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Exploration of HPTLC Technology for Rapid Chemical Fingerprinting and Simultaneous Determination of Bioactive Constituents from *Clitoria ternatea* Linn.

Jayanti Makasana,*[a] Narendra Gajbhiye,[b] Ashok Kumar Bishoyi,[c] Mehulkumar Savaliya,[d] Saravanan Raju,[e] Shrikant Bansod,[f] Lalji Baldaniya,[g] and Bharatkumar Dholakiya[h]

The analytical methods used for herbal analysis are need to be economic, fast and also produce minimum quantities of hazardous chemical waste. Presently analytical community put interest in the research area of non-hazardous and eco-friendly practices to develop various green chromatographic methods for routine quality analysis. High cost of phytochemical analysis and uses of hazardous chemicals with high-end sophisticated instrument, the attempt made to develop a simple analytical method for multiple samples with short time and less uses of solvents. A HPTLC method was developed for simultaneous determination of biological important constituents like β-sitosterol, taraxerol, clitorienolactone B and β-sitosterol glycoside from *Clitoria ternatea* Linn. The proposed method was validated and satisfied the ICH guidelines to demonstrate that the method is adaptable for its intended purpose. The method is simple, sensitive and economic it therefore embraces potential for detection, monitoring, and simultaneous quantification of the four bioactive compounds for *C. ternatea* and could also be apply to other species.

Introduction

The herbal drug is widely used as alternative medicine worldwide due to its healing properties. However, no measures are available for checking its quality in the majority of formulations. There is an urgent need to establish quality control standards for the herbal raw material or its bioactive compounds enriched formulated products. At present, chemical markers play a key role in the quality evaluation of herbal medicines which in turn is depending upon the availability of analytical tools.[1] The liquid chromatography methods are generally used with a conventional column, which produces waste of hazardous organic solvents.[2] Researchers applied many of chromatographic approaches to overcome the impact of harmful and toxic chemicals using the minimum quantity of unsafe organic solvents without changing the performance of the methods. Since the introduction of chromatography by Michael Tsweet, thin layer chromatography (TLC) was considered suitable for the separation and identification of plant components.[3] Presently TLC has become more sophisticated with reducing manual errors, reproducible results, and effective band separation with high resolution as HPTLC (High Performance Thin Layer Chromatography) technique.

Eco-friendly spectrophotometric and chromatographic methods are available for greenness profile assessment of pharmaceutical products via Eco-scale and GAPI tools.[4,5] HPTLC is a simple, flexible, reliable, and cost-effective separation technique for high-potential qualitative and quantitative herbal analysis compared to other chromatographic techniques like HPLC, LC–MS, LC–MS/MS, etc. The techniques also are considered as eco-friendly by minimizing the exposure hazard of toxic organic solvents and falling environmental pollution. It allows multi-sample analysis simultaneously which is generally not feasible with other available analytical techniques. Chemical fingerprinting generated by HPTLC gives a unique, specific and characteristic pattern of separated compounds agreed to their particular R_f (retention factor) values on the HPTLC plate and the chromatogram.[6,7] The fingerprint is a key feature in the quality control of complex herbal medicines and is already used by the American Herbal Pharmacopoeia for the identification of herbal products with fundamental chemical representations of "sameness" and "differences".[8] Moreover, the technique coupled with densitometry scanning was also accepted in the European Pharmacopoeias for routine quantitative herbal analysis.[9] HPTLC plates with smaller particle-sized silica and operated with an automated sample applicator,

[a] *Dr. J. Makasana*
Department of Chemistry, Marwadi University, Rajkot, Gujarat-360003, India
E-mail: jayantilal.makasana@marwadieducation.edu.in
[b] *Dr. N. Gajbhiye*
Division of Organic Chemistry, ICAR-DMAPR, Boriavi, Anand, Gujarat-387310, India
[c] *Dr. A. Kumar Bishoyi*
Department of Microbiology, Marwadi University, Rajkot, Gujarat-360003, India
[d] *Dr. M. Savaliya*
Department of Industrial Chemistry, Atmiya University, Rajkot, Gujarat-360005, India
[e] *Dr. S. Raju*
Division Crop Production, ICAR-CTCRI, Thiruvananthapuram, Kerala-695017, India
[f] *Dr. S. Bansod*
Department of Chemistry, Smt. Narsamma art commerce and science college Amravati, Maharashtra-444606, India
[g] *Dr. L. Baldaniya*
Faculty of Pharmacy, Marwadi University, Rajkot, Gujarat-360003, India
[h] *Dr. B. Dholakiya*
Department of Chemistry, SVNIT, Surat, Gujarat-395007, India

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digital image capturing by high-resolution cameras and computing software make the technique more hi-tech.[7]

β-sitosterol and taraxerol are phytocompounds broadly attributed in plant kingdoms owning their medicinal importance. β-sitosterol is mostly used in the treatment of coronary disease, hypercholesterolemia, cancer inhibition, arthritis, etc.,[10] while taraxerol showed a significant activity for anti-inflammatory, anti-cancer and Alzhiemer's and Parkinsonism, etc.[11] β-sitosterol glycoside is reported as potent anticancer agent[12] and Clitorienolactone B was reported as a memory enhancing agent.[13] Even though wide pharmacological applications of the *C. ternatea* principles, analytical methods are not available for its quality determination.

