

A Validated High-Performance Thin-Layer Chromatography Method for Determination of β -Sitosterol in *Indigofera cordifolia* Herb

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Research Article

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Abstract

Indigofera cordifolia B. Heyne ex Roth (family: Fabaceae), a wild annual to perennial herb, commonly known as 'heart leaf indigo', has been traditionally used to treat fever, pain, skin inflammation, arthritis, hepatitis, diabetes, and possesses aphrodisiac property. The present work was designed to develop and validate a simple, sensitive, rapid, and reliable high-performance thin-layer chromatography-densitometric method for the identification and quantification of β -sitosterol in the ethanolic extracts of aerial parts and roots of *I. cordifolia* herb. The precoated silica gel 60 F₂₅₄ HPTLC plates were used as stationary phase and toluene- ethyl acetate (85: 15, V/V) as mobile phase. β -sitosterol was eluted at R_f : 0.39 ± 0.004 . The CAMAG scanner 4 was used for the densitometric scanning at 530 nm wavelength using tungsten lamp. The proposed HPTLC method was validated in terms of linearity, limit of detection (LOD) and quantification (LOQ), precision, accuracy, robustness, and specificity. β -sitosterol showed linearity in 200–1000 ng band⁻¹ concentrations with correlation coefficient (r^2) of 0.9984 and LOD and LOQ values of 11.52 and 34.92 ng band⁻¹, respectively. The method demonstrated excellent intra- and inter-day precision and accuracy (%RSD < 2), robustness, and specificity under the optimized conditions of validation, confirming the presence of β -sitosterol (% w/w) in 1.193 ± 0.012 and 1.488 ± 0.023 , respectively, in the ethanolic extracts of aerial and root parts of *I. cordifolia* herb.

1 Introduction

Indigofera cordifolia B. Heyne ex Roth (family: Fabaceae), commonly known as 'Heart leaf indigo', is a wild annual to perennial herb, grows prostrate favorably in well drained but moist soil, branches spreads centrally, more or less hairy, forms gregarious colonies in grasslands in Central and Western India including Pakistan, Afghanistan, China, Myanmar, Saudi Arabia, etc. [1]. The green colored leaves are odd-pinnate or trifoliolate paired with pink colored flowers, typically arranged in axillary racemes or spikes directly to the main stems, and the pods are linear, oblong to globose [2, 3]. Literature review suggested the presence of primary and secondary plant metabolites as phytosterols, tannins, carbohydrates, flavonoids, terpenoids, saponins, steroids, triterpenes, and anthocynins in *I. cordifolia* herb [3, 4]. The gas chromatography- mass spectroscopy (GC-MS) study on the ethanolic extract of *I. cordifolia* entire herb showed the presence of mainly dodecane, tetradecane, phytol, 1,2-benzene dicarboxylic acid bis(2-methylpropyl), hexadecanoic and octadecanoic acid ethyl esters [5].

I. cordifolia herb has been traditionally used to treat fever, pain, skin inflammation, arthritis, hepatitis, diabetes, dyspepsia, and possesses aphrodisiac property [6]. Modern pharmacological screening studies revealed that *I. cordifolia* herb shows antioxidant [7, 8], antimicrobial and non-hemolytic [7], anti-inflammatory [4], anti-cancer in Ehrlich Ascites Carcinoma in mice [9] effects, owing to the presence of bioactive chemical constituents. It has also been reported to ameliorate streptozotocin induced type 2 diabetes mellitus in rats [10, 11].

β -sitosterol, a major bioactive of phytosterol, is abundantly present in dietary sources of lipids as nuts, seeds, legumes, vegetable oils, etc. Scientific researches demonstrates that β -sitosterol possesses

pharmacological effects to improve human health [12] and reported to possess anticancer [13], antiviral [14], antidiabetic [15], anti-ulcerative colitis [16] activities and ameliorates infective lungs inflammation [17] and osteoporosis [18], alleviates neuropathic pain [19], etc.

The World Health Organization (WHO) has recognized high-performance thin-layer chromatography (HPTLC) as an indispensable technique of chromatography. HPTLC has been therefore widely used to provide the most rapid, reliable, precise, accurate, reproducible, and robust analysis of the phytochemicals present in the herbal drugs.

