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ORIGINAL RESEARCH PAPER

A validated HPTLC method for quantification of cordifolioside A, 20-β-hydroxyecdysone and columbin with HPTLC–ESI–MS/MS characterization in stems of *Tinospora cordifolia*

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Abstract
The objective of the present work was to develop a simple, specific, and fast high-performance thin-layer chromatographic (HPTLC) method to identify and quantify cordifolioside A, 20-β-hydroxyecdysone and columbin with HPTLC−electrospray ionization−tandem mass spectrometry (ESI−MS/MS) for characterization in *Tinospora cordifolia* stem extracts. Chromatographic development was performed using a HPTLC aluminum plate, pre-coated with silica gel 60 F_{254} with hexane−chloroform−methanol−formic acid as the mobile phase. Densitometric quantification for 20-β-hydroxyecdysone and cordifolioside A was performed at 254 nm and for columbin at 600 nm after derivatization with anisaldehyde−sulfuric acid. The optimized mobile phase resulted in chromatographic separation of peaks for cordifolioside A, 20-β-hydroxyecdysone, and columbin at R_F of 0.12, 0.47, and 0.86, respectively. The linear concentration range was found to be 750−2250 ng/band for 20-β-hydroxyecdysone and cordifolioside A and 675−1875 ng/band for columbin with ($r^2 > 0.99$). The methodology showed good recoveries as 98.96−101.43% for cordifolioside A, 98.15−101.56% for 20-β-hydroxyecdysone, and 98.06−98.80% for columbin. The limit of detection was found for columbin, 20-β-hydroxyecdysone, and cordifolioside A as 53.86 ng/band, 40.90 ng/band, and 107.05 ng/band, while the limit of quantification was found to be 163.21 ng/band, 123.94 ng/band, and 324.38 ng/band, respectively. The relative standard deviation for precision and robustness study for all the markers was found to be within 2%. Three markers were identified and confirmed in *T. cordifolia* stem extracts by ESI−MS/MS. Compounds were assigned as norditerpene furan glycosides, ecdysteroids, and diterpenoid furanolactone: cordifolioside A (*m/z* = 527 $[M+Na]^+$; UV λ_{max} 221 nm), 20-β-hydroxyecdysone (*m/z* = 481.30 $[M+H]^+$; UV λ_{max} 247 nm), and columbin (*m/z* = 359 $[M+H]^+$; UV λ_{max} 210 nm). The optimized method was found accurate, reproducible, robust, and specific and can be applied for the quantification of cordifolioside A, 20-β-hydroxyecdysone, and columbin for quality control of extracts of *T. cordifolia*.

Keywords High-performance thin-layer chromatography (HPTLC) · *Tinospora cordifolia* · Guduchi · Giloy · Amrita · Immunomodulatory

1 Introduction

The regulatory requirements of the World Health Organization (WHO) emphasized proving the scientific validity of nutraceuticals or botanical drugs based on the application of sound scientific knowledge, thereby ensuring the identity, purity, and quality of herbal drugs [1–3]. The wide popularity of herbals or botanicals is due to their easy availability and patient acceptability. But the heterogeneous nature of the botanical drugs along with their complexity in terms of varied active constituents is critical for ensuring their therapeutic efficacy. For this, standardization is essential as it is a valuable tool for identifying and quantifying various chemical constituents of botanicals and drugs, thereby also differentiating related species [4]. Marker-based standardization is one of the important methods of characterization of botanical drugs. Standardization also aids in defining the amount of constituents that are attributable to biological activity [5, 6]. Additional measures for quality include botanical raw material control, quality control by chemical

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test and analytical test, biological assay along with supporting clinical data.

Tinospora cordifolia, belonging to the Menispermaceae family, is widely used in the indigenous system of medicine [5]. *T. cordifolia* family is widely distributed throughout the tropical India subcontinent, Sri Lanka, and China, ascending to 1200 m. *T. cordifolia* is also known as Amrita (Guduchi) in Sanskrit; 'Giloy' in Hindi and is widely used in the Ayurvedic system of medicine "Rasayanas" for strengthening the immune system and improving the body resistance against infections [7]. The stem is succulent with long fili form fleshy aerial roots, and the bark is creamy white to grey.

Many research communications report the scientific evaluation of efficacy of *T. cordifolia*. The immunomodulatory activity of the plant is very well-known in traditional and modern science. Moreover, it has a wide variety of other uses, including the treatment of general weakness, fever, dyspepsia, dysentery, gonorrhea, urinary diseases, viral hepatitis, anemia, antineoplastic, hypoglycemic, anti-inflammatory, and antioxidant activities [8, 9]. In previous clinical trials, the water extract of *T. cordifolia* stem was found to be effective for allergic rhinitis, immunomodulation in HIV positive patients, and burn patients [10–13].

T. cordifolia plant mainly reveals the presence of terpenoids, alkaloids, lignan, carbohydrates, bitters, steroids, glycosides, and sesquiterpenoids, aliphatic compounds, essential oils, fatty acids, and polysaccharides [14]. The aerial parts, mainly stems of *T. cordifolia,* various constituents including phenyl propanoid glycosides like cordifolioside A and B, syringin with diterpenoids tinosporaside, tinosporin, tinosporidine, tinocordifolioside, and alkaloids columbin, isocolumbin, berberine, magnoflorine have been reported [14–16]. These chemical constituents possess different therapeutic activities.

