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Assessment of chemical diversity in *Clitoria ternatea* accessions by an improved and validated HPTLC method

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ABSTRACT

Evaluation of chemical diversity among genotypes of medicinal plants plays a pivotal role for its improvement and large scale cultivation. Chemical variability of bioactive principles, viz. taraxerol and β-sitosterol are analyzed in 11 populations of *Clitoria ternatea* L., an important memory enhancer used in Ayurveda. The study revealed high diversity among the collected samples with concentrations (mg/g on dry weight basis) ranging from 0.358±0.006 to 1.04±0.024 mg/g and 0.183±0.004 to 0.334±0.009 mg/g for taraxerol and β-sitosterol, respectively. The chemical variation was examined for different accessions under the same cultivation and environmental conditions during the years 2014 and 2015 at DMAPR, Anand, Gujarat, India. Selection of elite accession was made possible by screening germplasm collected from various parts of India. The results of the experiment refute the popular belief that plants with white flowers are superior to blue flower on phytochemical content basis. Further a validated HPTLC method was developed for the simultaneous determination of taraxerol and β-sitosterol from the species. The method was also applied to study the effect of solvent and plant parts to achieve enhanced active ingredient extract. Results showed that petroleum ether is the most effective solvent for extraction of the two major pharmacologically active constituents, viz. taraxerol (126±4.368 µg/mg of root extract) and β-sitosterol (13.2±1.706 µg/mg of root extract) due to enriched content with high purity root extract of *C. ternatea*.

Key words: β-sitosterol, *Clitoria ternatea*, Chemical diversity, HPTLC, Taraxerol

Clitoria ternatea L. (Aparajita) is a perennial herbaceous climbing plant belonging to the family Fabaceae. It is an important and medicinally valued herb with healing properties and memory enhancing qualities. Phytochemical screening of *C. ternatea* showed the presence of alkaloids, flavanoids, glycosides, carbohydrates, proteins, resins, starch and tannins. A wide range of biological active secondary metabolites are isolated and identified from the different part of *C. ternatea* which include triterpenoids, flavonol glycosides, anthocyanins and steroids (Mukherjee *et al.* 2008, Uma *et al.* 2009). The major phytoconstituents isolated from *C. ternatea* were taraxerol, taraxerone and β-sitosterol (Banerjee and Chakravarti 1963, 1964; Tiwari and Gupta 1959). β-sitosterol (Fig 1) is a main phytosterol found in plant kingdom, reported to show anti-inflammatory, antipyretic, anticancer, antidiabetics and immunomodulating activities (Von Holtz *et al.* 1998, Gupta *et al.* 2011, Fraile *et al.* 2012). A pentacyclic triterpenoid taraxerol was reported with antigiardial activity, analgesic, anticancer and anti-inflammatory activities (Hernández *et al.* 2012, Biswas *et al.* 2009, Yao *et al.* 2013).

Considering the importance of particular components and the plant, only two methods are reported for estimation of β-sitosterol (Rout *et al.* 2014) and taraxerol (Kumar *et al*. 2008) as an individual component analysis from *C. ternatea*. Hence in the present study, a simple, rapid, accurate and

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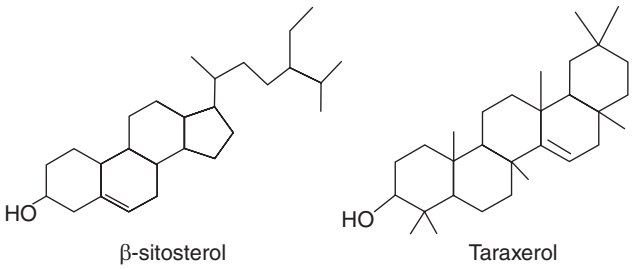

Fig 1 Chemical structure of bioactive compounds of *C. ternatea.*

precise High Performance Thin Layer Chromatography (HPTLC) method was developed and validated as per ICH guidelines for rapid screening and simultaneous determination of these two major pharmacologically active compounds, viz. β-sitosterol and taraxerol from *C. ternatea.* HPTLC is a standardized methodology that gives fingerprints of herbs for initial screening, possible adulteration screening of herbal products and quantitative evaluation (Reich and Schibli 2011). This is also applicable for simultaneous analysis of multi-components which make is a more cost effective tool (Gajbhiye *et al.* 2012). Bioactive secondary metabolites from herbs are increasingly being targeted for their health effects and potential for pharmaceutical uses. However, extraction of active principles from biological matrices and recovery from different sources requires standardization. The complete extraction depends on right choice of solvent and active plant parts (Ghasemzadeh *et al.* 2011). The present experiment was carried out to study the influence of various solvent and plant parts (root, stem and leaf) on extraction yield of these two investigated compounds.