I. cordifolia herb is so far not analyzed both qualitatively and quantitatively for any of its phytochemicals using a validated HPTLC method. In continuation of our previous works on the phytochemical analysis of the herbal drugs using validated HPTLC methods [20–23], the present work was attempted to identify and quantify β -sitosterol in the ethanolic extracts of aerial and root parts of *I. cordifolia* herb by a validated HPTLC method employing linearity, limits of detection and quantitation, intra- and inter-day precision, accuracy, robustness, and specificity validation parameters as per the guidelines of International Conference on Harmonization (Q2R1) [24] and in accordance to Reich and Schibli (2007) [25].

2 Experimental

2.1 Materials and chemicals

The chemicals and organic solvents, i.e. chloroform, methanol, ethanol, anisaldehyde, glacial acetic acid, concentrated sulphuric acid, toluene, ethyl acetate, etc. (Loba Chemie Pvt. Ltd., Mumbai, India) of analytical-grade were used. Precoated silica gel 60 F₂₅₄ aluminum HPTLC plates (10 cm × 20 cm; 1.05548.0001; Merck, India) were used. β -sitosterol (98 % purity) was procured from the YUCCA Enterprises, Mumbai, Maharashtra, India.

2.2 Collection and authentication of *I. cordifolia* herb

I. cordifolia whole herb was collected at flowering stage from Jayakwadi Dam, Paithan, Chhatrapati Sambhajnagar, Maharashtra state during September 2025 and immediately washed with water to remove dust, earthy matter, and dried under shade. For authentication, some of the freshly collected whole herbs, with flowers, were pressed between papers and weighted, and after 7 days the pressed whole herbs were prepared in the form of herbarium specimens, and authenticated by Dr. Arvind Dhabe, Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Chhatrapati Sambhajnagar, Maharashtra wherein the herbarium specimen of number 1304/2025 is kept for the future reference.

2.3 Drying and powdering of aerial and root parts *I. cordifolia* herb

After collection, the aerial parts and roots were separately allowed to shade dry until complete drying (for 15 - 20 days), finely powdered (passed through mesh sieve # 40) using a grinder, further dried in an oven at 50 °C for 3 h, and packed in plastic bags until further use.

2.4 Extraction of dried and finely powdered aerial and roots parts of *I. cordifolia* herb

A 100 g each of dried and finely powdered aerial parts and roots of *I. cordifolia* herb were separately subjected for extraction using ethanol in 1: 10 (W/V) proportion employing a reflux extraction method for 30 min at 78 ± 2 °C. The resulting extracts were filtered through Whatman filter paper # 42, and the marc (air dried residual plant material) was re-extracted with fresh ethanol to ensure complete extraction. The filtrates of both the ethanolic extracts of aerial parts and roots of *I. cordifolia* were concentrated and allowed to evaporate until complete dryness in an oven at 50 °C. The extractive values of the extracts of aerial parts and roots of *I. cordifolia* were 7.02 and 4.53 % w/w.

2.5 Preliminary phytochemical screening

The ethanolic extracts of aerial parts and roots of *I. cordifolia* herb were separately subjected to qualitative preliminary phytochemical screening employing different chemical tests, i.e., Dragendorff's and Mayer's reagent test (alkaloids), Shioda test (flavonoids), filter paper spot test (lipids), ferric chloride test (tannins and phenolics), carbohydrates (Fehling's A and B solution test), Liebermann-Burchard test (triterpenes and steroids) were employed [26, 27].

2.6 Preparation of sample solutions of the extracts of aerial and root parts of *I. cordifolia* herb

Sample solutions of both the extracts of aerial and roots of *I. cordifolia* were prepared separately by accurately weighing a 100 mg quantity and dissolving in 4 mL methanol (25 mg/mL) followed by vortexing the sample extract and solvent mixture for 2-3 min followed by ultra-sonication for 15 min. The mixture was then centrifuged at 2500 rpm for 15 min (to remove any extraneous matter), and the supernatant was transferred into a vial for application in 25, 50 and 75 $\mu\text{g band}^{-1}$ concentrations in 1, 2, and 3 μL volumes for the proposed HPTLC study.