The chemical and biological marker compounds from stems of *T. cordifolia* have shown a promising role in immunomodulatory and protective properties. Cordifolioside A, a glycoside from phenyl propanoid class from *T. cordifolia,* showed immunostimulant with cardio and radioprotective activities. At the same time, a phytosterol 20-β-hydroxyecdysone has been studied in a large number of studies with many potential beneficial health effects, including the immunoprotective wound-healing and anti-osteoporosis effects [17–23]. It has also been studied as an alternative therapeutic option for the prevention and alleviation of cardiometabolic syndrome. The study reveals its anti-inflammatory, anti-fibrotic, and cardioprotective role in the restoration of renin−angiotensin system balance and improves the health of COVID-19 patients who have severe pneumonia [24]. Columbin is a diterpenoid furanolactone; it displays anti-inflammatory, anti-cancer, and antioxidant activity, protects from azoxymethane-induced rat colon carcinogenesis and possess a trypanocidal mechanism [25, 26]. Hence, *T. cordifolia* has been drawn interest in research as a COVID-19 preventive measure [27, 28].

Due to widespread use, chromatographic standardization of *T. cordifolia* is an important aspect for assuring quality, safety, and efficacy [29]. Chromatographic fingerprinting is the most widely used method for controlling the quality of botanical drugs and extracts using techniques such as thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), and other hyphenated techniques, TLC−mass spectrometry (MS), HPLC−MS. Among these, HPLC and HPTLC are used routinely for the analysis of nutraceuticals and botanicals. In comparison, HPTLC−MS/MS, LC−MS or LC−MS/MS are analytical techniques that enable rapid identification with higher specificity analyzing complex herbal samples [30]. Moreover, the applicability of LC−MS in herbal samples includes the various characteristics; mobile phase selection, stationary phase, high resolution, accurate mass measurements, selectivity and fragmentation behavior along with characterization of structure. LC−MS as a powerful tool is used for analyzing multicomponents that are present in complex herbal matrix [31].

Some of the analytical methods have been reported in the literature for *Tinospora* plants [32–45]. The literature reports some of the HPLC methods for study of morpho-anatomies of leaves and stems of *Tinospora* species. The presence of 20-β-hydroxyecdysone, tinosporaside, cordioside, and columbin has been confirmed in various species by HPLC method. HPTLC method for determination of different markers like tinosporaside, berberine has been reported. However, still, a validated and specific marker-based HPTLC method using markers like cordifolioside A, 20-β-hydroxyecdysone, and columbin is lacking and is desirable for the quality control of *Tinospora* species. Hence, the present research is the first one that describes the development and validation of the HPTLC−densitometry (DS) method for standardization and simultaneous quantification of columbin, 20-β-hydroxyecdysone, and cordifolioside A with confirmation by HPTLC−MS/MS data in *T. cordifolia*. Thus, the objective of this research study was to develop and optimize cordifolioside A, 20-β-hydroxyecdysone, and columbin (Fig. 1) in extracts of *T. cordifolia* followed by validation of methodology as per International Council for Harmonisation (ICH) guidelines for linearity, accuracy, precision with a limit of detection (LOD), limit of quantification (LOQ). This methodology will be useful for qualitative fingerprinting and quantitative marker-based standardization by HPTLC for extracts of stem of *T. cordifolia*. Moreover, characterization of cordifolioside A, 20-β-hydroxyecdysone, and columbin in the stem extracts of *T. cordifolia* can be confirmed by triple quadrupole (TQ)−*electrospray ionization* (ESI)−MS.

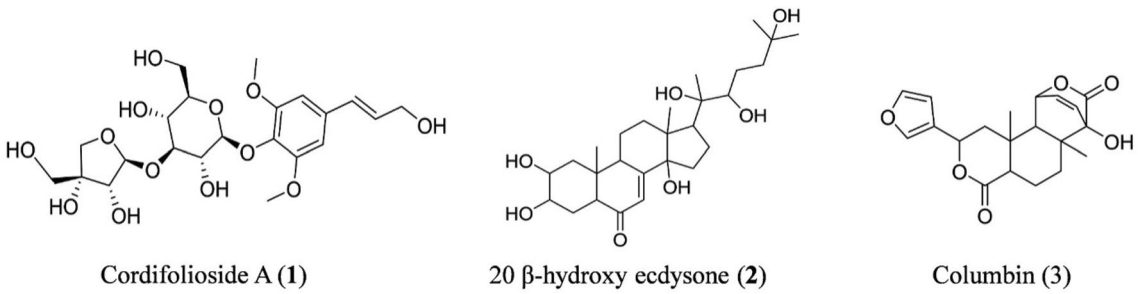

Fig. 1 The chemical structures of marker compounds from *Tinospora cordifolia.* (**1**) cordifolioside A; (**2**) 20-β-hydroxyecdysone, and (**3**) columbin

2 Experimental

2.1 Materials

T. cordifolia plant was collected from Ratnagiri (Maharashtra, India). The voucher specimen was submitted and authenticated by the Botanical Survey of India, Jodhpur, India.

The markers, cordifolioside A, 20-β-hydroxyecdysone, and columbin, were isolated in-house from dried and powdered stems of *T. cordifolia* by reported methodologies. All the isolated compounds were identified and characterized using ultraviolet (UV), Fourier-transform infrared spectroscopy (FTIR), HPLC−photodiode array (PDA), MS, and nuclear magnetic resonance (NMR) [23, 46, 47]. The minimum purity of the compounds was confirmed by analysis of HPLC in 1 mg/mL concentration in isocratic elution, and it was found to be greater than 90%. All chemicals and reagents used in the study were of analytical grade and purchased from Merck Specialities Pvt. Ltd. (Mumbai, India).

The dried and powdered stems of *T. cordifolia* (1 kg) were refluxed with 4 L water and water−ethanol (1:1, *V/V*) under stirring at 60 ± 5 °C for 3 h, and the filtrate was collected. The process was repeated two more times, and the obtained filtrate was dried under vacuum to get powdered extract (yields: 100 g and 123 g) for aqueous and hydroalcoholic extract.