Production of secondary metabolites in plants is influenced by environmental, geographical and genotypic factors (Sanwal *et al.* 2013, Chen *et al.* 2013). Influence of environmental, seasonal and genotypic characteristics on active principle content of medicinal plant can be helpful in the establishment of breeding improvement and conservation strategies (Facanali *et al*. 2015). Studies of genotypic variations based on chemical markers would be quite helpful for the selection of elite genotypes for commercial cultivation practices and pharmaceutical purpose with maximum yield of desired ingredients (Showkat *et al*. 2015). A systematic study on bioactive principles in diverse ecotypes of *C. ternatea* has not been reported in India. Considering the immense medicinal value and lack of proper chemical composition status of the species and floral varieties, the present investigation was aimed to evaluate the chemical diversity present within and among the populations of *C. ternatea* collected from different populations of India, using HPTLC method. This was an effort to explore the chemical variability in *C. ternatea* accessions for identification of potential areas with high content of taraxerol and β-sitosterol compounds.

MATERIALS AND METHODS

Root, stem and leaf parts of *C. ternatea* used for the extraction study were collected during 2014-15 from general plot of DMAPR, Boriavi, Anand, Gujarat, India which lies in latitude of 22.5° and longitude 73.0° and having average rainfall 800 mm, maximum temperature and minimum temperature ranges between 12.7° and 42° C. For the chemical variability study of *C. ternatea* accessions, seeds were collected from 11 different states of India (Table 1). Blue (B) and white (W) flowered accessions were collected from each collection sites except from Maharashtra (POP 4), Bihar (POP 7), Asom (POP 8) and Tamil Nadu (POP 10) from where only one flower types could be collected (Table 1). POP 4 (Maharashtra) and POP 8 (Asom) included an intermediate flower colour type, i.e. white petals with blue tinge on the petals (T) and POP 6 (Odisha) having lilac colour (L) (Fig 2). Hence based on flower colour and ecological collection, the populations were classified into 20 accessions (Table 1). The seeds of *C. ternatea* were placed in poly bags for germination and transplanted in the Field Gene Bank of the ICAR-Directorate of Medicinal and Aromatic Plants Research, Anand, Gujarat, India. The accessions were harvested after 9 months of planting and roots were collected from plants selected randomly (N = 3) from each accession. Collected fresh roots were washed by tap water, air dried in an oven below 45° C, and then made into fine powder in cyclone mill. In this study solvents and chemicals with analytical and higher purity grade were used. Standard reference chemicals β-sitosterol and taraxerol were purchased.

Table 1 Details of *C. ternatea* accessions included in the study

Locality	Population code	Accessions	Flower colour
Udaipur, Rajasthan, Northern India	POP 1	RAJ-W	White
		RAJ-B	Blue
Anand, Gujarat, Western India	POP 2	GUJ-W	White
		GUJ-B	Blue
Mandsaur, Madhya Pradesh, Central India	POP 3	MP-W	White
		MP-B	Blue
Akola, Maharashtra, Western India	POP 4	MH-T	White with blue tinge
Kalyani, West Bengal, Eastern India	POP 5	WB-W	White
		WB-B	Blue
Bhubaneswar, Odisha, Eastern India	POP 6	ODB-W	White
		ODB-B	Blue
		ODG-L	Lilac
		ODG-B	Blue
Pusa, Bihar, Eastern India	POP 7	BH-B	Blue
Gauvahati, Asom, Eastern India	POP 8	AS-T	White with blue tinge
Venkataramannagudem, Andhra Pradesh, Southern India	POP 9	AP-W	White
		AP-B	Blue
Coimbatore, Tamil Nadu, Southern India	POP 10	TN-W	White
Ernakulum, Kerala, Southern India	POP 11	KL-W	White
		KL-B	Blue

