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Iridoid glycoside-based quantitative chromatographic fingerprint analysis: A rational approach for quality assessment of Indian medicinal plant Gambhari (*Gmelina arborea*)

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ABSTRACT

A sensitive, selective and robust qualitative and quantitative densitometric high-performance thin layer chromatographic method was developed and validated for the determination of iridoid glycoside in the aerial part of Gambhari (*Gmelina arborea*). Iridoid gycoside 6-*O*-(2″,3″-dibenzoyl)-α-L-rhamnopyranosylcatalpol (IG) was used as a chemical marker for the standardization of *G. arborea* plant extracts. The separation was performed on aluminum Kieselgel 60F_{254} TLC plates using chloroform–methanol as mobile phase. The quantitation of IG was carried out using the densitometric reflection/absorption mode at 240 and 430 nm after post-chromatographic derivatization with vanillin–sulphuric acid reagent. A precise and accurate quantification can be performed in the linear working concentration range of 1000–5000 ng/spot with good correlation ($r^2 = 0.994$). The method was validated for peak purities, precision, robustness, limit of detection (LOD) and quantitation (LOQ), etc., as per ICH guidelines. Specificity of quantitation was confirmed using retention factor (R_f), UV–vis spectral correlation and ESI-MS spectra of marker compound (IG) in sample track.

1. Introduction

Gmelina arborea (family Verbenaceae), an important commercial timber species has been used in Ayurveda since ancient times. Its decoction is used as a diuretic for loosening phlegm, as an appetite stimulant and in the treatment of various stomach disorders, fevers, skin problems and liver disorders [1–3]. *G. arborea* is an important ingredient of generic Ayurvedic formulation "*Dashamularishta*" prescribed for several gynaecological disorders and used in several commercial ayurvedic preparations [4]. *In vitro* studies on bark and fruit extracts showed antioxidant activity and protected liver slice culture cells by alleviating oxidative stress-induced damage to liver cells [5]. *Ex vivo* studies of the extract on perfused isolated rabbit jejunum and *in vivo* studies based on castor oil-induced model proved to have activity against diarrhea in mice but at low doses [6]. Luteolin [7], indole alkaloids [8], acylated iridoid glycosides [9] in the aerial part and octacosanol [10], cluytyl ferulate [11], lignans [12–16] and iridoid glycosides [17] in the heartwood and coumarin glycoside [18] in the root have been reported in *G. arborea*.

Iridoid glycosides belong to an important class of compounds due to their structural link between terpenoids and indole alkaloids therefore possessing spectrum of biological activities [16–17,19–20]. Iridoid esters are labile to transform into other isomers and isolation of these types of constituents in pure form was prevented by instability problems [21]. Our continued efforts by repeated preparative HPLC has resulted in the isolation of the only stable isomer, i.e. 6-*O*-(2″,3″-dibenzoyl)-α-L-rhamnopyranosylcatalpol (IG) in pure form for quantitative chromatographic fingerprint analysis of Gambhari. The isolated and authenticated iridoid glycoside (IG) from aerial part of *G. arborea* has been used to develop a validated analytical procedure for its quality assurance. Analytical techniques like high-performance thin layer chromatographic (HPTLC) fingerprint looks at a suite of compounds; including their respective ratios, provide a more rational approach to the authentication and quality assessment of crude drug as well as formulation [22–24]. Our continued interest on the development of rapid HPTLC method for quality assessment of medicinal plants [25–28] led us to develop an analytical procedure for quality assessment of *G. arborea* for the first time.

Abbreviations: HPTLC, high-performance thin layer chromatography; IG, iridoid glycoside.

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The objective of present study was to optimize, develop and validate a rapid, sensitive and accurate high-performance thin layer chromatographic method for the determination of iridoid glycoside 6-*O*-(2″,3″-dibenzoyl α-L-rhamnopyranosylcatalpol) in aerial part of *G. arborea*.