2.2 Instrumentation and software

A CAMAG HPTLC system (Muttenz, Switzerland) comprising of a CAMAG Linomat V semi-automatic sample applicator, CAMAG TLC Scanner IV, CAMAG TLC visualizer, flat bottom and twin-trough developing chamber (10 × 10 cm), UV cabinet with dual-wavelength UV lamp, Hamilton syringe (100 µL; Bonaduz, Switzerland), ultrasonic bath (Frontline FS-4, Mumbai, India) and CAMAG winCATS software was used in the study. Calculations were performed by the use of Microsoft Excel 2007 software for linear regression analysis for quantification of extracts and determination of validation parameters, accuracy, precision, robustness. *Bartlett's* test was also performed for determining homoscedasticity of variance for data of linearity in MS Excel 2007.

2.3 Instrumental and chromatographic conditions

The samples were spotted in the form of bands having a band width of 8 mm with a 100-µL microsyringe (Linomat syringe 659.0014) on pre-coated silica gel aluminum HPTLC plate 60 F_{254} (10 cm × 10 cm, 100 µm thickness, E. Merck, Darmstadt, Germany) using a CAMAG Linomat V sample applicator. Then, the plates were dried with a cold air dryer. Densitometric scanning was done in absorbance−reflectance mode at 254 nm using a deuterium lamp and 600 nm using a halogen tungsten lamp. The slit dimensions were set at 0.60 mm × 0.45 mm, the scanning speed at 20 mm/s, and the data resolution at 100 µm/step. The results were evaluated to achieve an optimum separation between spots and migration of spots to ensure separation reproducibility.

2.4 Preparation of standard solutions of cordifolioside A, 20-β-hydroxyecdysone, and columbin

Accurately weighed 3.0 mg of each analyte (cordifolioside A, 20-β-hydroxyecdysone, and columbin) individually was transferred into an 5 mL Eppendorf tube, dissolved in 2 mL of methanol, and mixed well. This solution was used for qualitative analysis. For validation, an aliquot of 5.0 mg of each marker was dissolved in 5 mL of volumetric flask for preparation of stock solution with a concentration of 1000 µg/mL for each standard marker in volumetric flask. From the stock solutions, the working standard solution was prepared to have a concentration of 375 µg/mL of each marker. These stock and working standard solutions were stored at 4−6 °C.

2.5 Mobile phase optimization

The mobile phase was optimized for TLC using solvents of different polarities, consisting of chloroform, methanol, toluene, hexane, ethyl acetate in different proportions and combinations with saturation of chamber for 10 min. Working standard solutions were applied to the HPTLC plates as 8 mm bands using Linomat 5. Prior to use, the mobile phase components were mixed. Before each run, the development chamber was allowed to saturate with mobile phase vapor for 10 min.

2.6 HPTLC fingerprinting

An aqueous and hydroalcoholic extract of stems of *T. cordifolia* was applied on TLC plate, and the plate was developed in optimized mobile phase and further dried at room temperature in air. The plate was scanned at 254 nm before spraying and at 600 nm after spraying with detection reagent (anisaldehyde−sulfuric acid reagent), and the plate was heated at 110 °C for 5 min. The retardation factor (R_F) values and color of the resolved bands were noted.

2.7 Method validation

The method was validated as per ICH guidelines Q2 (R1) for the determination of validation parameters; linearity, precision, accuracy, LOD and LOQ, and robustness [48, 49]. The linear relationship between peak area and concentration of all three markers was evaluated by regression coefficient over the concentration range of 750−2250 ng/band for 20-β-hydroxyecdysone and cordifolioside A (at 254 nm before derivatization) and 675−1875 ng/band for columbin (at 600 nm after derivatization) by making five replicate measurements using different calibration sets. The standard deviations of slope and the intercept were calculated using ordinary least squares. Further linearity was verified by *Bartlett's* test for confirming homoscedasticity of variance. The precision of the developed method was evaluated by performing repeatability and intermediate precision studies, and the peak area measured was expressed in terms of percent relative standard deviation (*%RSD*). The sample application's repeatability was evaluated by applying the same concentration six times and further scanning and accumulating results as *%RSD*. The intra-day precision study was carried out by performing three replicates of three different concentrations (750, 1500, and 2250 ng/band for 20-β-hydroxyecdysone and cordifolioside A; 675, 1275, and 1875 ng/band for columbin) on the same day. Similarly, an intermediate precision study was performed on different days.

The accuracy was assessed by the methodological recovery studies to check the recovery at different levels: 80%, 100%, and 120% by adding a known amount of standard to the extracts and analyzed by the proposed method, in triplicate. As per the ICH guideline, the LOD and LOQ were computed from the standard deviation of the response, peak area and slope of the calibration curve of markers using the formulas $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$, where σ is the standard deviation of response, peak area, S is the slope of the calibration curve. The specificity of the method was ascertained by analyzing the standard marker and extracts. The band for cordifolioside A, 20-β-hydroxyecdysone, and columbin in extract was confirmed by comparing the R_F and spectra of the band with those of standard and further, peak purity index was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M), and peak end (E) positions of the band. The robustness of the proposed HPTLC method was evaluated on the response, R_F, and peak area by small changes in different method conditions: mobile phase ratio (±0.1 mL in the volume of methanol); saturation time (±0.5 min); solvent front (85 ± 2 mm); wavelength change (254 ± 2 nm) and (600 ± 2 nm).

2.8 Fingerprinting and analysis of the *T. cordifolia* extracts

From the *T. cordifolia* extracts, an aliquot of 100 mg of each was accurately weighed and transferred into a volumetric flask, dissolved in 2 mL water, and sonicated further for 10 min. The solution was then filtered through Whatman filter paper. After preparation as previously described, the sample solution (4 µL) was spotted on a plate, developed under the same conditions as described for the standard. After the development and drying of the plates, all three markers were found to be completely separated. The bands at the respective R_F value were scanned for detecting the peak of cordifolioside A, 20-β-hydroxyecdysone at 254 nm, and after derivatization with anisaldehyde−sulfuric acid for columbin at 600 nm, and the concentration of each marker in the extract was determined from the calibration curve plotted by linear regression analysis.