It has been substantial consideration to develop a rapid analytical technique for analysis of the plant material and pharmaceutical products due to the innumerable pharmacological benefits of the said constituents in the plants. Still, to the best of our knowledge, a method has not been reported for simultaneous determination of the compounds for the plant. Considering the qualitative and quantitative applications of HPTLC techniques and the need for a reliable and multi marker-based rapid analytical method for quality control of *C. ternatea.* The proposed study focused on the development of a green eco-friendly HPTLC method with highly efficient approaches for simultaneous separation and determination of the four bioactive compounds i.e., β-sitosterol, taraxerol, clitorienolactone B and β-sitosterol glucoside from the plant. The developed HPTLC fingerprint may use in rapid screening of raw drugs of *C. ternatea* or their various extracts and finished products.

Results and Discussion

In this investigation, a validated HPTLC protocol was developed for the simultaneous quantitative determination of the bioactive contents in *C. ternatea* by optimization of mobile phase, chamber saturation time, detection wavelength, visualizing agents and plate derivation time, etc., with effective separation and resolution of bioactive compounds of the plant samples.

Optimization of HPTLC chromatographic conditions for rapid analysis of four bioactive phytoconstituents from *C. ternatea*

Mobile phase optimization was carried out to resolve the four marker compounds of *C. ternatea* i.e., taraxerol, β-sitosterol, clitorienolactone B and β-sitosterol glucoside on TLC plate. The chemical structure of the four bioactive compounds is presented in Figure 1.

In the beginning of method development, all four compounds were tried to separate by earlier reported individual estimation methods viz., sterols[14,15] taraxerol[16–19] and β-sitosterol glucoside.[20,21] These methods failed in the simultaneous separation and evaluation of all the compounds. Therefore, in the standardization of the new HPTLC method, the four standards were applied on a TLC plate and then developed by various polarity ranged solvents like petroleum ether, chloroform, toluene, ethyl acetate, acetone and methanol.

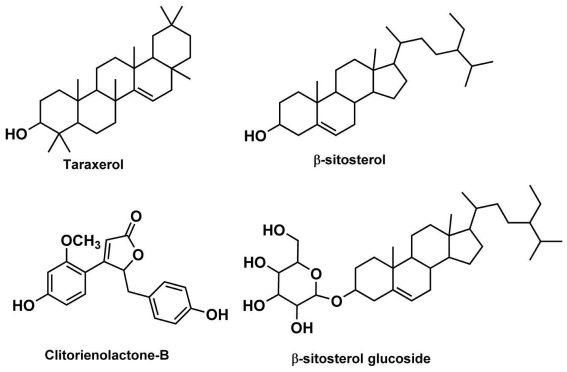

Figure 1. Structure of reference standard chemicals.

According to preliminary observation, combinations of these solvents were checked as binary, ternary and quaternary solvent systems to separate the compounds within the plate. A combination of chloroform: methanol (9:1 and 8.5:1.5 v/v) was tried but the results were not found clear, so the same solvents with formic acid were performed in several ratios like 8:2:0.1, 8.5:1.5:0.2, 7:3:0.1 and 9:1:0.5 (v/v) which also could not achieve the adequate separation. Likewise, other solvent systems with combinations also failed, while in the solvent system of chloroform: toluene (6:4 v/v) the taraxerol, β-sitosterol and clitorienolactone B were separated from each other however, β-sitosterol glucoside was not moved from its TLC application point. Adding a small quantity of methanol in this solvent system with an altered proportion of chloroform and toluene, the separation of all four compounds was found better with good peak resolutions. Finally, the solvent system of chloroform: toluene: methanol in the ratio of 7:3:1 (v/v) was selected as the best for effective resolution with symmetrical and reproducible peaks of each compound as; taraxerol (R_f 0.86 ± 0.004), β-sitosterol (R_f 0.75 ± 0.005), clitorienolactone B (R_f 0.35 ± 0.004) and β-sitosterol glucoside (R_f 0.22 ± 0.004) as shown in Figure 2. As per the GSK's solvent sustainability guidelines toluene and methanol fall in amber zone; chloroform in red zone.[22] Nonetheless the method required tiny amount of above-mentioned solvents compared to other analytical methods. TLC plate observed under UV light at 366 nm was found the best for visual detection of clitorienolactone B (Figure 3), while the other three compounds like taraxerol, β-sitosterol and β-sitosterol glucoside could not be detected under these conditions. They were made visualized using vanillin-sulfuric acid derivatization reagent and produced purple-coloured bands under white light (Figure 3).