2. Experimental

2.1. Plant materials

Aerial parts of Gambhari (*G. arborea* Roxb.) were collected locally from Lucknow and identified by the Botany and Pharmacognosy department of our Institute. A voucher specimen (CIMAP No. 7656) has been deposited in the herbarium of the institute.

2.2. Chemicals and reference compound

All solvents and reagents used were either analytical or HPLC grade (E. Merck Ltd., Mumbai, India). Before use, the solvents were filtered through a 0.45 μm Millipore membrane (Millipore, Billerica, MA) after sonication for 15 min. The standard compound, iridoid gycoside 6-*O*-(2″,3″-dibenzoyl)-α-L-rhamnopyranosylcatalpol (IG) was isolated (purity > 99% using area normalization method of HPLC) and characterized by spectral analysis in our laboratory.

2.3. Apparatus

Preparative HPLC (Shimadzu, Japan) consisting column (Supelcosil LC-18, 21.2 mm × 250 mm, 12 μm), pumps LC-8A and PDA detector was used for isolation and purification of reference compound (IG). Precoated TLC silica gel $60F_{254}$, (10 cm × 10 cm, 20 cm × 10 cm, E. Merck, Darmastadt, Germany) were used for optimization of analytical protocol. Vario system, TLC Scanner winCATS-III, Reprostar 3, twin trough chamber, immersion device III, TLC plate heater (Camag, Muttenz, Switzerland) were used for digital image scanning for HPTLC method development and validation. NMR spectra were recorded in pyridine with TMS as internal standard using 300 MHz spectrometer (Avance, Bruker, Switzerland). ESI-MS spectra were obtained on LCMS-2010EV (Shimadzu, Japan) hyphenated to LC system (LC-20AD, CTO-20A, SIL-10AF, SPD-M20A, and ABM-20A) for authenticity and purity of the reference compound.

2.4. Extraction and Isolation of reference compound (IG) from G. arborea

Air-dried and finely powdered aerial parts of the plant (1.45 kg) were exhaustively extracted at room temperature (25 ± 3 °C) using the solvent methanol (3× 10 L) in a percolator and the pooled extracts then obtained were concentrated under vacuum to give methanolic extract. Methanolic extract was partitioned with *n*-hexane (34.75 g), chloroform (13.65 g) and *n*-butanol (68 g). The *n*-butanol fraction was concentrated in vacuum to afford a brown residue. This residue was chromatographed over a Silica gel column eluting with chloroform followed by increasing concentrations of methanol. Fraction 145–150 (chloroform:methanol, 90:10) resulted in a viscous solid. Preparative HPLC (MeOH:H_2O, 65:35; flow rate, 15 mL/min) was used to achieve the desired marker (RT: 11.00 min). The structure elucidation of 6-*O*-(2″,3″-dibenzoyl α-L-rhamnopyranosylcatalpol (Fig. 1) was performed with the help of UV, ^{1}H NMR, ^{13}C NMR, 2D experiments (COSY and NOESY) and mass (ESI-MS) analysis and confirmed as reported earlier [9].