2.7 Preparation of standard solution of β -sitosterol

A standard stock solution of β -sitosterol (200 ng/ μL) was prepared by dissolving in methanol in a 10 mL volumetric flask. Different volumes of stock solution, i.e., 1, 2, 3, 4, 5 μL were applied on the precoated silica gel F254 aluminum HPTLC plate in order to apply 200, 400, 600, 800, 1000 ng band^{-1} concentrations. The data of peak area were plotted vs the corresponding concentration, treated by linear regression analysis.

2.8 HPTLC system and chromatographic conditions

A CAMAG high-performance thin-layer chromatography (HPTLC) system comprised of (a) Linomat 5 sample applicator, (b) an automatic developing chamber 3 (ADC 3), (c) a semi-automatic derivatizer, (d) a plate heater, and (e) a visualizer 3 was employed. The visionCATS software was used. Precoated silica gel 60 F₂₅₄ aluminum HPTLC plates (10 cm × 20 cm; 1.05548.0001; Merck, India), prewashed with methanol, were used as stationary phase. β -sitosterol, procured from the YUCCA Enterprises, Mumbai, India, was used as a phytochemical standard to standardize the ethanolic extracts of aerial parts and

roots of *I. cordifolia* by the proposed HPTLC method. β -sitosterol was used in 200, 400, 600, 800, and 1000 ng band⁻¹ concentrations and the ethanolic extracts of aerial parts and roots (both in 25, 50, and 75 μ g band⁻¹ concentrations) of *I. cordifolia* were applied on the HPTLC plates in the form of bands of 6 mm width through Linomat V applicator and the slit dimension was kept as 4 $\times$ 0.30 mm. The mobile phase, consisting of toluene-ethyl acetate (85: 15, V/V), was transferred in a CAMAG twin trough chamber and allowed to saturate for 20 min at room temperature, 25 °C $\pm$ 2 °C, and relative humidity, 55% $\pm$ 5%. The chromatograms were developed in an ascending mode upto 70 mm height. After development of the chromatograms, the HPTLC plates were dried using warm air of hair dryer for 10 min. Post-chromatographic derivatization of the chromatograms was done using anisaldehyde- sulphuric acid reagent [prepared by transferring 170 mL methanol in 200 mL glass bottle and cooled it in water-ice cube bath. To the ice-cold methanol, 20 mL acetic acid and 10 mL sulphuric acid was carefully added with constant stirring. This mixture was allowed to cool to room temperature and then 1 mL anisaldehyde was added; note- this colorless reagent has stability of only 2-3 days, which on later changes to red-violet] [28] with heat treatment of the chromatograms at 100 °C for 7 min. Densitometric scanning was done using CAMAG scanner at 530 nm using tungsten lamp. Chromatographic evaluation was done by the assessment of concentration of the analyte vs peak area, using regression analysis in linear mode.

2.9 HPTLC method validation

The guidelines of International Conference on Harmonization, Q2(R1)] [24] and reports of Reich and Schibli, 2007 [25] were employed to validate the proposed HPTLC method in terms of linearity, limits of detection and quantitation, intra- and inter-day precision, accuracy study, robustness of the method, and specificity validation parameters.

2.9.1 Linearity study

It was performed by analyzing the five point calibration curves of β -sitosterol (200–1000 ng band⁻¹ concentrations). Linear regression analysis was performed to interpret the results of linearity study, i.e., expressed by the linear regression equation with its coefficient of correlation (r^2), Y-intercept, and slope.

2.9.2 Sensitivity Study

Measurement sensitivity, i.e., the LOD and LOQ of β -sitosterol were determined on the basis of standard deviation (SD) and slope (S) of the calibration plot, with the formula: LOD = 3.3σ / S and LOQ = 10σ / S, where S= slope of the calibration plot, and σ = standard deviation of the Y-intercept.

2.9.3 Precision study

Both the inter-day (intermediate precision, i.e., same-day study) and the intra-day (repeatability, i.e., three consecutive days study) precision studies for β -sitosterol were performed at three different concentrations, i.e., 400, 600 and 800 ng band⁻¹, analyzed in six replicates (n=6).