Densitometry scanning for quantitative purpose revealed the maximum absorption of clitorienolactone B was found at 366 nm before derivatization of the plate. While taraxerol, β-sitosterol and β-sitosterol glucoside showed the maximum absorption at 551 nm after derivatization of the same plate. In similar to the present study the vanillin-sulfuric acid used for visualization of terpenoids phytocompounds in TLC.[7]

If densitometry scanning of TLC plate is carried out at a single wavelength, the high throughput of a particular

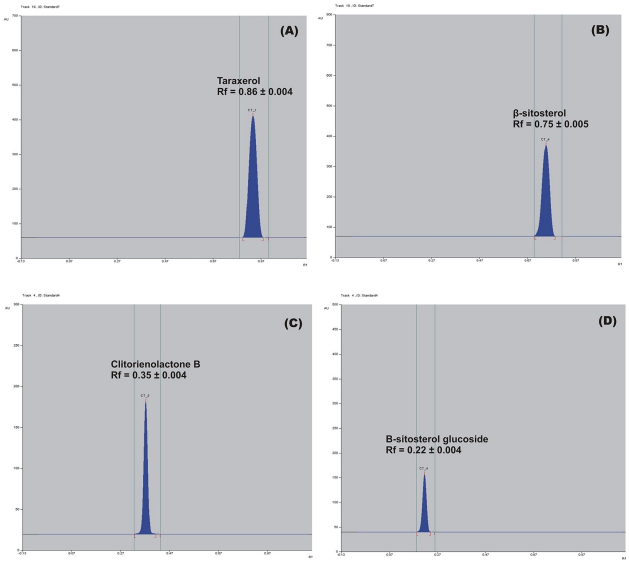

Figure 2. HPTLC chromatograms of reference compounds; (A) Taraxerol, (B) β-sitosterol, (C) clitorienolactone B and (D) β-sitosterol glucoside.

compound may be lost in multi-compound analysis due to diverse absorption properties of compounds or their different concentration in plant extracts.[23] Hence, the developed plate needed multi wavelengths scanning in the range of 200 to 700 nm with assigning all track bands to find out a suitable wavelength for the best absorption of the targeted compounds.

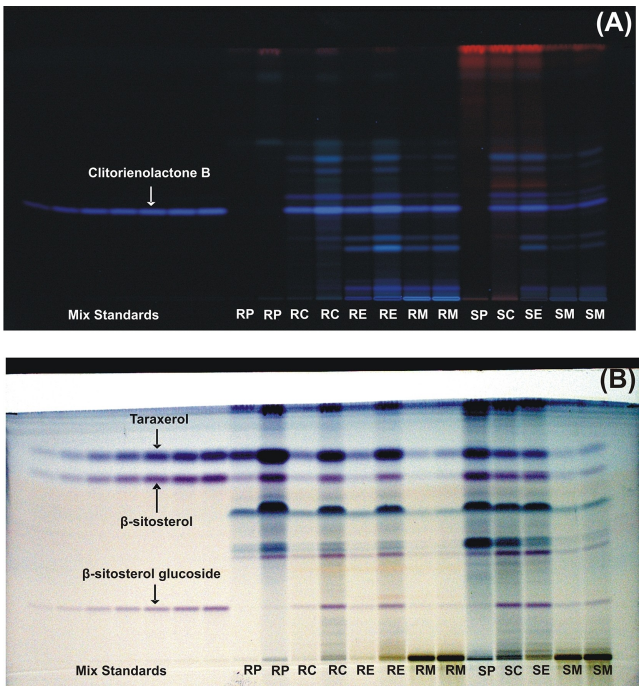

Figure 3. TLC chemical profile of root and stem extracts of *C. ternatea* with mixture of standards; Detection of plate (A) at 366 nm before derivatization and (B) under white light after derivatization.

Under these optimized conditions root and stem extracts of *C. ternatea* were analyzed and their respective HPTLC chromatograms (Figure 4 and 5) showed the resolved peaks of reference compounds and as well as the extracted sample. The HPTLC chemical profiling accomplished in different solvent extract of *C. ternatea*, which revealed the occurrence of various phytochemicals with diverse amount. The R_f values of the analytes in the plant extracts confirmed by comparing them with the reference standards. The chromatograms (Figure 4; E–H and Figure 5; E–H) indicated the peak of Clitorienolactone B was found in all the root and stem extracts except petroleum ether extract of each. While the chromatogram (Figure 4; A–D and Figure 5; A–D) represent the separation of taraxerol, β-sitosterol and β-sitosterol glucoside. The peak of β-sitosterol glucoside was not found in the petroleum ether extract of root while the peaks of all these three compounds were observed in all the extracts. Marker compound and 3D densitogram based fingerprints of the plant extracts were established as shown in Figure 6 and 7.

The densitogram shows that the separation permitted for simultaneous determination of each analyte with utmost sensitivity assigned by the recorded spectrodensitogram. The similar study also reported for pharmaceutical formulations.[24,25]