6-*O*-(2″,3″-Dibenzoyl)-α-L-rhamnopyranosylcatalpol (IG) obtained as white amorphous powder (18 mg), $[\alpha]_D^{25} = -139.9$ °C (*c* 0.62, MeOH); UV (MeOH) λ_{max} 223, 276 nm; ^{1}H NMR (300 MHz, pyridine)–aglycon moiety δ 5.52 (1H, H-1), 6.43 (1H, H-3), 5.24 (1H, H-4), 2.80 (1H, H-5), 4.25 (1H, H-6), 3.78 (1H, H-7), 2.82 (1H, H-9), 4.4 (1H, H-10A), 4.5 (1H, H-10B), glucosyl moiety δ 5.48 (1H, H-1′), 4.1 (1H, H-2′), 4.2 (1H, H-3′), 4.2 (1H, H-4′), 3.9 (1H, H-5′), 4.3 (1H, H-6′A), 4.5 (1H, H-6′B), rhamnosyl moiety δ 5.53 (1H, H-1″), 4.9 (1H, H-2″ 6.1 (1H, H-3″), 6.3 (1H, H-4″), 4.5 (1H, H-5″), 1.26 (3H, H-6″), benzoyl moiety δ 8.11 (2H, H-2‴,6‴), 7.44 (2H, H-3‴,5‴), 7.4 (1H, H-4‴), 8.01 (2H, H-2⁗,6⁗), 7.3 (2H, H-3⁗,6⁗), 7.3 (1H, H-4⁗); ^{13}C NMR (75 MHz, pyridine)–aglycon moiety 95.05(C-1), 141.69(C-3), 103.12(C-4), 36.96(C-5), 84.22(C-6), 58.81(C-7), 66.53(C-8), 43.58(C-9), 60.64(C-10), glucose moiety 100.76(C-1′), 75.18(C-2′), 78.50(C-3′), 71.86(C-4′), 79.07(C-5′), 62.99(C-6′), rhamnose moiety 100.41(C-1″), 69.94(C-2″), 73.04(C-3″), 74.16(C-4″), 67.96(C-5″), 18.09(C-6″), benzoyl moiety 130.63(C-1‴), 130.11(C-2‴, 6‴), 129.02(C-3‴,5‴), 133.67(C-4‴), 166.51(C=O), 130.50(C-1⁗), 130.07(C-2⁗,6⁗), 128.73(C-3⁗,5⁗), 133.36(C-4⁗), 166.36(C=O); ESI-MS *m*/*z* 739 $[M+Na]^+$, 740 $[M+H+Na]^+$, 355 [dibenzoylrhamnose-oxonium]$^+$ ion (calculated for $C_{35}H_{40}O_{16}$, 716).

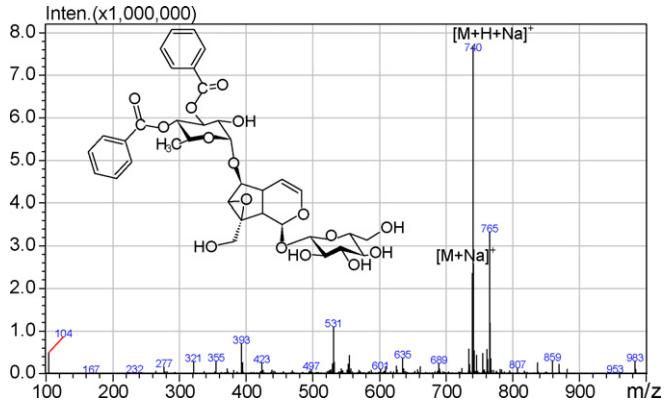

Fig. 1. ESI-MS in +ve mode and structure of iridoid gycoside 6-*O*-(2″,3″-dibenzoyl)-α-L-rhamnopyranosylcatalpol (IG) isolated from *G. arborea*.

2.5. Standard stock solution and sample preparation

Standard stock solution of reference compound (IG) was prepared by dissolving 5.0 mg/mL in methanol and filtered through 0.45 μm filters. Working stocks for calibration studies were prepared by dilution using Hamilton syringe. Dried and finally milled aerial part (100 mg) of *G. arborea* were extracted with various organic solvents, viz. *n*-hexane, chloroform, ethyl acetate, methanol and *n*-butanol (3× 5 mL, 10 h for extraction every time) separately and exhaustively extracted residues were collected and dissolved in 1.0 mL of respective solvents and centrifuged at 10,000 rpm for 10 min. The supernatants were filtered with 0.45 μm filter and used for TLC analysis. The results of extraction efficiencies of solvents are summarized in Table 1.

Table 1
Extraction efficiency of different solvent for chemical marker (IG) from the aerial parts of *G. arborea* on dry weight basis

Solvents[a]	Amount of compound quantified (%, w/w)
n-Hexane	ND
Chloroform	0.113
Ethyl acetate	0.136
Methanol	0.355
n-Butanol	0.246

[a] Cold percolation method as per description of sample preparation section.