2.9.4 Accuracy study

The accuracy (recovery) study was assessed by evaluating the recovery of β -sitosterol spiked (50%, 100% and 150% of the β -sitosterol present in the samples) in the pre-analyzed samples, i.e., the ethanolic extracts of aerial parts and roots of *I. cordifolia* herb, in triplicate, and re-analyzed to determine the recovery of β -sitosterol in terms of average recovery and %RSD values calculated from the theoretical and the measured values.

2.9.5 Specificity study

At a concentration level of 600 ng band⁻¹ of β -sitosterol, the robustness of the method was performed using three different compositions of the mobile phase, i.e., toluene-ethyl acetate (84: 16; 85:15; and 86:14, V/V), and at scanning wavelengths of 510, 520, 530, 540, and 550 nm using tungsten lamp. For studying specificity of the proposed HPTLC method, the bands of β -sitosterol standard and that of bands of β -sitosterol present in the ethanolic extracts of aerial parts and roots of *I. cordifolia* samples (post-chromatographic derivatization with anisaldehyde-sulphuric acid reagent) were examined by comparing the R_F and spectral peaks.

2.10 Stability study of β -sitosterol in methanol

For confirming stability of β -sitosterol in methanol for 24 h, its methanolic solution in 600 ng band⁻¹ concentration was analyzed by the proposed HPTLC method at different time intervals, i.e., at 0, 2, 4, 6, 8, 12, 24 h at 25 $\pm$ 2 °C. The amount of β -sitosterol at each time interval was determined using the derived regression equation ($y = 1.5607x + 141.53$), and the results are expressed in terms of %RSD.

2.11 Statistical analysis

Microsoft Excel 2010 software was employed for studying linear regression analysis, i.e., for quantification of β -sitosterol in the extracts of *I. cordifolia* and determination of the results of validation parameters, i.e., mean $\pm$ standard deviation, standard error, as well as %RSD values.

3 Results and discussion

3.1 Preliminary phytochemical screening

Both the ethanolic extracts of aerial parts and roots of *I. cordifolia* have shown the presence of flavonoids, triterpenes and steroids/sterols, lipids, carbohydrates, and phenolic types of chemical constituents by employing different chemical tests.

3.2 Optimization of the mobile phase

Based on the polarity of β -sitosterol and other chemical constituents present in the ethanolic extracts of aerial and root parts of *I. cordifolia* herb, different mobile phases containing varying proportions of the

organic solvents, i.e., n-hexane-ethyl acetate, petroleum ether (60–80°C)-chloroform, toluene-ethyl acetate, toluene-ethyl acetate-methanol, n-hexane-chloroform-acetone, etc. were used on trial and error basis. However, as shown in Fig. 2, the mobile phase consisting of toluene-ethyl acetate (85: 15, V/V) provided acceptable resolution of β -sitosterol at $R_F=0.37 \pm 0.016$ (purple spot under visible light, post-chromatographic derivatization with anisaldehyde-sulphuric acid reagent followed by heating of the HPTLC plates at 100°C for 7 min).

3.3 Calibration curves and linearity study of β -sitosterol

For proving linearity, five calibration points were analyzed over the range of 200–1000 ng band⁻¹ of β -sitosterol. The data were analyzed by linear regression least square model and showed a good linear relationship over the tested range ($r^2 = 0.9984$). The linear regression equation was derived as $y = 1.5607x + 141.53$ where y is the peak area response and x is the concentration in ng band⁻¹. A non-significant difference was observed in the slopes and intercepts of standard curves as shown in Table 1. The image of HPTLC chromatograms of β -sitosterol and the ethanolic extracts of *I. cordifolia* are shown in Fig. 2. β -sitosterol was found in both the aerial parts and roots of *I. cordifolia* herb, respectively, with content (% w/w, mean $\pm$ SD) of 1.193 ± 0.012 and 1.488 ± 0.023 .