Analysis work was carried out on HPTLC equipment, M/s CAMAG made (Muttenz, Switzerland) and consisted of Linomat-V sample applicator fitted with a 100-µl syringe, TLC Scanner-3 operated with win CATS software (version 1.4.2), Reprostar-3 TLC documentation with CCD camera. Analysis was performed by using TLC pre-coated silica gel 60 F_{254} aluminium plates (20 × 10 cm) with petroleum ether: acetone (9.1:0.9, v/v) as mobile phase and linear ascending development was carried out in a twin-trough glass chamber (20 × 10 cm). Standard and sample solutions

Fig 2 Different petal colour variants of *C. ternatea* (a) Blue flower, (b) White flower, (c) White with blue tinge flower, (d) lilac flower.

were applied to the plates as 8 mm bands at 15 mm from the left edge and 12 mm from the lower, with 10 mm space between two bands. The chamber saturation time for the mobile phase was 5 min at temperature (25 ± 2°C) and relative humidity of 50% ± 5%. The TLC plates run up to 8 cm. The developed plate was derivatized by immersing in 5% sulphuric acid prepared in methanol for 2 sec and air dried then heated in oven at 110°C for 10 min. The derivatized plate was photographed in white light and 366 nm. The plate was scanned and quantified by densitometrically at 601 nm in absorbance mode by TLC scanner-3, which was controlled by winCATS software. The slit dimensions were 6 mm × 0.45 mm, and the scanning speed was 20 mm/s. Quantitative evaluation of the plate was performed using polynomial regression equations of respective compounds.

Stock solutions (1 000 ppm) of the reference compounds of β-sitosterol and taraxerol were prepared in methanol by accurately weighing each standard into a volumetric flask. The further above stock was diluted in appropriate concentration and mixed in equal volume in one flask used as a working standard solution. The aliquots (4, 8, 12, 16, 20, and 24 µL) of mixed working standards solution had concentration ranges of 100–600 ng/spot for β-sitosterol and taraxerol applied on the TLC plates for calibration curve.

Powdered samples of root, stem and leaf parts of *C. ternatea* (5 g) were extracted with petroleum ether, chloroform, ethyl acetate, acetone, methanol and ethanol (3×150 ml, 1 hr), under reflux condition on a boiling water-bath at 85 ± 5°C. Each solvent extract was filtered and then evaporated under reduce pressure. The residue (10 mg) was quantitatively transferred to a 10 ml volumetric flask with appropriate solvent. The root sample (2 g) of *C. ternatea* accessions were extracted in methanol (3×50 ml, 1 hr), filtered and dried it, after that the residue was dissolved in 20 ml methanol. The further required dilutions of each sample extracts were done according to standard calibration curve. The prepared sample was centrifuged at 10 000 rpm for 10 min and supernatants were filtered through a 0.45 µm membrane before loading to HPTLC analysis used for the quantification of β-sitosterol and taraxerol. Suitably diluted sample (10 µl) of each extract was applied in triplicates on a TLC plate. The plates were developed, derivatized and scanned as mentioned above. The peak areas were recorded, and the amount of β-sitosterol and taraxerol was calculated using the calibration curve.

RESULTS AND DISCUSSION

Development of TLC procedure

TLC procedure was developed based on quantification of two major bioactive compounds in plant sample. The analysis was carried out on normal phase TLC. An effective mobile phase optimized for the separation of β-sitosterol and taraxerol using different system wherein various compositions consisting of different ratios of solvents of varying polarity were tried like toluene–ethyl acetate–methanol, chloroform and methanol, petroleum ether– ethyl acetate. The mobile phase of petroleum ether– acetone (9.1:0.9, v/v) gave the best resolution, with a sharp and well defined peak of β-sitosterol at $R_F = 0.22 \pm 0.04$ and taraxerol at $R_F = 0.38 \pm 0.05$ from the other components of the sample extracts and enabled their simultaneous quantification. The developed method was found to be quite selective with good baseline resolution of each reference compounds. The 3D densitogram patterns of the test samples and standards showed that the peak corresponding to R_F value of each reference compounds were super imposable in all the samples. The peak purity and identification of the bands of compounds in the test sample were confirmed by overlaying their absorption spectra with those of the standards and the spectrum analysis found to correlate exactly, indicating the compounds at R_F value of 0.22 and 0.38 of the standards and the test samples to be similar. The derivatized plates were visualized in white light and at 366 nm, as the compounds were found to absorb at variable spectrum range. The generated fingerprint data could be enhanced visual identification of individual compounds from the plant samples.