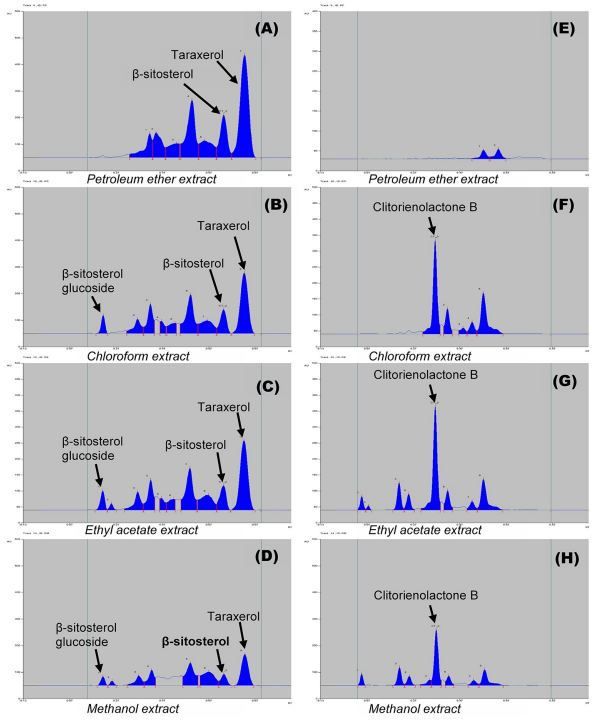

Figure 4. HPTLC chromatograms of root extracts of *C. ternatea* at 551 nm after derivatization and 366 nm before derivatization; Figures A–D chromatograms obtained at 551 nm after derivatization of TLC plate and E–H chromatograms acquired at 366 nm before derivatization.

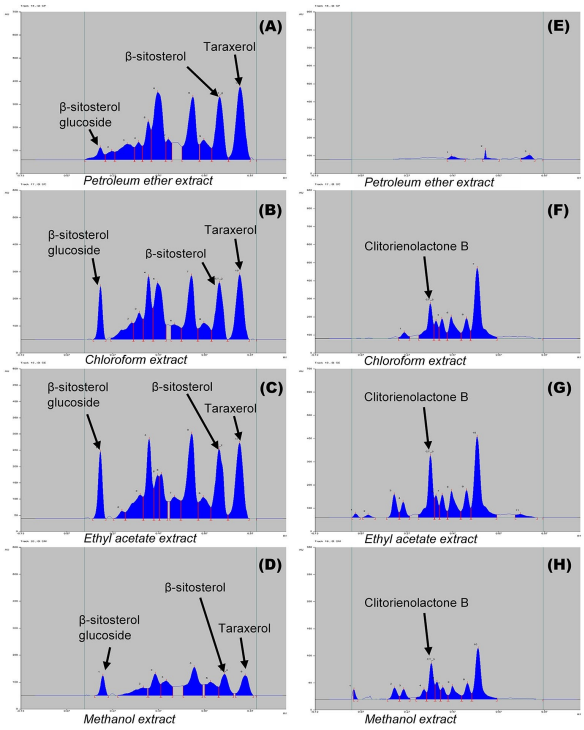

Figure 5. HPTLC chromatograms of stem extracts of *C. ternatea* at 551 nm after derivatization and 366 nm before derivatization; Figures A–D chromatograms acquired at 551 nm after derivatization of TLC plate, E–H chromatograms found at 366 nm before derivatization.

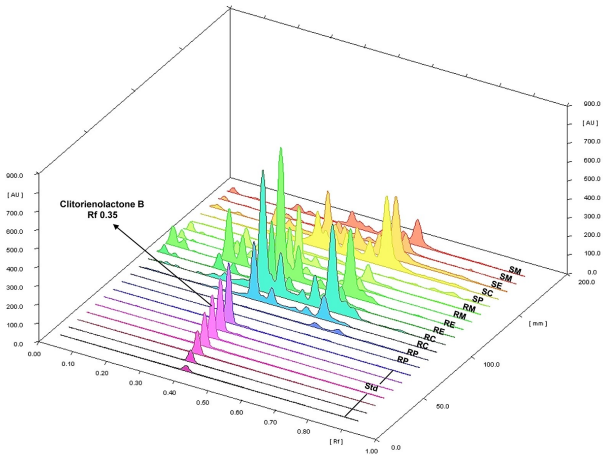

Figure 6. 3D densitogram of root and stem extracts of *C. ternatea* and standards mixture at 366 nm before derivatization of plate.

Method Validation

System suitability

A system suitability test was performed before the validation process to confirm the reproducible results. The test was carried out by the standard mixture with concentrations of 400 ng μL^{-1} for taraxerol and β-sitosterol; 200 ng μL^{-1} for clitorienolactone B and β-sitosterol glucoside. It was assured by

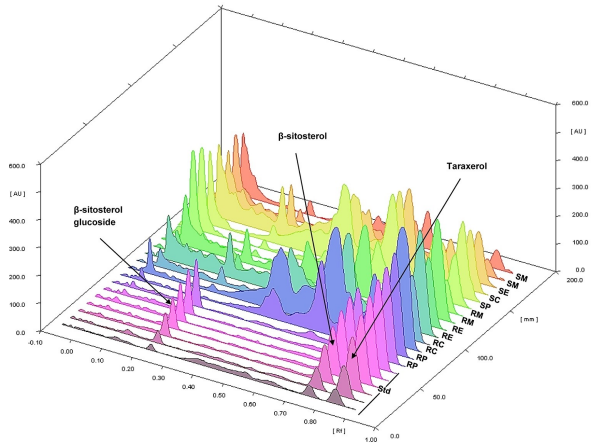

Figure 7. 3D densitogram of root and stem extracts of *C. ternatea* and standards mixture at 551 nm after derivatization of plate.

six successive spotting of the standard mixture and then analysed under optimized conditions. Peak areas and R_f values were measured for each compound and expressed as %RSD to assess the system suitability. According to Table 1, resultant RSD values (< 2.0 %) indicated that the system was suitable for estimation of the compounds.