2.6. Analytical procedure

Chromatography was performed on preactivated silica gel $60F_{254}$ HPTLC plates (20 cm × 10 cm). The application parameters were identical for all the analyses performed. The plates were dried in a current of air and the layers developed with a mobile phase of chloroform:methanol (80:20) under laboratory conditions (temperature 25 ± 3 °C and relative humidity 35–40%). Developed plates were immersed in freshly prepared vanillin–sulphuric acid derivatizing reagent and plates were then heated at 110 °C for 15 min. In order to reveal the analytes, the analyte spots were scanned at 430 nm using a slit size of 6.00 mm × 0.45 mm.

2.7. Chromatographic experiments

Aluminum Kieselgel $60F_{254}$ TLC plates (Merck) were activated at 60 °C for 5 min prior to chromatography. Standard and sample solutions were applied in the form of spot at 15 mm from both the lower and left edge, and 20 mm space between two spot, with a microliter syringe (Hamilton, Bondauz, Switzerland). Linear ascending development was carried out in pre-saturated (optimized to 2 min for better resolution) vertical twin trough glass chambers (10 cm × 10 cm or 20 cm × 20 cm Camag, Switzerland) saturated with the mobile phase. The mobile phase selection and optimization was carried out using the Vario System wherein different compositions consisting of different ratios of solvents of varying polarity with stationary phases were tried. Finally, a mobile phase consisting of chloroform–methanol (80:20, v/v) was found suitable for satisfactory separation and quantitation of IG with interfering components of sample matrix. After development, the plates were dried and the components were visualized by UV radiation at 240 nm and after dipping the plates into vanillin–sulphuric acid reagent (vanillin:EtOH:H_2SO_4, 5.0 gm:475 mL:25 mL) using the Immersion device (dipping time 2 s, dipping speed 5 cm s^{-1}) followed by air dying for 5 min. The plates were then heated for 3 min at 110 °C using TLC plate heater-III (Camag) and quantified densitometrically at 430 nm. TLC Scanner-III controlled by winCATS 1.4.2.8121 software (Camag) was used for quantitative evaluation. The scanning wavelengths 240 nm and 430 nm, respectively were selected on the basis of maxima of UV or vis spectra scanning of the standard IG in pre- and post-derivatization of the spot on TLC plate. The densitometry scanning was performed in the reflectance/absorbance mode slit width 6.00 mm × 0.45 mm, scanning speed 20 mm s^{-1} and data resolution 10 μm $step^{-1}$. Savitsky-Golay-7 was used for data filtering and the lowest slope for baseline correction in order to integrate the area. For recording of characteristic UV absorption spectra (200–400 nm) of sample track, deuterium lamp was used, while for derivatized spot of compounds IG and sample tracks in the range 400–800 nm, tungsten lamp was used. Reprostar 3 with cabinet cover and mounted digital camera (Canon PowerShot G5 with Neck Strap NS-DC2, Canon, Japan) was used for imaging and archiving the thin layer chromatograms. Quantitation was performed using peak area with linear regression of amount (μg/spot).

2.8. Method validation

Validation of quantitative TLC method includes the evaluation of following performance parameters such as linearity, limit of sensitivities, specificity, precision and accuracy, recovery and robustness according the guidelines of the International Conference on Harmonization (ICH) [29] and the IUPAC [30].

