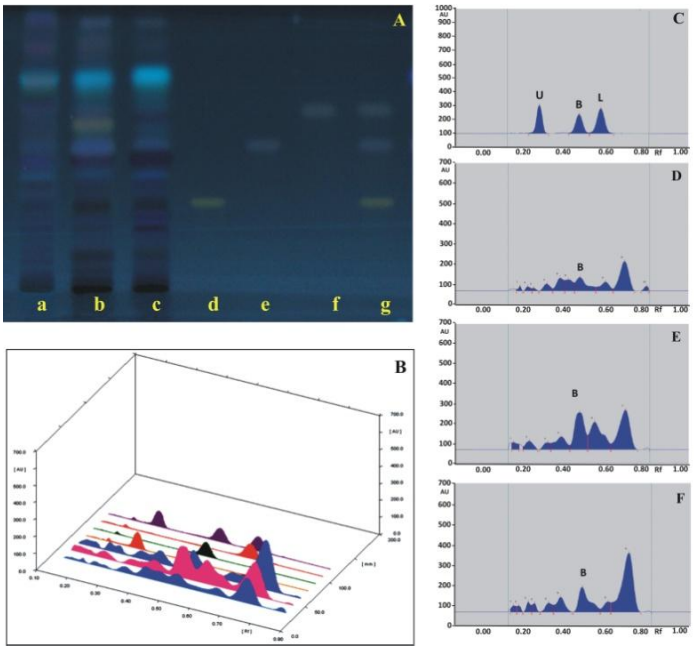

Fig. 6: TLC plate photo (A) and overlay of the chromatograms (B) showing the presence of a terpenoid - β-sitosterol at 366 nm in *Dendrobium chrysotoxum* leaves, roots and stem.
Track details for plate A: a) *Dendrobium chrysotoxum* leaves, b) *Dendrobium chrysotoxum* roots, c) *Dendrobium chrysotoxum* stem, d) ursolic acid (10 µg/mL), e) β-sitosterol (50 µg/mL), f) lupeol (10 µg/mL), and g) mixture of ursolic acid, β-sitosterol and lupeol (10 µg/mL, 50 µg/mL and 10 µg/mL respectively). C represents the chromatogram of three terpenoids - ursolic acid, β-sitosterol and lupeol. D represents the chromatogram showing β-sitosterol in *Dendrobium chrysotoxum* leaves. E represents the chromatogram showing β-sitosterol in *Dendrobium chrysotoxum* roots. F represents the chromatogram showing β-sitosterol in *Dendrobium chrysotoxum* stem.

All the therapeutically active compounds (ursolic acid, β-sitosterol, and lupeol) have been detected for the first time from the methanolic extract of *D. nobile*, *D. fimbriatum*, *D. moschatum*, *D. chrysanthum* and *D. chrysotoxum* following the reported extraction procedure and optimized HPTLC conditions.

Although, ursolic acid, β-sitosterol, lupeol and gallic acid are not the plant specific markers, they were chosen for the quality evaluation of five different *Dendrobium* species because of their proven therapeutic efficacy against various ailments. As simultaneous marker-based quantitative methods would be complementary as well as additive approaches for the quality control and stability assessment of herbal preparations (Shailajan *et al.*, 2013), the results of the present research work could be used for the chemical characterization of different *Dendrobium* species in order to check their uniformity.

The results of present research work suggest that, on the basis of maximum marker content, stem of *D. fimbriatum* could be the alternative source of *D. nobile* which should be equally supported by evaluation of safety and pharmacological profile.

Table 1: Results of method validation experiment for simultaneous estimation of three terpenoids (ursolic acid, β-sitosterol and lupeol) and a phenolic compound (gallic acid).

Parameters	Results for each phytochemical marker			
	Ursolic acid	**β-sitosterol**	**Lupeol**	**Gallic acid**
R_f	0.28 ± 0.02	0.48 ± 0.02	0.58 ± 0.02	0.53 ± 0.02
LOD (µg/mL)	2.5	1.0	2.0	10.0
LOQ (µg/mL)	5.0	5.0	5.0	20.0
Linearity (µg/mL)	5.0 – 100.0	5.0 – 60.0	5.0 – 75.0	20.0-150.0
Regression equation	y = 30.80x + 132.5	y = 39.76x + 194.0	y = 24.81x + 35.10	y= 56.67x-56.74
Coefficient of determination (r^2)	0.999	0.993	0.995	0.992
Instrumental precision(% RSD), n=7	1.82	1.63	1.57	1.12
Repeatability(% RSD), n=5	1.09	0.96	1.28	1.12
Intraday Precision(% RSD)	1.73	0.50	0.89	1.37
Interday Precision(% RSD)	1.99	0.93	1.15	1.94
Recovery (%)	97.23	96.54	98.16	98.12
Specificity			Specific	
Ruggedness			Rugged	

Table 2: Content of terpenoids (ursolic acid, β-sitosterol and lupeol) and a phenolic compound (gallic acid) in different parts of five *Dendrobium* species.

Species	Parts	Content of phytochemical markers (in mg/g) (Mean ± SD, n=3)			
		Ursolic acid	**β-sitosterol**	**Lupeol**	**Gallic acid**
Dendrobium nobile	Roots	0.85 ± 0.011	3.43 ± 0.008	ND	ND
	Stem	1.19 ±0.013	2.86 ± 0.004	ND	ND
Dendrobium fimbriatum	Leaves	ND	2.62 ± 0.005	ND	ND
	Roots	ND	2.81 ± 0.009	ND	ND
	Stem	ND	3.84 ± 0.012	ND	ND
Dendrobium moschatum	Leaves	ND	1.64 ± 0.005	ND	ND
	Roots	ND	1.83 ± 0.012	ND	ND
	Stem	ND	1.95 ± 0.009	0.92 ± 0.009	ND
Dendrobium chrysanthum	Leaves	ND	3.47 ± 0.013	ND	ND
	Roots	ND	0.90 ±0.015	ND	ND
	Stem	ND	2.86 ± 0.010	ND	ND
Dendrobium chrysotoxum	Leaves	ND	1.75 ± 0.008	ND	ND
	Roots	ND	3.25 ± 0.006	ND	ND
	Stem	ND	2.23 ± 0.011	ND	ND

(ND: not detected)

CONCLUSION

The demand for the use of *Dendrobium* for medicinal properties has increased considerably over the period of time. In the present research work, quality of *D. nobile*, *D. fimbriatum*, *D. moschatum*, *D. chrysanthum* and *D. chrysotoxum* has been evaluated in terms of three terpenoids viz. ursolic acid, β-sitosterol, and lupeol using validated HPTLC technique.

Findings of this chromatographic analysis revealed that, *D. nobile*, *D. fimbriatum*, *D. moschatum*, *D. chrysanthum* and *D. chrysotoxum* are a good source of β-sitosterol (a therapeutically active compound) and may also be used as a potential alternative to *D. nobile* which is one of the endangered *Dendrobium* species. The use of other *Dendrobium* species as an alternative source of *D. nobile* should also be supported by its therapeutic evaluation.

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