Specificity

Method specificity was confirmed by R_f values and absorption spectra of all four standards and studied plant extracts. The peak purity of the compounds was assessed by comparing the spectra acquired at the start (S), apex (M) and end (E) positions of the peak. Subsequently, r (S, M) and r (M, E) were found (> 0.99) for each compound in the sample and standards. There was no interference of other peaks from the plant extracts, thereby confirming the method was specific. In addition to this, absorption spectra of standards and various solvent extracts were compared and superimposed on each other and it also confirmed the specificity of the developed method as shown in Figure 8.

Linearity

Linearity of the method was evaluated by linear regression equation and correlation coefficient calculated from calibration curves of each compound. Mix standard solution of all four studied compounds was applied with increasing application volume ranging from 2 μL to 24 μL to determine a linear range. Seven-point calibration curves were found linear for taraxerol and β-sitosterol in the range of 100–1200 ng/spot, and clitorienolactone B and β-sitosterol glucoside in the range of 50–600 ng/spot. Their respective regression equations and correlation coefficients were established as follows: $Y = 1191.574 + 7.678X$ ($r^2 = 0.99350$) for taraxerol, $Y = 966.618 + 11.692X$ ($r^2 = 0.99451$) for β-sitosterol, $Y = 315.126 + 13.205X$ ($r^2 = 0.99788$) for clitorienolactone B and $Y = 126.705 + 5.662X$ ($r^2 = 0.99931$) for β-sitosterol glucoside. It revealed a good

Table 1. System suitability parameters for the proposed HPTLC method.

Sr. No.	Taraxerol		β-sitosterol		Clitorienolactone B		β-sitosterol glucoside	
	Peak area	R_f	Peak area	R_f	Peak area	R_f	Peak area	R_f
1	2879	0.85	1780	0.75	963	0.35	866	0.22
2	2781	0.86	1759	0.75	990	0.36	857	0.22
3	2784	0.86	1761	0.76	973	0.35	874	0.22
4	2813	0.85	1789	0.76	977	0.35	851	0.23
5	2831	0.86	1796	0.75	997	0.35	846	0.22
6	2753	0.86	1798	0.75	979	0.35	873	0.22
Mean	2806.88	0.86	1780.22	0.75	979.78	0.35	861.17	0.22
SD	44.42	0.004	17.09	0.005	12.12	0.004	11.74	0.004
%RSD[a]	1.58	0.48	0.96	0.68	1.24	1.16	1.36	1.84

[a] %RSD calculated as (SD/Mean)×100, SD means standard deviation.

correlation coefficient ($r^2 > 0.99$) for each compound in the developed method and is presented in Table 2 and Figure 9. These values were supported by acceptable method guidance described in Health Canada that the coefficient should be ≥ 0.95 for biological samples.[26]

Sensitivity (LOD & LOQ)

LOD (limit of detection) and LOQ (limit of quantification) were determined based on the signal-to-noise ratio (S/N), for different concentrations of all four the studied compounds applied on the TLC plate. LOD and LOQ were observed by S/N of 3:1 and 10:1, respectively. LOD was found at 30 ng/spot for taraxerol and β-sitosterol, 10 ng/spot for clitorienolactone B and for β-sitosterol glucoside it was 20 ng/spot (Table 2). While LOQ was observed at 100 ng/spot for taraxerol and β-sitosterol, 25 ng/spot for clitorienolactone B and 50 ng/spot for β-sitosterol glucoside (Table 2). These recorded lower LODs and LOQs confirmed a good sensitivity of the method for studied compounds.

Accuracy

The accuracy of the method was measured by performing recovery test at three different concentration levels with known amounts of standards viz., taraxerol, β-sitosterol, clitorienolactone B and β-sitosterol glucoside to be spiked in pre-analyzed plant sample (Table 3). Recovery (%) and average recovery (%) values for the compounds were calculated and the results of the spiked samples were compared with non-spiked samples.

Percentage recoveries for the compounds were found in the range of 97.65 to 101.49% and their %RSD ranged from 0.55 to 2.11% (Table 3). Results were found nearby the acceptable criteria (90 to 110%) for accuracy of analytical method and agreeable in herbal analysis.[26,27]

Precision

Instrumental precision was evaluated by repeated scanning seven times of the same spot of each standard (taraxerol;

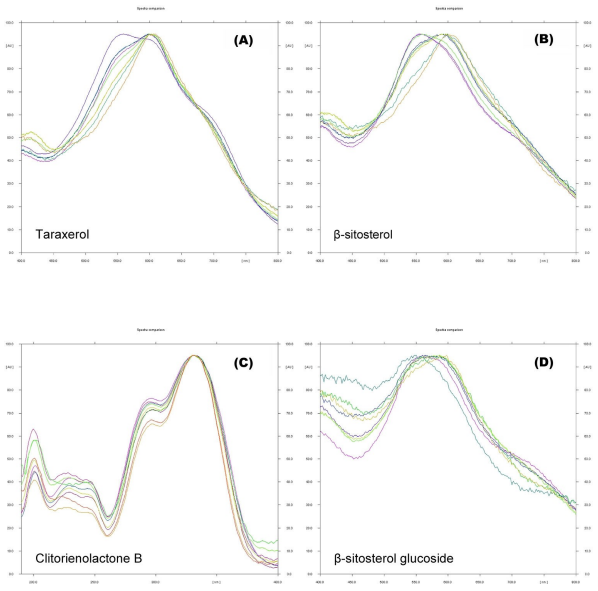

Figure 8. Overlay absorption spectra of standards with root and stem extracts of *C. ternatea*; (A) Taraxerol, (B) β-sitosterol, (C) clitorienolactone B and (D) β-sitosterol glucoside.