Table 1
Linear regression equation and validation parameters for quantification of β -sitosterol in the aerial parts and roots of *I. cordifolia* herb by the proposed HPTLC method

Linear regression parameters	β -sitosterol
Linearity range (ng band ⁻¹)	200–1000
R_F	0.398 ± 0.004
Linear regression equation	$y = 1.5607x + 141.53$
Correlation coefficient (r^2)	$r^2 = 0.9984$
Slope $\pm$ SD	1.5607 ± 0.010
Confidence limit of slope ^a	1.5505–1.571
Y-intercept $\pm$ SD	141.53 ± 5.450
Confidence limit of intercept ^a	136.5–146.7
LOD (ng band ⁻¹)	11.524
LOQ (ng band ⁻¹)	34.921

Figure 2 Representative HPTLC chromatographs of β -sitosterol (200–1000 ng band⁻¹), ethanolic extracts of aerial parts (25, 50, 75 μ g band⁻¹; track 3, 7, 11, 13) and roots (25,50,75 μ g band⁻¹; track 4, 8, 12 and

14) of *I. cordifolia* herb with R_f : 0.371, captured at 530 nm. Mobile phase: toluene- ethyl acetate (85:15, V/V).

3.4 HPTLC method validation

3.4.1 Limits of detection (LOD) and quantification (LOQ) studies

The LOD and LOQ values for β -sitosterol were found to be 11.52 ng band⁻¹ and 34.92 ng band⁻¹ respectively, suggesting, it's enough sensitive to determine by the proposed HPTLC method.

3.4.2 Precision study

The intra-day (repeatability) and inter-day (intermediate) precision studies were performed using three concentrations of β -sitosterol, i.e., 400, 600 and 800 ng band⁻¹ of linearity and range concentrations (200–1000 ng band⁻¹). Peak area of corresponding concentrations was measured using the six replicates of the same band. It was expressed in terms of percent relative standard deviation (%RSD) and standard error of mean (SEM). The low values of %RSD (< 1) and SE suggested an excellent precision of the method as shown in Table 2.

Table 2
Inter- and intra-day precision of the HPTLC method (n = 6) for β -sitosterol

Amount (ng band ⁻¹)	Inter-day precision				Intra-day precision			
	(Intermediate precision) ^a				(Repetability) ^b			
	Mean	SD	%RSD	SEM	Mean	SD	%RSD	SEM
400	784.91	7.29	0.93	2.98	774.13	6.73	0.87	2.75
600	1088.25	7.27	0.66	2.96	1082.30	9.71	0.87	3.96
800	1429.57	12.16	0.85	4.96	1418.80	13.32	0.93	5.43
Inter-day ^a ; inter-day ^b (n = 6)								

3.4.3 Recovery study

Standard addition method was employed to assess recovery of β -sitosterol, i.e., the pre-analyzed ethanolic extracts of aerial parts and roots of *I. cordifolia* were spiked with extra 50, 100, and 150% of β -sitosterol and re-analysis was done by the proposed HPTLC method in triplicate in view to check the recovery of β -sitosterol at different levels in the samples. The results showed that the percent recoveries of β -sitosterol were in the range of 97.80–98.88%. The results are shown in Table 3.

Table 3

Recovery studies of β -sitosterol from the ethanolic extracts of *I. cordifolia* aerial parts and roots by the proposed HPTLC method.

Amount of β -sitosterol (ng) present	Amount of β -sitosterol (ng) added	Theoretical added value (ng)	Amount of β -sitosterol found	Recovery (%) ^a	Average recovery (%) ^a	SD	%RSD	SEM
Aerial parts extract (25 $\mu\text{g band}^{-1}$)								
571	250	821	803	97.80	98.44	0.56	0.57	0.32
	500	1071	1059	98.87				
	750	1321	1303	98.63				
Roots extract (25 $\mu\text{g band}^{-1}$)								
735	350	1085	1063	97.97	98.39	0.46	0.46	0.27
	700	1435	1419	98.88				
	1050	1785	1755	98.31				
^a Mean (n = 3)								

3.4.4 Robustness study

By incorporating small intentional changes in the solvent system composition and the scanning wavelength, the effects on the results were evaluated. This study was performed using β -sitosterol at a concentration level of 600 ng band⁻¹ (n = 3). A scanning wavelength of 530 nm produced maximum peak area (1085.3 $\pm$ 9.90) of β -sitosterol. Low values of %RSD, SD and SEM obtained after introducing these intentional changes in the proposed HPTLC method indicated its robustness (Table 4).