Table 2
Summary of method validation data for the quantitation of chemical marker compound (IG) in *G. arborea*

Parameters	Value
Retention factor (R_f)	0.44 ± 0.02
Densitometric relationship[a]	
Working concentration range (digital scanning at 240 nm)	1–5 μg/spot
	Linear fit: $Y = 2359 \pm 109.1$ $X + 4410 \pm 361.9$
	Goodness of fit ($S_{y.x}$) = 345.0
	Correlation coefficient (r^2) = 0.994
Working concentration range (digital scanning at 430 nm)	1–5 μg/spot
	Linear fit: $Y = 991.6 \pm 54.27$ $X + 104 \pm 180.0$
	Goodness of fit ($S_{y.x}$) = 171.6
	Correlation coefficient (r^2) = 0.991
Sensitivities	
Limit of detection (LOD)	0.439 μg (scanning at 240 nm)
	0.519 μg (scanning at 430 nm)
Limit of quantitation (LOQ)	1.462 μg (scanning at 240 nm)
	1.731 μg (scanning at 430 nm)
Specificity	
Peak purity	
$R(s, m)$[b]	
Standard track (marker compound IG)	0.9999
Sample track (methanol extract)	0.9999
$R(m, e)$[c]	
Standard track (marker compound IG)	0.9999
Sample track (methanol extract)	0.9998
Precision and accuracy[d]	
Intra-day (coefficient of variance, R.S.D. %)	1.54 and 1.77
Inter-day (coefficient of variance, R.S.D. %)	3.19 and 3.36

[a] Digital scanning at 240 nm and 430 nm post-derivatization with vanillin–sulphuric acid reagent and statistical relationship were established considering five data point each in triplicate; *X*, amount of compound (μg/spot); *Y* area under peak in AU.

[b] Correlation of spectrum at start of peak with spectrum at the centre of peak at 430 nm scanning.

[c] Correlation of spectrum at center of peak with spectrum at the end of peak at 430 nm scanning.

[d] $n = 5$ at 1500 and 2000 ng/spot, respectively (data analysis was performed at 430 nm scanning).

2.8.1. Linearity and quantification

Working stock solutions were prepared by dilution to give solutions containing IG in the concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL. Two microliters of each standard solution was spotted on the TLC plate to obtain absolute amounts of 1, 2, 3, 4, and 5 μg/spot. Each concentration was spotted thrice on TLC plates. The calibration curves were prepared by the least-squares method using absolute amount (μg/spot) as independent variable (*X*) and the peak area of IG as dependent variable (*Y*). Regression analyses test of the compound was performed by Graph PAD Prism 3.0. The curves confirm the significant linear relationship between the concentration and the peak area (Table 2). Two microliters of each sample solution from different locations were taken and applied on TLC plates in triplicate with similar procedure (as described earlier in Section 2.7). The experimental parameters were identical for all the above analysis. A calibration curve of standard as prepared

Table 3
Results of recovery study

Marker compound IG in sample (ng absolute)	Spiked amount (ng absolute)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)	Average recoveries (%)	R.S.D. (%, $n = 3$)
794	1000	1794	1701	90.700	90.333	1.969
			1678	88.400		
			1713	91.900		
	2000	2794	2611	90.850	92.517	1.583
			2666	93.600		
			2656	93.100		
	3000	3794	3674	96.000	94.567	1.339
			3602	93.600		
			3617	94.100		

above was used to calculate the percent content of analyte in the sample.

2.8.2. Limit of detection and quantification

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated for IG using the linear regression equation (Table 2). Following equations were applied: LOD = $3S_{y,x}/b$ and LOQ = $10S_{y,x}/b$, where $S_{y,x}$ is the standard deviation of the Y-value distribution around the regression line and b is the slope of the calibration curve.

2.8.3. Specificity

The specificity of the method was ascertained by co-analyzing standard and sample. The band for IG in sample was confirmed by comparing the R_f (0.44) and absorption spectra of the spot to that of reference compound. The peak purity of IG peak in sample track was assessed by comparing the spectra at peak start, peak apex and peak end positions of the band. Good correlation was also obtained between standards and sample overlay spectra ($r^2 > 0.99$). Further to confirm the specificity from the center of spot corresponding to IG in both standard track and sample track silica were scraped. ESI-MS (Fig. 1) was obtained by direct introduction into mass detector through restriction coil (without any column). Mass spectra of the IG from sample track were stored in the library of LCsolution software and matched with that of sample track.