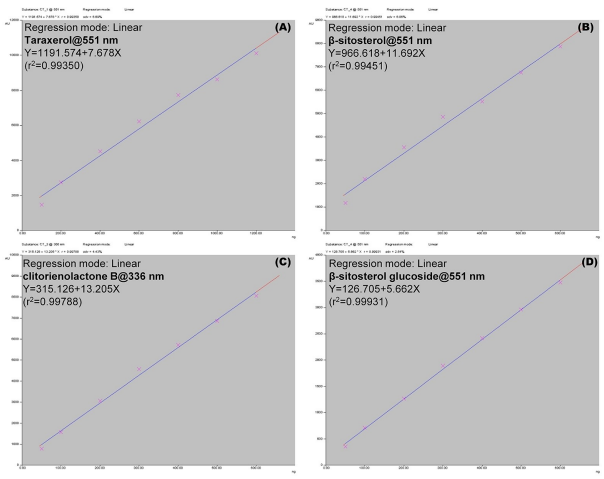

Figure 9. Calibration curves for standard compounds; (A) taraxerol, (B) β-sitosterol, (C) clitorienolactone B, and (D) β-sitosterol glucoside.

Table 2. Method performance parameters for estimation of four marker compounds by the developed HPTLC method.

Parameter	Taraxerol	β-sitosterol	Clitorienolactone B	β-sitosterol glucoside
Specificity	Specific	Specific	Specific	Specific
R_f	0.86 ± 0.004	0.75 ± 0.005	0.35 ± 0.004	0.22 ± 0.004
Linearity range (ng/spot)	100–1200	100–1200	50–600	50–600
Linear regression equation[a]	Y = 1191.574 + (7.678) X	Y = 966.618 + (11.692) X	Y = 315.126 + (13.205) X	Y = 126.705 + (5.662) X
correlation coefficient (r^2)	0.9935	0.99451	0.99788	0.99931
Instrumental precision (%RSD)[b]	0.89	0.34	0.26	0.76
Average recovery%[c] (n = 3)	97.65	98.63	101.48	99.49
LOD (ng)	30	30	10	20
LOQ (ng)	100	100	25	50

[a] Linear regression equation presented as Y=C + mX where Y is peak area and X is concentration of standard,
[b] %RSD is calculated from (Standard deviation /Mean) × 100,
[c] recovery (%) = (recovered amount/added amount) × 100, n = 3 means replications in triplets.

400 ng/spot, β-sitosterol; 400 ng/spot, clitorienolactone B; 200 ng/spot and β-sitosterol glucoside; 200 ng/spot) and their peak area was measured. The observed %RSD values were 0.89, 0.34, 0.26 and 0.76 for taraxerol, β-sitosterol, clitorienolactone B and β-sitosterol glucoside, respectively which revealed the system was precise (Table 2). Precision of the method was assessed by analysing the mixed standard solution, prepared with three different concentrations of each standard 200, 400 and 800 ng/spot for taraxerol and β-sitosterol, respectively and 100, 200, and 400 ng/spot for clitorienolactone B and β-sitosterol glucoside, respectively. According to the results presented in Table 4,

The intraday variability (%RSD) was found in the range of 1.50–2.43, 1.24–2.74, 0.36–0.57 and 0.96–1.29 for taraxerol, β-sitosterol, clitorienolactone B and β-sitosterol glucoside, respectively. Inter-day variability (%RSD) was observed in the range of 1.88–2.55, 1.20–2.89, 0.44–0.83 and 1.54–1.77 for taraxerol, β-sitosterol, clitorienolactone B and β-sitosterol glucoside, respectively. The %RSD values were found within the acceptable criteria ($\leq$ 5 %) and confirmed the proposed HPTLC method as precise.[27]

Robustness

To test the robustness of the method, small changes in chromatographic parameters were made deliberately and changes in the detection amount of all four compounds were measured (Table 5). The performance of the method was evaluated by some modifications of optimized conditions such as mobile phase composition [± 0.1 mL (v/v)], mobile phase volume (± 2.0 mL), solvent migration distance (± 2 mm) and chamber saturation time (± 1 min). %RSD were calculated and presented in Table 5 for each parameter. Results of the small changes in the proposed chromatographic conditions indicated that the measured contents of compounds were not affected much (RSD < 2.0 %). Therefore, the developed HPTLC method was found as robust.

Determination of four bioactive markers from *C. ternatea* using the proposed HPTLC method

It is the first report on the simultaneous determination of four bioactive phytocompounds like taraxerol, β-sitosterol, clitorienolactone B and β-sitosterol glucoside from *C. ternatea* using HPTLC technique. The analysis result of root and stem extract

Table 3. Recovery study of the proposed HPTLC method.