Table 4

Robustness study for β -sitosterol ($n = 3$, 600 ng band⁻¹) by the proposed HPTLC method.

Parameter(s)	Change parameter(s)	^a Mean peak area $\pm$ SD	%RSD	SEM
Minor changes in the proportion of solvents used in solvent system (toluene: ethyl acetate, V/V)	84:16	1085.4 $\pm$ 10.05	0.92	5.80
	86:14	1075.2 $\pm$ 20.37	1.89	11.76
	85:15	1089.6 $\pm$ 10.24	0.93	5.88
Scanning wavelength (nm)	510	785.8 $\pm$ 7.77	0.98	4.49
	520	1047.3 $\pm$ 9.65	0.92	5.57
	530	1085.3 $\pm$ 9.90	0.91	5.71
	540	1017.8 $\pm$ 11.20	1.10	6.46
	550	997.1 $\pm$ 9.75	0.97	5.62
^a Mean ($n = 3$)				

3.4.5 Specificity study

For interpreting the results of the specificity study by the proposed HPTLC method, the peaks (spectra) of β -sitosterol standard and β -sitosterol present in the samples were compared at three different positions, i.e., the spectrum start, spectrum center, and spectrum end. As shown in Fig. 3, β -sitosterol spectral peaks (both standard and samples) at 400–700 nm were observed well resolved indicating there was no interference from the other chemical constituents present in the ethanolic extracts of aerial parts and roots of *I. cordifolia* herb.

3.5 Stability study β -sitosterol in methanol

As shown in Table 5, the methanolic solution of β -sitosterol was found to be stable up to 24 h at $25 \pm 2^\circ\text{C}$. The results are indicated by non-significant difference in the peak area of β -sitosterol (600 ng band⁻¹).

Table 5
Stability study of β -sitosterol solution (600 ng band⁻¹) in methanol.

Time (h)	Peak area	Amount found (ng band ⁻¹)
0	1087.31	604.72
2	1082.85	607.61
4	1070.29	611.07
6	1078.49	600.37
8	1074.70	597.93
12	1079.89	594.15
24	1083.61	596.59
Mean $\pm$ SD	1079.59 $\pm$ 5.74	601.78 $\pm$ 3.68
% RSD	0.53	0.61

4 Conclusions

This is so far the first report on the HPTLC-based standardization of *I. cordifolia* herb using β -sitosterol as a bioactive phytochemical standard. A simple, rapid, economical, and reliable HPTLC-densitometric method has been developed and validated for determining β -sitosterol in the ethanolic extracts of aerial and root parts of *I. cordifolia* herb, confirming the presence of β -sitosterol in $1.193 \pm 0.012\%$ w/w and $1.488 \pm 0.023\%$ w/w content, respectively. Henceforth, the developed HPTLC method proves to be reliable, sensitive, precise, accurate, robust, and specific tool to standardize *I. cordifolia* herb using β -sitosterol for its quality control.

Declarations

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Author Contribution

BG had designed the present work. BG and GK collected, authenticated, extracted aerial parts and roots of *I. cordifolia* herb; developed and validated HPTLC method. BG evaluated and interpreted the data and prepared article for communication.