2.8.4. Precision and accuracy

The repeatability of measurement ($n = 5$) of peak area of IG was expressed in terms of percent coefficient of variation (R.S.D. %). The intra- and inter-day variations were also evaluated at two concentration levels: 1500 and 2000 ng/spot. The results depicted in Table 2 showed that the method is accurate and precise for the analysis of chemical marker IG in *G. arborea.*

2.8.5. Recovery

The accuracy of quantitation was assessed in a recovery study. For this purpose, *G. arborea* samples of known amounts (3.962 g/kg) of marker compound IG, was extracted with methanol (as per procedure of sample preparation described above Section 2.5) and applied in triplicate onto the plate (2 μL) and individually spiked with the three different amount of IG marker to reach a final content in the lower, middle and upper range of working linear calibration. Two microliters of each and every IG standard solution (0.5, 1.0, 1.5 mg/mL) were used to calculate recovery (Table 3).

2.8.6. Robustness

To test the robustness of the method deliberately small changes in the chromatographic parameters which may affect the performance of the method, i.e. mobile phase composition, plate treatment time, delay between spotting and plate development, plate heating time and delay in digital scanning after derivatization, were made and changes in the peak area of marker compound IG was evaluated. Mobile phase compositions of two solvent, i.e. chloroform and methanol in the ratio of 80:20 (v/v) was tried with variation of 0.5% (v/v) in each solvent. Time gap between spotting to chromatography, from chromatography to scanning and derivatization time (plate heating time) was varied from 0, 10, 30 min. Robustness was performed at two levels, 1500 and 2000 ng/spot. At a time only one parameter was varied while the rest were kept constant. The coefficient of variation (R.S.D. %) of peak areas was calculated for each parameter. The overall low values coefficient of variation as shown in Table 4 indicated the robustness of the method. Quantitation was not significantly effected by changing scanning wavelength ±5 nm.

3. Results and discussion

3.1. Sample preparation, fingerprint and densitometric scanning mode for quantitative HPTLC

The effect of extracting solvents was studied with respect to the percent content of IG in *G. arborea*. Various extracts, viz. *n*-hexane, chloroform, ethyl acetate, methanol and n-butanol were tried and chromatographed to evaluate the extraction efficiency as well as the interferences due to co-eluted compounds. All the sample tracks were scanned at 240 nm (Fig. 2a) and 430 nm wavelength after vanillin–sulphuric acid derivatization (Fig. 2b). It is clearly evident that no interfering compound eluted in the sample tracks to affect the quantitation of the targeted marker IG (Fig. 2a and b) but the extraction efficiency varied. Methanol as extraction solvent was proven to be the most suitable and exhaustive solvent for sample preparation (Table 1). The selection of scanning mode for qualitative analysis, i.e. peak profiling could be performed either by UV or vis after derivatization (Fig. 3a and b). Both the scanning modes provide equal peak purity, which ruled out the possibility of merging of closet components. Also, the spectra of the IG in both standard and sample track are highly correlated (Fig. 4) which confirms that both densitometric modes are equally sensitive and effective. For quantitative analysis of IG in *G. arborea*, densitometric scanning at 430 nm after derivatization is more suitable. Though, the response

Table 4
Robustness testing to access the stability of the HPTLC method ($n = 5$)

Parameters	Coefficient of variance (R.S.D. %) of peak area of marker compound IG
Mobile phase composition	2.89
Plate treatment time	1.43
Time gap between spotting and plate development	0.67
Derivatization time (plate heating time)	1.38
Time gap between derivatization and scanning	1.22