Compound	Amount present (ng)	Amount added (ng)	Observed amount (ng)	Recovery (%)[a]	%RSD[b]	Average recovery (%)
Taraxerol	380	150	516.33	97.42	1.78	97.65
		300	655.11	96.34	1.05	
		450	823.36	99.20	2.11	
β-sitosterol	320	150	464.69	98.87	1.45	98.64
		300	591.67	95.43	1.22	
		450	782.63	101.64	1.94	
clitorienolactone B	120	50	175.51	103.24	0.75	101.49
		100	219.30	99.68	0.78	
		150	274.16	101.54	0.55	
β-sitosterol glucoside	90	50	143.11	102.22	0.98	99.49
		100	186.73	98.28	1.08	
		150	235.10	97.96	1.36	

[a] recovery (%) = (recovered amount/added amount) × 100,
[b] %RSD = (SD/Mean) × 100, SD is standard deviation.

Table 4. Intraday and inter-day precision of the proposed HPTLC method.

Compound	Concentration (ng/spot)	%RSD[a]	
		Intraday	Inter-day
Taraxerol	200	1.64	1.88
	400	1.50	2.04
	800	2.43	2.55
β-sitosterol	200	1.24	1.20
	400	1.96	2.07
	800	2.74	2.89
Clitorienolactone B	100	0.42	0.44
	200	0.36	0.83
	400	0.57	0.45
β-sitosterol glucoside	100	1.04	1.54
	200	1.29	1.77
	400	0.96	1.60

[a] %RSD = (SD/Mean) × 100, SD represents standard deviation.

of *C. ternatea* in various solvents is summarized in Table 6. The extracted biomarker contents vary ($p < 0.05$) in different polarity solvents. The highest content of Taraxerol and β-sitosterol were observed in petroleum ether extract, while clitorienolactone B and β-sitosterol glucoside was found highest in ethyl acetate extract of root and stem parts of the plant. Results also indicated that the content of clitorienolactone B in *C. ternatea*, was predominantly high in roots and stems in comparison to other three analysed compounds. The resulting phytochemical variation in stem and root parts of the plant was in accordance with earlier reported data wherein secondary metabolites widely varied within the plant parts.[28–30]

Overall efficacy of herbal medicine may not be responsible due to any single active constituent and their quality analysis remains a challenge as they contain multi-compounds.[31] Therefore, the simultaneous chromatographic separation of secondary metabolites from plant materials is required in quality analysis.[32] The present result also offers a simultaneous determination of four bioactive phytocompounds like taraxerol, β-sitosterol, clitorienolactone B and β-sitosterol glucoside from *C. ternatea* using the HPTLC technique.

Conclusion

The simultaneous measurement of four bioactive compounds from *Clitoria ternatea*, including taraxerol, β-sitosterol, clitorienolactone B, and β-sitosterol glucoside has been accomplished using a simple, accurate, exact, and reliable validated technique. The newly developed HPTLC method is an environmentally friendly, quick analysis that uses just small (20 mL)

Table 5. Robustness studies of the proposed HPTLC method.

Parameter	Taraxerol (Amount spotted 400 ng) Detected amount (ng) ± RSD (%)[a]	β-sitosterol (Amount spotted 400 ng) Detected amount (ng) ± RSD (%)[a]	Clitorienolactone B (Amount spotted 200 ng) Detected amount (ng) ± RSD (%)[a]	β-sitosterol glucoside (Amount spotted 200 ng) Detected amount (ng) ± RSD (%)[a]
Solvent migration distance (78 mm)	398.01 ± 1.08	401.31 ± 1.23	198.47 ± 0.98	200.78 ± 1.84
Solvent migration distance (82 mm)	400.19 ± 0.98	400.64 ± 1.28	199.78 ± 1.55	200.73 ± 1.99
Chamber saturation time (9 min)	400.93 ± 1.63	399.91 ± 1.64	201.07 ± 1.95	203.10 ± 1.86
Chamber saturation time (11 min)	398.48 ± 1.42	398.65 ± 1.72	201.72 ± 1.89	199.94 ± 1.83
Mobile phase composition (6.9 : 2.9 : 0.9 v/v)	398.46 ± 1.71	399.46 ± 1.72	201.04 ± 1.78	197.61 ± 1.25
Mobile phase composition (7.1 : 3.1 : 1.1 v/v)	403.27 ± 0.89	395.13 ± 1.76	202.86 ± 1.53	200.66 ± 1.69
Mobile phase volume (23 mL)	401.04 ± 1.26	392.88 ± 1.67	198.60 ± 1.80	201.64 ± 1.90
Mobile phase volume (27 mL)	396.78 ± 1.98	398.05 ± 1.90	200.79 ± 1.02	198.35 ± 1.67

[a] RSD (%) calculated as (SD/Mean) × 100, SD means standard deviation.

Table 6. Determination of four marker compounds in various solvent extracts of *C. ternatea*.