Method validation

The developed HPTLC method was validated as per ICH guidelines for different parameters like specificity, accuracy, precision, and sensitivity (ICH 2005). The specificity of the method was confirmed by analysing the bands of β-sitosterol and taraxerol in sample solution were ascertained by comparing the R_F and spectra pattern with

the reference standards.The six-point calibration curves for each standard plotted in the range of 100–600 ng/spot. Regression equation and correlation coefficient for the reference compound were as follows: Y= 55.568+3.631*X + (-0.002*X2) (r = 0.9999) for β-sitosterol and Y= (-199.047)+5.268*X + (-0.003*X2) (0.9991) for taraxerol, which revealed a good correlation coefficient for developed method and are presented in Table 2. Accuracy of developed analytical method was examined by recovery test, spiking known amount of standard reference compound. The samples were spiked with three different concentrations (100, 200, and 300 ng) of β-sitosterol and taraxerol. The experiment was repeated in triplicate and then analysed by proposed HPTLC method. Good recoveries were obtained by the fortification of the sample at three concentration levels for each compound. It is evident from the results that the average per cent recovery was 98.23% for β-sitosterol and 98.41% for taraxerol as shown in Table 2. The sensitivity of the method was studied with respect to limit of detection (LOD) and limit of quantitation (LOQ). LOD was defined as lowest concentration detected by the instrumentof standard compound (S/N ratio 3:1) and LOQ was lowest concentration quantified in the sample (S/N ratio 10:1) determined by analytical method. LOD obtained were 30 ng/spot for β-sitosterol and 35 ng/spot for taraxerol. While the LOQ obtained was 90 ng/spot for both compound (Table 2). This indicated that the proposed method exhibits a good sensitivity for the quantification of above compounds. The precision of the developed method was checked by measurement of peak area in repeated scanning in six replicates of same spot (300 ng/spot) of both standards. The repeatability of the sample application and measurements of peak area was expressed in terms of percentage relative standard deviation (%RSD). The precision was found to be 0.45 for β-sitosterol and 0.58 for taraxerol (Table 2). The TLC method were found precise with % RSDs for intra-day and inter-day (five replications in the same plate) in three different concentration of (100, 300 and 500 ng/spot) of both standards in the range of 1.37-2.24, 2.12-3.04 for β-sitosterol and 1.76-2.12, 1.96-3.15 for taraxerol, respectively, (Table 3) which demonstrated the good precision of proposed method.

Table 2 Method validation parameters of β-sitosterol and taraxerol

	β-sitosterol	Taraxerol
Rf	0.22 ± 0.04	0.38 ± 0.05
Linearity range (ng/spot)	100-600 ng/spot	100-600 ng/spot
Regression equation	Y=55.568+3.631* X + (-0.002*X2)r = 0.9999, sdv = 0.22%	Y=(-199.047)+ 5.268*X + (-0.003*X2)r = 0.9991, sdv = 3.58%
Instrument precision (% RSD) (n = 6)	0.45	0.58
Specificity	Specific	Specific
LOD (ng)	30	35
LOQ (ng)	90	90
Average % recovery (n=3)	98.23	98.41

Extraction optimization study in different polarity solvent and within the plant parts