2.9 Extraction and characterization of markers by TLC−MS interface

The targeted analytes cordifolioside A, 20-β-hydroxyecdysone have been eluted by TLC−MS interface based on R_F values of the standard before derivatization, whereas columbin was observed and eluted after derivatization. The bands of markers were eluted with methanol by using TLC−MS interface 2 (CAMAG) and analysis with the help of ESI detector by using the LC−MS 8045 (Shimadzu, Kyoto, Japan) controlled by LabSolutions software, with mobile phase A: 0.1% formic acid in water and B: acetonitrile, flow rate (1.0 mL/min), nebulising gas flow (1.5 L/min),

desolvation line (DL) temperature (250 °C), and detector voltage (0.95 kV). The mass spectrum was recorded at positive and negative ion mode using ESI source [50, 51].

3 Results and discussion

3.1 Optimization of detection wavelength and mobile phase

Spectra of the cordifolioside A, 20-β-hydroxyecdysone, and columbin were recorded in the wavelength range of 200 to 700 nm using the scanner, where cordifolioside A showed maximum intensity at 240 nm, 20-β-hydroxyecdysone at 254 nm. Columbin gave lower intensity at 210 nm (before derivatization), while columbin gave high intensity after derivatization with anisaldehyde−sulfuric acid at 600 nm. Hence, the detection wavelength for two markers, cordifolioside A and 20-β-hydroxyecdysone, for validation, was selected as 254 nm before derivatization, and for columbin, detection was performed after derivatization at 600 nm (Fig. S1).

Mobile phase optimization using trial and approach method was performed for separation of marker compounds bands (cordifolioside A, 20-β-hydroxyecdysone and columbin). The initial mobile phase composition of chloroform and methanol in the ratio of 6:4 (*V/V*) was unable to achieve adequate separation. Further, modification of mobile phase composition with addition of toluene showed separation of marker compound with broad band. Hence, toluene was replaced by hexane and formic acid as modifier was added for better separation of bands. Hexane−chloroform−methanol−formic acid (4:4:2:0.1%, *V/V*) as optimized mobile phase resulted in a good separation of bands at R_F of 0.12, 0.47, and 0.86 for cordifolioside A, 20-β-hydroxyecdysone and columbin, respectively (Fig. S2).

3.2 HPTLC fingerprinting

HPTLC fingerprint development showed dark brown band, greenish blue band at 254 nm for cordifolioside A (R_F 0.12), 20-β-hydroxyecdysone (R_F 0.43), whereas bluish violet band for columbin (R_F 0.85) in white light after derivatization with anisaldehyde−sulfuric acid was observed in aqueous and hydroalcoholic extract of stems of *T. cordifolia.* HPTLC fingerprint was developed for identification of marker compounds band in aqueous and hydroalcoholic extract in comparison with reference standard band (Fig. 2).

3.3 Method validation

The method was validated in accordance with ICH guidelines Q2 (R1) by determination of validation parameters. The linearity of the calibration curve was established by

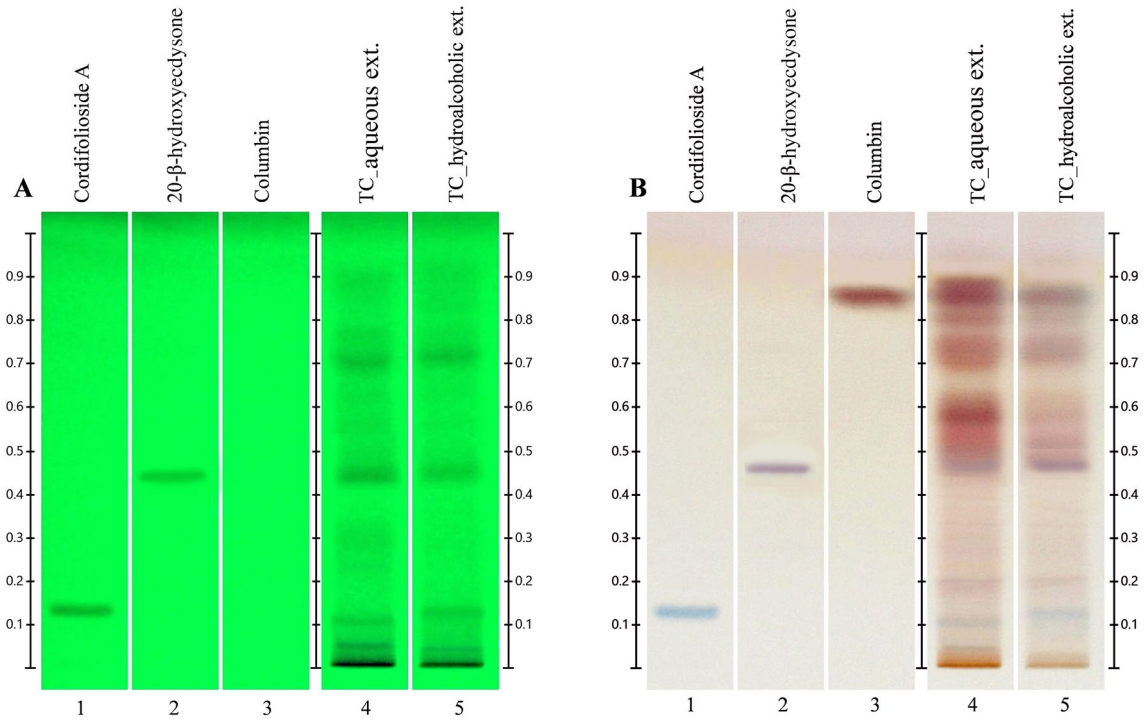

Fig. 2 Developed HPTLC plate photograph of water and hydroalcoholic extracts of *T. cordifolia* stem with marker compounds. **A** 254 nm before derivatization. **B** Visible mode after derivatization

a high correlation coefficient and residuals measured for different calibration sets (Figs. 3, 4). Further linearity was verified by *Bartlett's* test confirming homoscedasticity of variance that was exemplified by Chi-square value less than tabulated value (9.488) (Table 1). As per ICH guidance, determination of LOD and LOQ was performed on standard deviation of the response, peak area measured for five different calibration sets. LOD for columbin,