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Figures

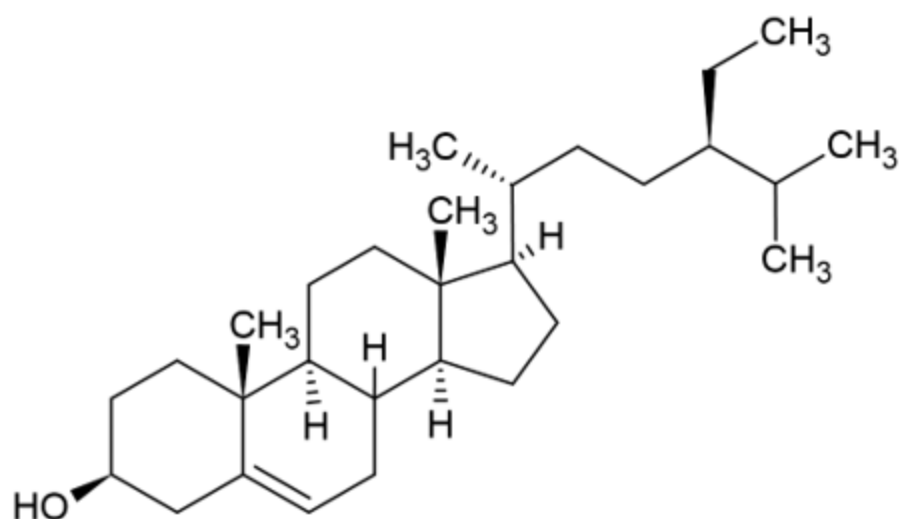


Figure 1

The chemical structure of β -sitosterol

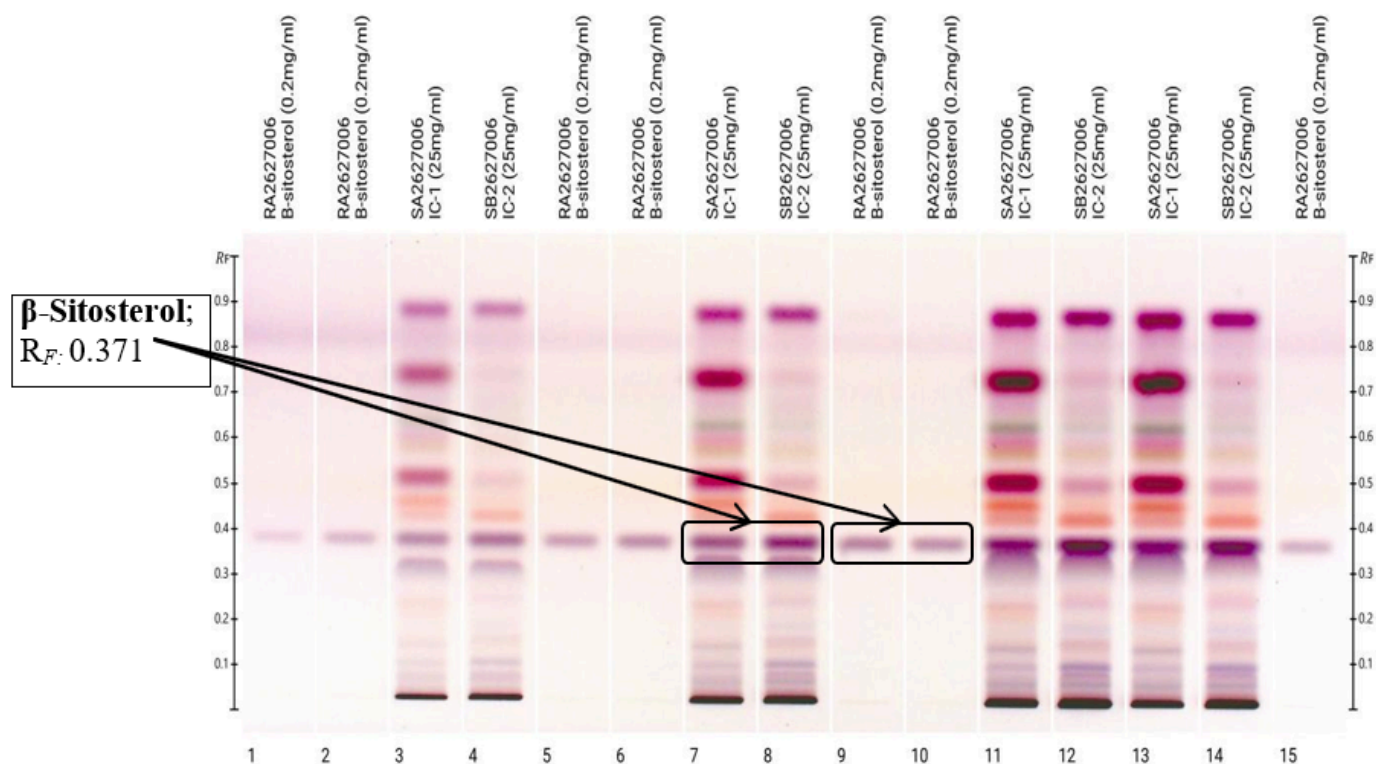


Figure 2

Representative HPTLC chromatographs of β -sitosterol (200 – 1000 ng band⁻¹), ethanolic extracts of aerial parts (25, 50, 75 μ g band⁻¹; track 3, 7, 11, 13) and roots (25,50,75 μ g band⁻¹; track 4, 8, 12 and 14) of *I. cordifolia* herb with R_f: 