The extraction yield varied by chemical nature of sample, solvent, time and temperature used for the extraction process. Among that the chemical property and solvent polarity will play a key role in increasing the extraction yield (Iloki-Assanga *et al.* 2015, Sulaiman *et al.* 2011). Different parts of the same plant may synthesize and accumulate different compounds or different amounts of a particular compound (Gajbhiye *et al.* 2015). Considering that, the effect of various solvents (Petroleum ether, chloroform, ethyl acetate, acetone, methanol and ethanol) on extraction yield of β-sitosterol and taraxerol from root, stem and leaf parts of *C. ternatea* studied. From the results of present report, indicated a wide variation in the taraxerol and β-sitosterol content of different solvent extracts, taraxerol ranging from 1.0 to 126 µg/mg of extract and β-sitosterol ranging from 1.5 to 13.3 µg/mg of extract. It showing the highest taraxerol content was found in petroleum ether extract of root (126 ± 4.368 µg/mg extract) and the highest β-sitosterol content in leaf (13.3 ± 0.962 µg/mg extract). Kumar *et al.* (2008) reported the taraxerol was found to be 12.4 mg/g (w/w) in hydro-alcoholic extract of *C. ternatea* root, which was lower comparatively to the present reported yield in petroleum ether (126 µg/mg of root extract). The experimental results proved that the petroleum ether to be the most effective solvent for extraction due to enriched content of taraxerol and β-sitosterol compounds (126 and 13.2 µg/mg of root extract) from samples of *C. ternatea*, whereas much lower yields were obtained from ethanol extract (1.0 µg/mg of taraxerol from leaf and 2.55 µg/mg of β-sitosterol from stem).The order of effectiveness in extraction of taraxerol was found in petroleum ether (126 µg/mg extract) >chloroform (54.4 µg/mg extract) >acetone (30 µg/mg extract)≥ethylacetate (28 µg/mg extract) >methanol (6.2 µg/mg extract) >ethanol (5.6 µg/mg extract) from root parts of *C. ternatea.* The order of β-sitosterol extraction in petroleum ether (13.2 µg/mg extract) >chloroform (11.1 µg/mg extract) >ethylacetate (4.6 µg/mg extract)≥acetone (4.55 µg/mg extract)>methanol (1.775 µg/

Table 3 Intra-day and inter-day precision of the developed HPTLC method

Compounds	Amount (ng)	Intra-day precision % RSD	Inter-day precision %RSD
β-sitosterol	100	1.86	2.12
	300	1.37	2.27
	500	2.24	3.04
Taraxerol	100	2.12	1.96
	300	1.76	2.28
	500	1.80	3.15

(*n*=5), RSD, Relative standard deviation.

mg extract) >ethanol (1.5 µg/mg extract) from leaf parts of *C. ternatea*. The present study revealed that the distribution of taraxerol and β-sitosterol differed significantly between different parts of *C. ternatea*. From the results it was observed that the content of taraxerol found higher in root as compare to stem and leaf, while β-sitosterol occurs higher in leaf part more than the root and stem parts of the plant. It is for the first time that a simple, accurate, and rapid HPTLC method has been developed and validated for the simultaneous determination of β-sitosterol and taraxerol in different polarity solvent and plant parts of the species.

Assessment of chemical diversity bases on taraxerol and β-sitosterol in different accessions of C. ternatea

Twenty different *C. ternatea* accessions were analyzed for extraction yield of two major bioactive principles taraxerol and β-sitosterol. The concentrations of both components were calculated based on HPTLC study and presented in Table 4. Among all the accessions the taraxerol yield was found higher as compare to β-sitosterol which is in accordance with the literature (Rout *et al.* 2014 and Swain *et al.* 2012). The taraxerol content ranged from 0.358 ± 0.006 to 1.04 ± 0.024 mg/g, with a mean of 0.586 ± 0.026 mg/g in all accessions. The β-sitosterol concentration ranged from 0.183 ± 0.004 to 0.334 ± 0.009 mg/g, with a mean of 0.256 ± 0.011 mg/g among the all accessions. The results also supported by evidence of sterol and petacyclic terpenoid compounds distribution in different geological collected samples of *Prunus africana* (Kadu *et al.* 2012). In similar the phytochemical (kaempferol content) variability was found among different geographical accessions of *C. ternatea* (Zahid *et al.* 2013). The study also take attention towards the phytoconstituents distinction among the studied samples even though a very low genetic diversity was reported among different populations of *C. ternatea* from diverse geographical locations (Bishoyi *et al.* 2014). The highest taraxerol content observed was in KL-W (1.04±0.024 mg/g), whereas AS-T had the lowest (0.358±0.006 mg/g) and β-sitosterol content was found highest in RAJ-B (0.334±0.009 mg/g), whereas lowest recorded in AP-B (0.183±0.004 mg/g). This kind of variability in chemical constituents may be due to the variability in reaction of diverse genotypes to similar environment conditions in between collected accessions from different regions (Sanwal *et al.* 2010).