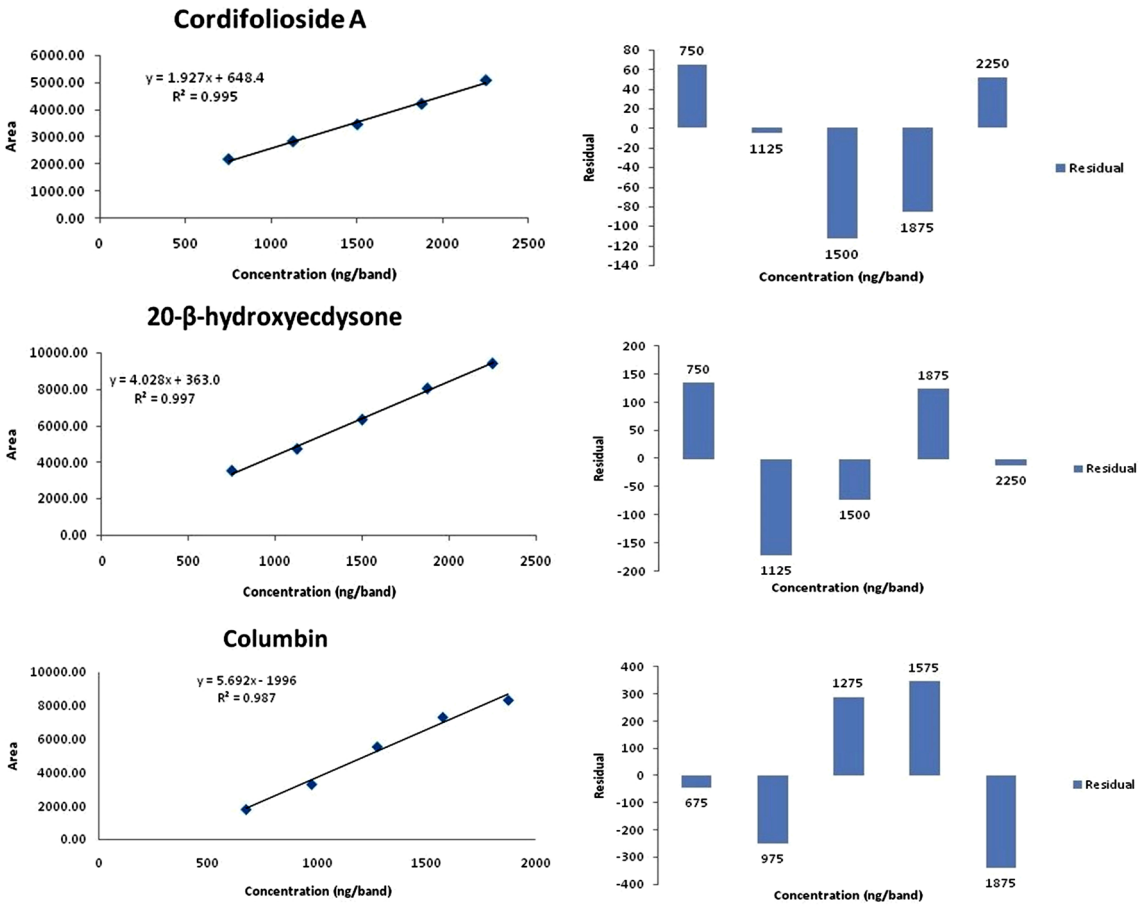

Fig. 3 Calibration plot and plot of residuals for cordifolioside A, 20-β-hydroxyecdysone and columbin in the linear calibration range

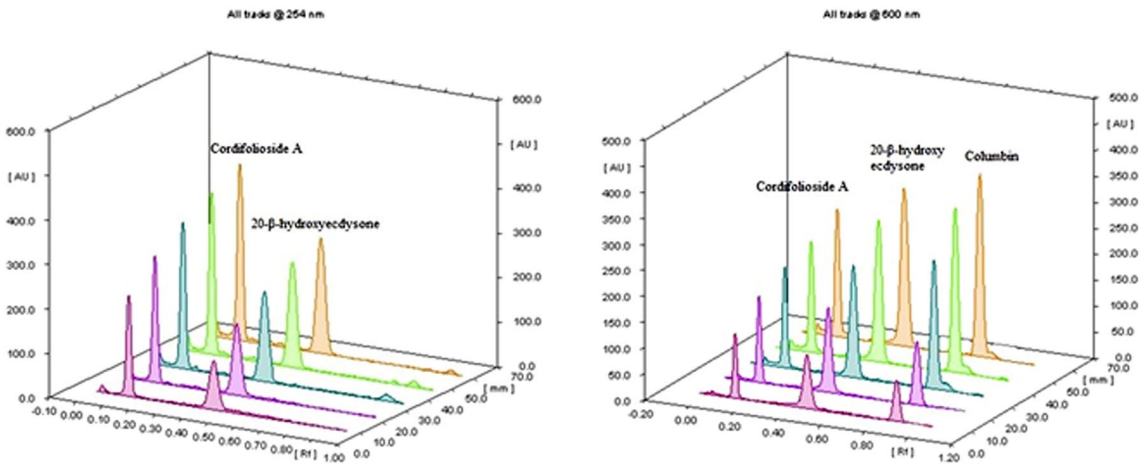

Fig. 4 Three-dimensional densitogram for linearity. **A** Cordifolioside A and 20-β-hydroxyecdysone at 254 nm. **B** Columbin at 600 nm

Table 1 Linear regression parameters for cordifolioside A, 20-β-hydroxyecdysone, and columbin in optimized DS based HPTLC studies