0.371, captured at 530 nm. Mobile phase: toluene- ethyl acetate (85:15, V/V).

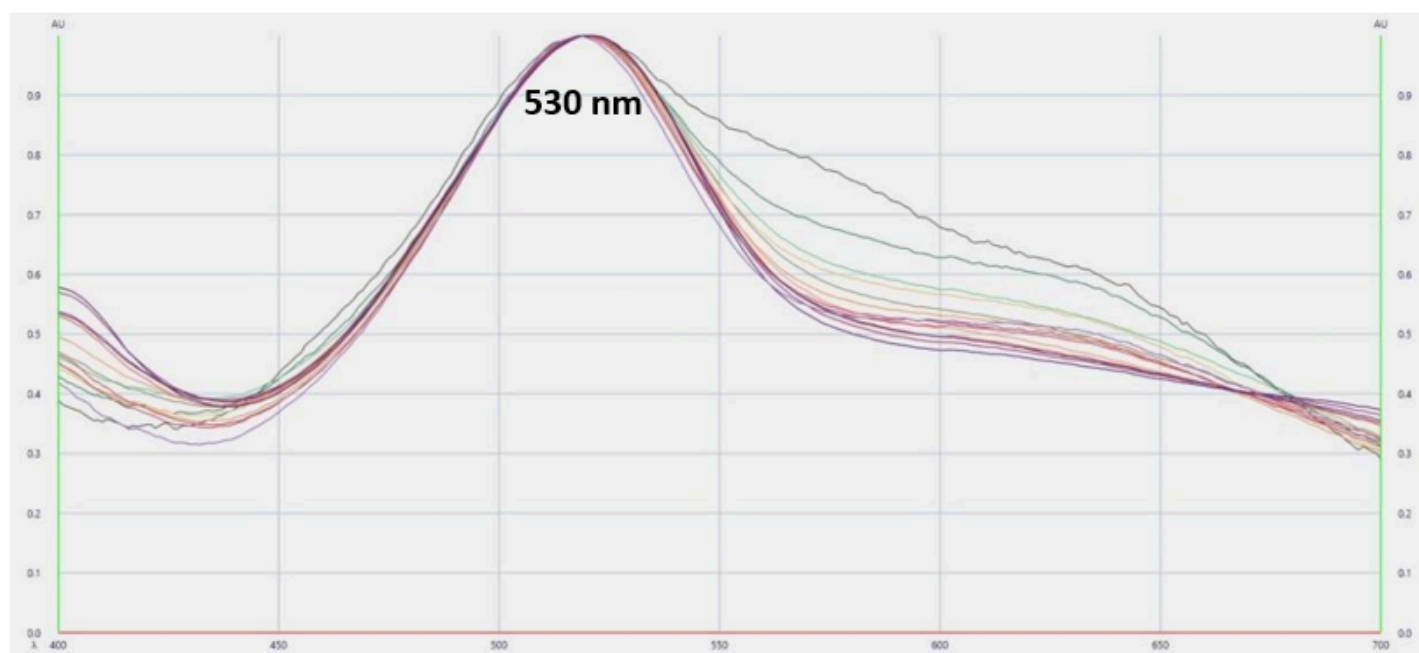


Figure 3

Overlaid spectrum of β -sitosterol (200 - 1000 ng band⁻¹) along with the spectra of β -sitosterol detected in the ethanolic extracts (25, 50, and 75 μ g band⁻¹) of aerial parts and roots of *I. cordifolia*, post-chromatographic derivatization with anisaldehyde-sulphuric acid reagent followed by scanning at 400 - 700 nm.