Table 4 Phytochemical content in different accessions of *C. ternatea* (each value is represented as mean ± SD) (n=3)

Accessions	Taraxerol (mg/g)	β-Sitosterol(mg/g)
RAJ-W	0.561±0.032	0.326±0.074
RAJ-B	0.494±0.018	0.334±0.009
GUJ-W	0.520±0.020	0.239±0.033
GUJ-B	0.538±0.011	0.256±0.008
MP-W	0.426±0.044	0.254±0.017
MP-B	0.510±0.045	0.260±0.003
MH-T	0.483±0.004	0.253±0.001
WB-W	0.661±0.039	0.205±0.015
WB-B	0.579±0.039	0.196±0.001
ODB-W	0.480±0.035	0.249±0.003
ODB-B	0.466±0.040	0.348±0.003
ODG-L	0.736±0.007	0.238±0.002
ODG-B	0.504±0.005	0.233±0.012
BH-B	0.609±0.006	0.270±0.006
AS-T	0.358±0.006	0.236±0.001
AP-W	0.569±0.033	0.224±0.011
AP-B	0.606±0.077	0.183±0.004
TN-W	0.591±0.017	0.260±0.008
KL-W	1.040±0.024	0.285±0.012
KL-B	0.979±0.027	0.276±0.006

Petal colour variabilities of *C. ternetea* were reported in this species. White flowered variety is believed to be therapeutically more potent and blue flowered types is perceived to be inferior (Mukherjee *et al.* 2008). Experiment was conducted to profile the phytochemicals from the plants with different petal colour and correlation study between flower colour and chemical constituents of *C. ternatea* was done. Seven populations were considered for the study of taraxerol and β-sitosterol content variability among the white and blue flowered variety collected from the same regions. From the results higher taraxerol content was observed in four different populations, viz. POP 11, POP 5, POP 1 and POP 6 in white flowered variety as compared to blue flowered, while in three populations POP 9, POP 2 and POP 3 reverse trend was found. The β-sitosterol concentration was found higher in white flowered variety in three populations POP 11, POP 9 and POP 5 while remaining in four populations, viz. POP 6, POP 3, POP 2 and POP 1 have higher β-sitosterol content in blue flowered one. Result revealed that there was no correlation between blue and white flowered accessions collected from the same region in respect of taraxerol and β-sitosterol content. The taraxerol content was comparable in two petal colour variants KL-W (1.04±0.024 mg/g) and KL-B (0.979±0.027 mg/g) of same population. Similar result was found in case of β-sitosterol content from RAJ-W (0.326±0.074 mg/g) and RAJ-B (0.334±0.009 mg/g). The study showed that POP 11 (1.009±0.025 mg/g) and POP 1 (0.33±0.041 mg/g) are suitable for cultivation for higher yield taraxerol and β-sitosterol respectively from *C. ternatea* with potential application in pharmaceutical industries.

The present investigation is a first report of assessment of petal colour variants and phytochemical diversity among the populations of *C. ternatea* from India using HPTLC analysis. The study revealed the broad chemical diversity in two major bioactive compounds, viz. taraxerol and β-sitosterol from different geographical samples. The study also focus on similar chemical composition in floral variants (blue and white flowered) of *C. ternatea* in respect of investigated compounds. Knowledge on chemical diversity from different populations would be helpful in selection of superior accessions for plant harvesting practice and industrial uses. Quality assessment of geographical accessions was carried out by a simple, precise, and accurate HPTLC method developed for simultaneous determination

of β-sitosterol and taraxerol from *C. ternatea* root extract. The proposed method was applied to study the extraction optimization and proved the petroleum ether extract from root part of the species to be the most effective due to enriched content of taraxerol and β-sitosterol. The validated method may be adopt in quality control for raw drug or formulations of *C. ternatea* and also applicable to other similar herbs.

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