Parameters	Cordifolioside A	20-β-hydroxyecdysone	Columbin
Linearity[a] (ng/band)	750–2250	750–2250	675–1875
Correlation coefficient (R^2)	0.995	0.997	0.987
Regression equation	$y=1.927x+648.4$	$y=4.028x+363.0$	$y=5.692x+1996$
LOD (ng/band)	107.05	40.90	53.86
LOQ (ng/band)	324.38	123.94	163.21
Bartlett's test[b] (χ^2)	0.0266	0.0004	0.0039

[a]Mean of five replicates, five concentrations

[b]Calculated value χ^2 less than critical value $\chi^2(0.05, 4)=9.488$

20-β-hydroxyecdysone, and cordifolioside A was 53.86, 40.90, and 107.05 ng/band, while LOQ was found to be 163.21, 123.94, and 324.38 ng/band (Table 1). The precision study was repeated three times in a day (intra-day precision) and on three different days (inter-day precision) with the determination of the average *%RSD* values for peak area of columbin, 20-β-hydroxyecdysone and cordifolioside A. Both intra-day and inter-day precision studies resulted in *%RSD* less than 2, confirming that the method is reproducible (Table 2). Recovery studies were carried out by spiking three different amounts of 20-β-hydroxyecdysone, cordifolioside A, and columbin (600, 750, and 900 ng/band) to the extract (750 ng/band) by the standard addition method. Recoveries for the three markers were found to be 98.96–101.43% for cordifolioside A, 98.15–101.56% for 20-β-hydroxyecdysone, 98.06–98.80% for columbin (Table 3). The densitogram of the extract obtained using the developed method showed peaks at R_F of 0.12, 0.47, and 0.85 for cordifolioside A, 20-β-hydroxyecdysone, and columbin, respectively, found to be at the R_F value similar to standard markers (Fig. 5). The peak purity of all markers in this botanical extract, when evaluated by comparing the overlaid spectra at peak start, peak apex, and peak end positions of the spot, showed a peak purity of more than 0.99 for all markers (Fig. 6, Fig. S4 and Table S3). Small, deliberate changes in different parameters like mobile phase composition, chamber saturation time, distance traveled, wavelength showed less than 2% RSD for a response, peak area, and R_F; the results reveal that there is only a slight effect on R_F and peak area, indicating that the method is robust (*%RSD* between 0.05 to 1.45).

3.4 Quantification of compounds (1–3) by HPTLC–DS in *T. cordifolia* extracts

The content ($n=3$, RSD < 5.0%) of cordifolioside A, 20-β-hydroxyecdysone, and columbin was found to be 0.134, 0.845, and 0.196% *w/w* in hydroalcoholic extract and 0.017, 0.499, and 0.284% *w/w* in aqueous extract by the validated HPTLC–DS method (Fig. 5 and Table 4).

Table 2 Precision studies as repeatability and inter-day precision for the determination of cordifolioside A, 20-β-hydroxyecdysone, and columbin by HPTLC–DS method

Amount (ng/band)	Repeatability		Inter-day precision			
			Day 1		Day 2	
	Peak area[a] ± SD	*%RSD*	Peak area[a] ± SD	*%RSD*	Peak area[a] ± SD	*%RSD*
Columbin						
675	1833.9 ± 28.8	1.57	1865.2 ± 15.2	0.81	1858.7 ± 34.0	1.83
1275	5596.0 ± 54.4	0.98	5515.3 ± 69.7	1.26	5557.2 ± 58.9	1.06
1875	8296.0 ± 27.7	0.57	8257.4 ± 39.0	0.45	8243.6 ± 35.5	0.41
20-β-hydroxyecdysone						
750	3512.4 ± 34.4	0.98	3486.3 ± 26.8	0.77	3549.9 ± 21.8	0.61
1500	6376.6 ± 16.1	0.25	6332.2 ± 27.6	0.44	6381.1 ± 27.4	0.43
2250	9476.8 ± 13.44	0.14	9480.1 ± 27	0.28	9454.4 ± 6.7	0.18
Cordifolioside A						
750	2121.0 ± 5.2	0.24	2213.7 ± 10.5	0.47	2213.5 ± 8.2	0.37
1500	3479.3 ± 9.3	0.27	3444.7 ± 4.1	0.12	3465.0 ± 4.2	0.12
2250	5208.4 ± 9.3	0.18	5208.5 ± 5.3	0.10	5226.3 ± 4.2	0.08

[a]Mean of five replicates

Table 3 Recovery studies for the determination of cordifolioside A, 20-β-hydroxyecdysone, and columbin by HPTLC–DS method

Percent of analyte spiked	Total amount in sample after spiking	Total amount found	% Recovery[a]	SD	*%RSD*
Cordifolioside A					
80	1350	1348.91	99.92	0.39	0.39
		1339.72	99.24		
		1340.09	99.27		
100	1500	1500.32	100.02	0.77	0.76
		1512.84	100.86		
		1523.36	101.56		
120	1650	1622.74	98.35	0.10	0.10
		1620.31	98.20		
		1619.54	98.15		
20-β-hydroxyecdysone					
80	1350	1342.86	99.47	0.515	0.51
		1349.87	99.99		
		1335.96	98.96		
100	1500	1493.62	99.57	0.328	0.33
		1484.74	98.98		
		1492.89	99.53		
120	1650	1649.25	99.95	0.279	0.28
		1657.03	100.43		
		1648.88	99.93		
Columbin					
80	1350	1335.00	98.80	0.39	0.39
		1327.13	98.22		
		1325.05	98.06		
100	1500	1485.00	98.80	0.23	0.23
		1478.52	98.37		
		1479.72	98.45		
120	1650	1635.00	98.80	0.35	0.35
		1627.13	98.32		
		1623.76	98.12		