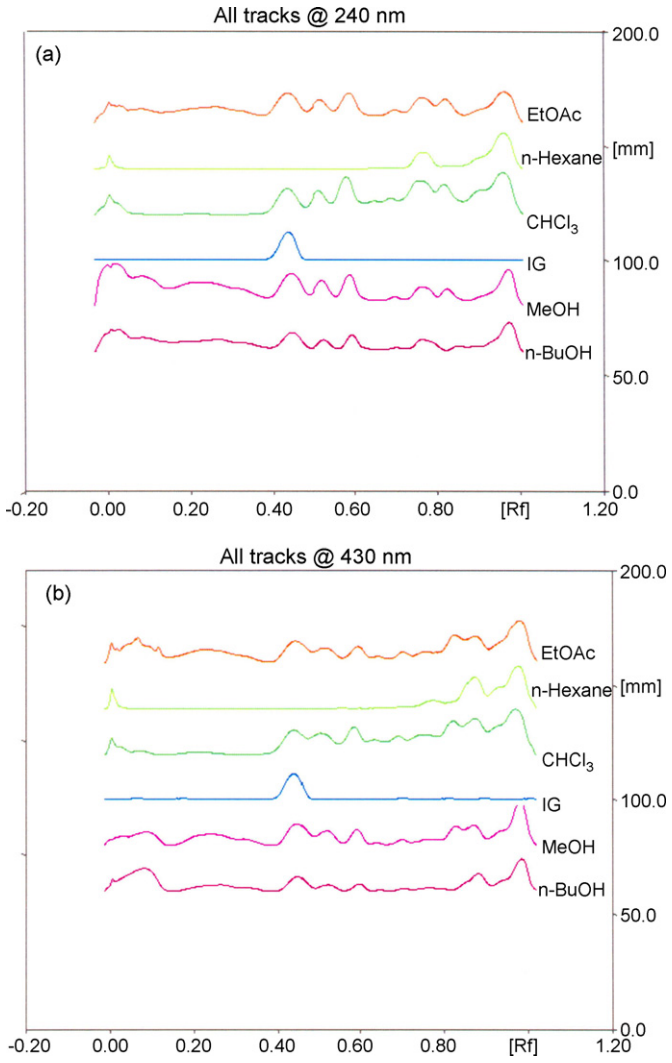

Fig. 2. HPTLC Fingerprint of various extracts of *G. arborea* at (a) 240 nm and (b) 430 nm after derivatization with vanillin–sulphuric acid reagent. The derivatization of TLC plates resulted into increased stability of chemical marker, iridoid glycoside (IG).

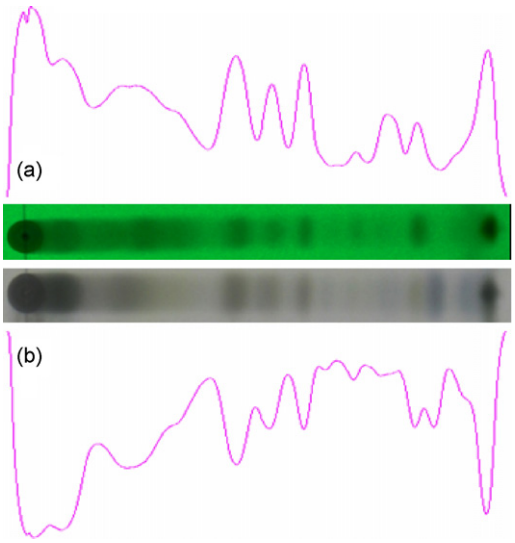

Fig. 3. HPTLC image and digital scanning profile of methanol extract of *G. arborea* at (a) 240 nm and (b) 430 nm after derivatization with vanillin–sulphuric acid reagent.