Plant Part	Solvent extract	Taraxerol (mg g^{-1} extract)	β-sitosterol (mg g^{-1} extract)	Clitorienolactone B (mg g^{-1} extract)	β-sitosterol glucoside (mg g^{-1} extract)
Root	Petroleum ether	74.40 ± 0.73	18.57 ± 0.21	n.d.	n.d.
	Chloroform	35.60 ± 0.42	10.33 ± 0.07	15.23 ± 0.41	8.67 ± 0.13
	Ethyl acetate	36.50 ± 0.24	8.70 ± 0.04	49.00 ± 0.11	7.50 ± 0.10
	Methanol	7.57 ± 0.08	2.48 ± 0.06	15.67 ± 0.09	1.12 ± 0.04
Stem	Petroleum ether	18.20 ± 0.13	17.57 ± 0.72	n.d.	2.00 ± 0.08
	Chloroform	10.50 ± 0.05	10.40 ± 0.56	7.83 ± 0.15	11.67 ± 0.25
	Ethyl acetate	9.27 ± 0.15	9.07 ± 0.61	11.87 ± 0.21	11.33 ± 0.31
	Methanol	2.38 ± 0.08	3.33 ± 0.10	5.00 ± 0.04	3.80 ± 0.07

volumes of organic solvents for the quantitative/qualitative examination of four substances at once. The HPTLC method is more widely used as a quick and affordable analytical instrument because it includes a procedure for multiple sampling that is economically viable. Conventionally, the chemical marker-based technique can be used as a diagnostic tool to identify and assess the quality and purity of *C. ternatea* for its medication standardization and to prevent any adulteration by other species.

Experimental Section

The analysis was performed by HPTLC equipment (CAMAG, Switzerland). The plant extracts were applied on TLC aluminum plates (20×10 cm, 0.2 mm thick) with pre-coated silica gel 60 F_{254} (Merck, Germany) in triplicates along with different concentration levels of the standards solutions. The solution of each reference standards was prepared in adequate amount of methanol. Sample solution of dried petroleum ether extracts of root (RP) and stem (SP), chloroform extracts of root (RC) and stem (SC), ethyl acetate extracts of root (RE) and stem (SE) and methanol extracts of root (RM) and stem (SM) were prepared in methanol with concentration of 2 mg mL^{-1}. The test solutions were loaded on TLC plates in 8 mm bands (15 mm from the left edge and 12 mm from the bottom, with 9.4 mm space between two bands) using Linomat-V sample applicator fitted with a 100-µL syringe, which sprayed samples with constant speed at 70 nL/s. Plates were developed about 80 mm in a pre-saturated twin trough glass chamber with 25 mL of chloroform: toluene: methanol in the ratio of 7:3:1 (v/v) as a mobile phase at room temperature (25±2 °C) and at 50±1% of relative humidity. Developed plates were dried by air dryer and viewed under UV light (254 and 366 nm) using TLC Reprostar-3. Same plates were derivatized by immersing in vanillin-sulphuric acid reagent for 2 seconds. The reagent was prepared by dissolving 1 g vanillin in 100 mL of 95% ethanol containing 5 mL concentrated sulfuric acid. The derivatized plate air-dried, then heated at 110 °C for 10 minutes on a TLC plate heater (IKA 3581801 C-MAG HP 7, M/s Cole parmer, India). After derivatization, plates were viewed under UV (366 nm) and white light. Quantification was done by densitometry scanning of the plates at proper wavelength using TLC scanner-3. The slit dimension of 6.0×0.45 mm was selected with scanning speed of 20 mm/s and data resolution carried out at 100 mm/step for densitometric performance. Peak areas of targeted compounds were recorded and their amounts were calculated using the standard calibration curve. WinCATS software (Version 1.2.1) was used to operate the HPTLC procedures. The optimized HPTLC method was validated as per the guidelines mentioned in the International Conference on Harmonization.[33,34]

Supporting Information Summary

The plant sample and chemicals used for this investigation, including the isolation method of taraxerol, clitorienolactone-B, and -sitosterol glucoside compounds, are mentioned in the summary of supporting information. Furthermore, included in that were the standard reference preparation and the plant sample.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: chemical screening · *Clitoria ternatea* · HPTLC · method development · method validation · simultaneous determination

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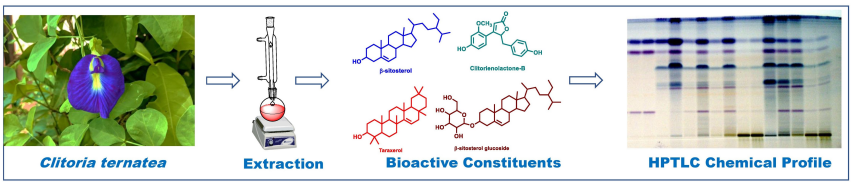

Dr. J. Makasana, Dr. N. Gajbhiye, Dr. A. Kumar Bishoyi, Dr. M. Savaliya, Dr. S. Raju, Dr. S. Bansod, Dr. L. Baldaniya, Dr. B. Dholakiya*

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Exploration of HPTLC Technology for Rapid Chemical Fingerprinting and Simultaneous Determination of Bioactive Constituents from *Clitoria ternatea* Linn.

The simplest, rapid, and reliable HPTLC method was developed and validated for fast chemical screening and quality standardization of *Clitoria ternatea*.