[a]Mean of three replicates / three concentration levels

3.5 Characterization of compounds by HPTLC–MS/MS

To confirm selected markers compounds **(1–3)** from *T. cordifolia,* TLC–MS interface combined with indirect profiling of LC–ESI–MS/MS analysis has been used. Compound **(1)** was eluted at Rt 4.71 min at the wavelength of 221 nm and was obtained in its sodium ion adduct $[M+Na]^+$ at $m/z = 527.0$ and potassium ion adduct $[M+K]^+$ at $m/z = 541.1$. Two prominent fragment ions of compound **(1)** were observed at $m/z = 312.4$ $[M+Na-C_{11}H_{15}O_2]^+$ with the loss of two sugar moieties, and at $m/z = 333.16$ $[M-C_6H_{15}O_9-2OH^-]^+$ with the loss of one sugar moiety and two −OH atoms from the parent molecule. The fragments collectively confirmed compound (**1**) using ESI–MS. Compound **(2)** was later eluted at the Rt 11.36 min, at the maximum wavelength of 247 nm. The compound was observed in positive ion mode with the prominent molecular ion peak at $[M+H]^+$ at $m/z = 481.30$ followed by the formation of its two adducts, namely, sodium adduct $[M+Na]^+$ at $m/z = 503.31$ and potassium adduct $[M+K]^+$ at $m/z = 519.23$. Two major fragment ions of compound **(2)** were observed at $[M-OH]^+$ at $m/z = 464.31$ (loss of an −OH ion), and another fragment was observed at $m/z = 426.13$ (with the loss of $[M-CH_2OH]^-$ ion). Subsequently, the mass spectra of compound **(3)** showed a protonated molecular ion peak $[M+H]^+$ at $m/z = 359$ followed by two prominent fragment ions observed as the deprotonated ions at $m/z = 340$ with loss of water molecule $[M-H_2O-H]^+$ and $m/z = 312$ with the deprotonated ion and loss of two carbonyl ions $[M-H-CO_2]^+$ at positive ESI mode. Compound **(3)** was obtained at Rt 24.49 min at 210 nm (Table 5, Fig. 7 and Fig. S5).

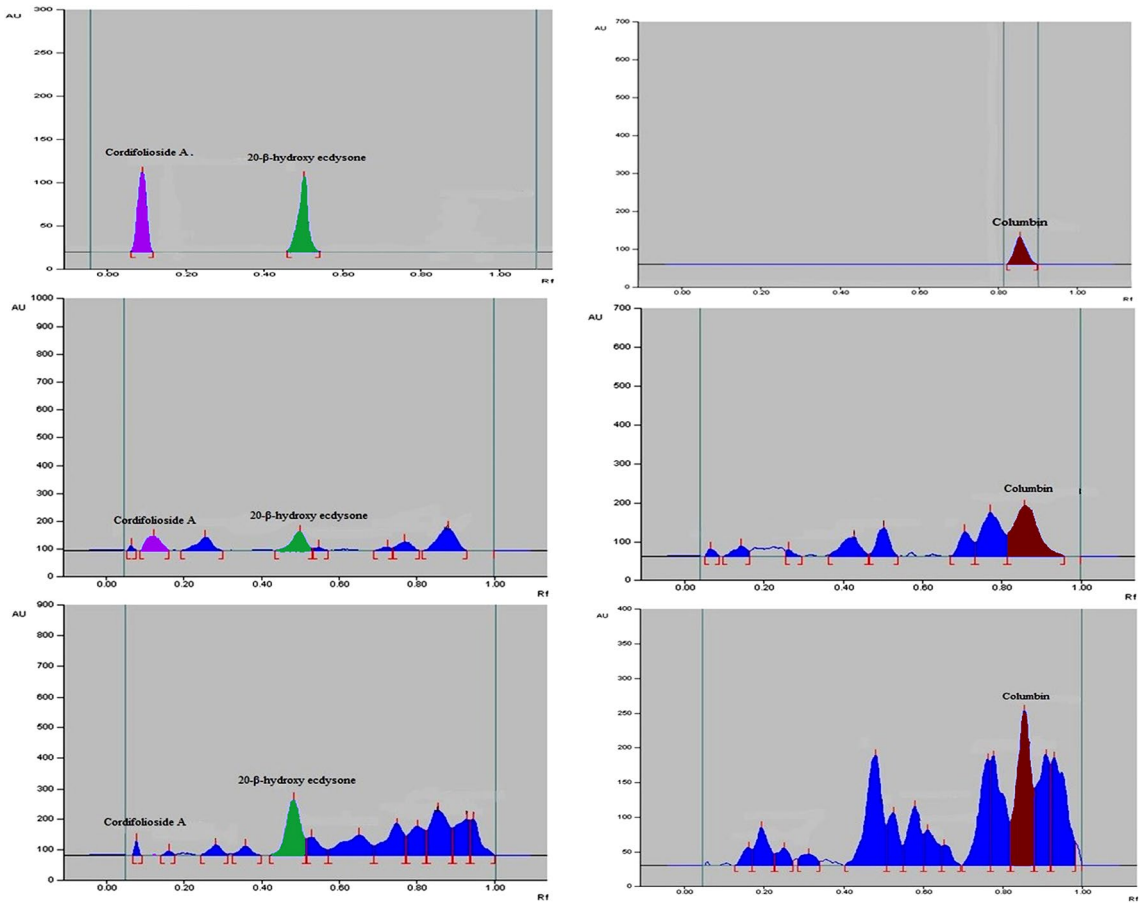

Fig. 5 Developed HPTLC densitograms of markers with hydroalcoholic and aqueous extracts. **A** at 254 nm. **B** After derivatization at 600 nm