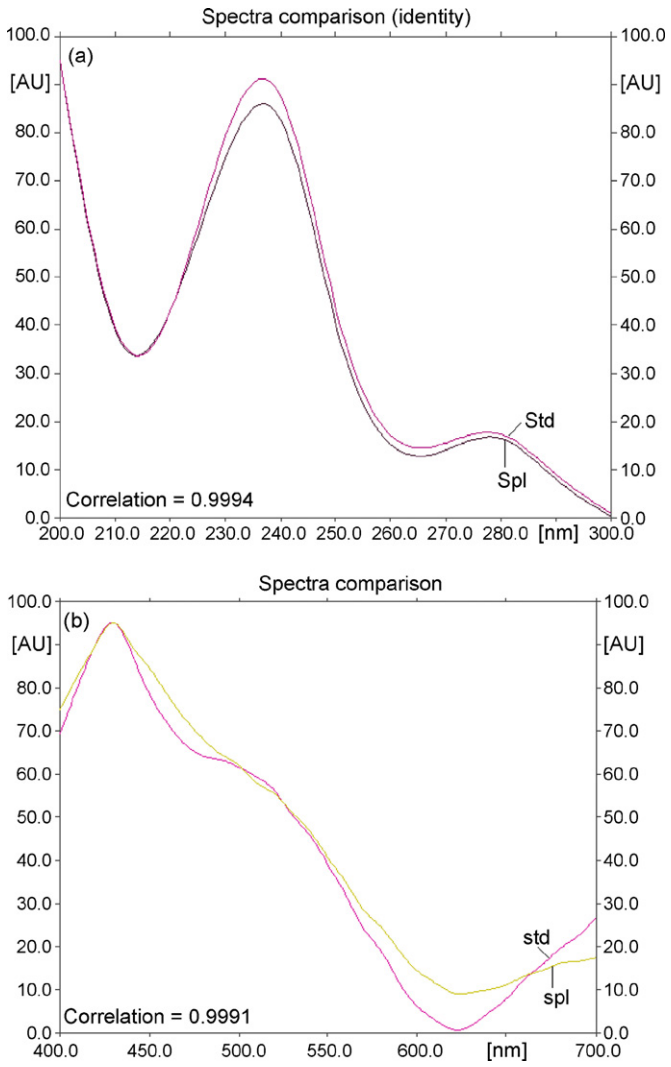

Fig. 4. Overlay spectra of standard IG and sample absorption mode in (a) UV range and (b) vis range post-derivatization with vanillin–sulphuric acid reagent.

of marker compound IG on TLC, i.e. peak area/μg of compound in UV mode is high but with greater variation, which resulted into higher variation in the result (R.S.D. 8.45%). The probable reason may be the unstable nature of iridoid glycoside without derivatization. The attachment of cinnamoyl and rhamnose moieties is labile to transform during plate development and evaporation process. The instability phenomenon of iridoid glycosides was described due to transacylation reaction [30]. Derivatization reagent stabilizes the molecule by blocking the rhamnose hydroxyls and thus preventing the possible transformations [30]. While derivatization step provide greater stability, the analytical sensitivities (LOD and LOQ) of the methods are not significantly different (Table 2).

3.2. Quantitative evaluation of marker in G. arborea

The samples of *G. arborea* from three different sources were collected and analyzed for the chemical marker iridoid glycoside. On the basis of best extraction ability methanol extract was chosen for quantitative analysis. In the densitograms of samples, the well-separated band of reference compound (IG) (R_f 0.44) was observed. No interference of the nearby components was observed in the quantitative analysis of IG. The amount of marker was source dependent and varied (Table 5).

Table 5
Percent content of marker compound IG in the aerial part of *G. arborea* (calculated on plant dry weight basis)

Locations	Marker compound IG (g/kg) in *G. arborea*
1	3.962 ± 0.022
2	3.557 ± 0.024
3	3.176 ± 0.022

4. Conclusion

Thin layer chromatography is a globally accepted rational and practical solution to characterize the crude plant drug, pharmacologically active constituent enriched standardized extracts and their formulations. TLC method on silica gel $60F_{254}$ with chloroform–methanol (80: 20, v/v) and densitometric evaluation at 430 nm after vanillin–sulphuric acid reagent derivatization is simple, specific, precise, accurate and robust for the determination of 6-*O*-(2″,3″-dibenzoyl)-α-L-rhamnopyranosylcatalpol (IG). This standardized TLC procedure may be used effectively for the screening analysis as well as quality evaluation of the plant or its derived herbal products.

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