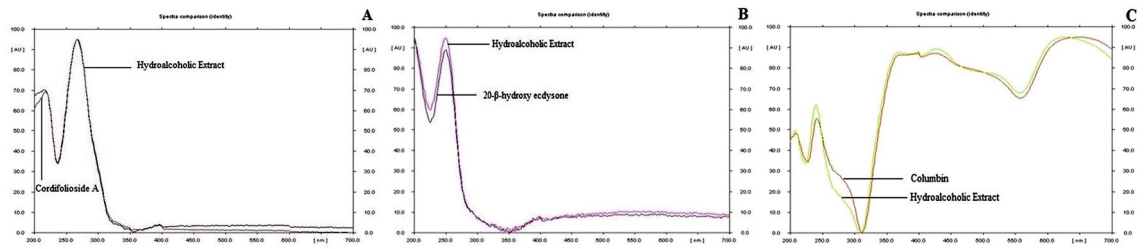

Fig. 6 Densitometric UV spectra of standard markers with hydroalcoholic extract. **A** Cordifolioside A before derivatization. **B** 20-β-hydroxy-ecdysone before derivatization. **C** Columbin after derivatization with anisaldehyde−sulfuric acid

Table 4 Quantification of cordifolioside A, 20-β-hydroxyecdysone, and columbin in aqueous extract and hydroalcoholic extract by the validated HPTLC–DS method

Extract	Mean percent of cordifolioside A[a] (%mg/mg) ± *%RSD*	Mean percent of 20-β-hydroxyecdysone[a] (%mg/mg) ± *%RSD*	Mean percent of columbin[a] (%mg/mg) ± *%RSD*
Aqueous extract	0.017 ± 0.66	0.499 ± 0.75	0.284 ± 0.80
Hydroalcoholic extract	0.134 ± 0.52	0.845 ± 0.43	0.196 ± 0.55

[a]Mean of three replicates

Table 5 ESI–MS/MS fragmentation of markers of *T. cordifolia*

Marker name	Molecular weight	Fragmentation ions (MS^n)	Precursor ion adducts
Cordifolioside A	527.00	211.20, 246.20	527.00 $[M+Na]^+$ 541.20 $[M+K]^+$
20-β-hydroxyecdysone	481.05	427.10, 445.40, 463.50	504.50 $[M+Na]^+$ 579.30 $[M+K]^+$
Columbin	359.00	341.00, 313.10	–

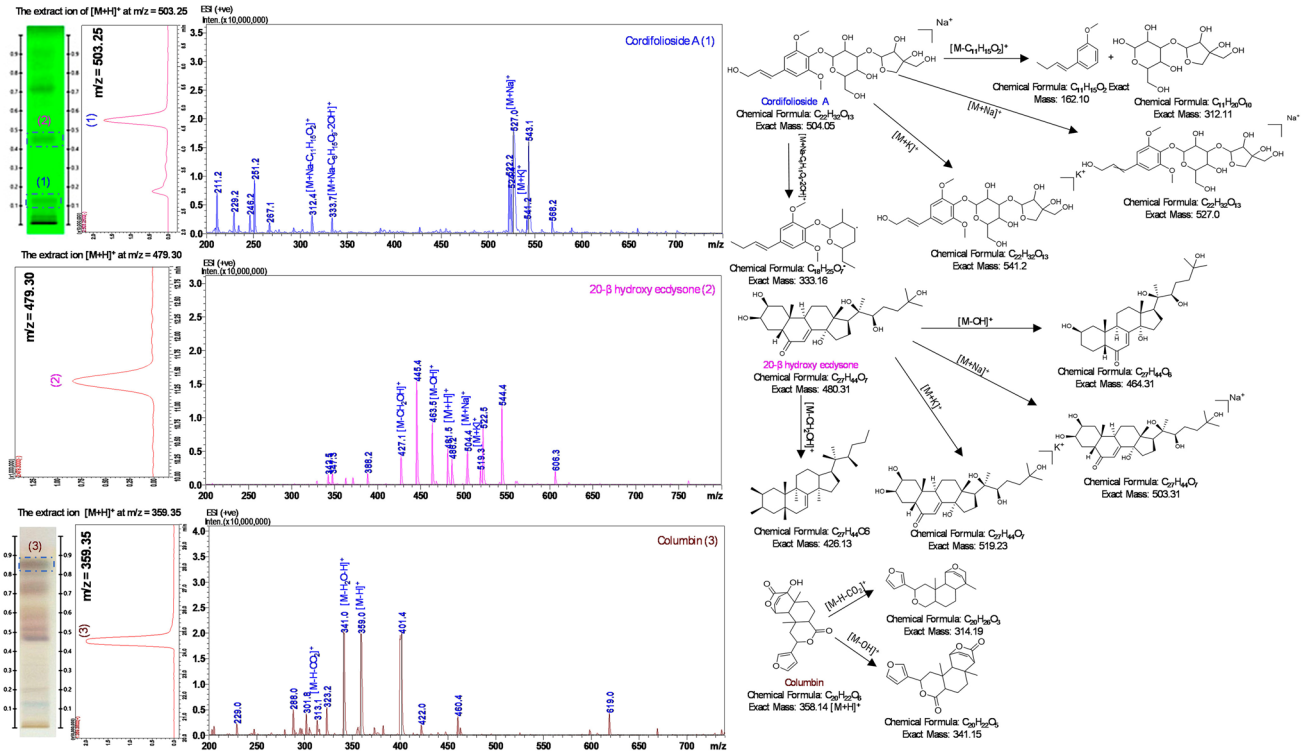

Fig. 7 HPTLC–ESI–MS/MS chromatogram of hydroalcoholic extracts of *T. cordifolia* stem identifying cordifolioside A, 20-β-hydroxyecdysone (254 nm before derivatization) and columbin (visible light after derivatization) with its mass fragmentation pattern

4 Conclusion

The TLC–MS interface technique was used for the separation of cordifolioside A, 20-β-hydroxyecdysone, and columbin from *T. cordifolia* extract. HPTLC–MS/MS analysis confirmed the presence of compounds **(1–3)** in stem extracts (hydroalcoholic and aqueous) of *T. cordifolia*. The developed and validated HPTLC method for estimation of three markers may be useful for the quality control and standardization of these extracts and its further role in traditional medicines and nutraceuticals.

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