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Original Paper

Separation and quantification of lignans in *Phyllanthus* species by a simple chiral densitometric method

A sensitive, selective, and robust high-performance TLC (HPTLC) method using chiral TLC plates for qualitative and quantitative analysis of phyllanthin (**A**), hypophyllanthin (**B**), niranthin (**C**), and nirtetralin (**D**), the active lignans of *Phyllanthus* species, was developed and validated. The effectiveness and role of various stationary phases *viz* TLC silica gel $60F_{254}$, HPTLC silica gel $60F_{254}$, and chiral TLC plates in the quantitation were evaluated. A precoated chiral TLC plate was found suitable for the simultaneous analysis of four pharmacologically active lignans. For achieving good separation, the optimized mobile phase of *n*-hexane/acetone/1,4-dioxane (9:1:0.5 by volume) was used (R_f = 0.30, 0.36, 0.41, and 0.48 for compounds **A**, **B**, **C**, and **D**, respectively). A densitometric determination of the above compounds was carried out in reflection/absorption mode at 620 nm. Optimized chromatographic conditions provide well-separated compact bands for the tested lignans. The calibration curves were found linear in the concentration range of 100 – 500 ng/band. Recoveries of **A** – **D** were 99.98, 100.51, 99.22, and 98.74%, respectively. The method was validated according to ICH guidelines. The method reported here is reproducible and applied for the quantitative analysis of the above lignans in the leaves of four *Phyllanthus* species, *i.e.*, *P. amarus*, *P. maderaspatensis*, *P. urinaria*, and *P. virgatus*.

Keywords: Chiral TLC / Hypophyllanthin / Niranthin / Nirtetralin / Phyllanthin

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1 Introduction

Lignans, representing a class of bioactive molecules in a large number of medicinal plants, are considered as an interesting source for lead structures toward new drugs and of interest as drugs in the area of cancer chemotherapy [1]. Their chemistry and biological activity as well as therapeutic potential have been reviewed by many workers [2 – 5].

Commonly distributed species of *Phyllanthus* in India are *P. amarus*, *P. fraternus*, *P. urinaria*, *P. virgartus*, *P. maderaspatensis*, and *P. deblis*. *P. amarus* is highly valued in the treatment of liver ailments [6, 7]. Phyllanthin and hypophyllanthin have been shown to be antihepatotoxic [8]. Nirtetralin and niranthin are reported to possess hepatoprotective and anti-inflammatory activities [9 – 11]. Cytotoxic effect of nirtetralin, niranthin, and phyllanthin on two human leukemia cell lines, K-562 and Lucena-1, suggests a potential action of *Phyllanthus* lignans as multidrug resistance (MDR) reversing agents [12].

Few analytical procedures based on TLC [13 – 17] and high performance chromatography [18 – 22] are reported for the quantitation of two lignans phyllanthin and hypophyllanthin. Recently, the use of high-performance TLC (HPTLC) has gained importance in plant analysis because of its simplicity, low cost, and rapid analytical capabilities [23 – 27]. During careful qualitative analysis, it was observed that in *Phyllanthus* species, mainly *P. amarus*, there were four major peaks of lignans. This led us to isolate four major lignans **A** – **D** by column chromatography followed by preparative HPLC and then structures were confirmed by spectral analysis. Here, a simple, validated TLC separation and simultaneous quantitation of four major isomeric lignans **A** – **D** is reported for the first time. A well-resolved separation was achieved on a chiral TLC plate. The method was applied for the quantitation of these lignans in leaves of different *Phyllanthus* species. Various validation aspects of the analysis such as linearity, specificity, recovery, precision, robustness, LOD, LOQ, *etc.* have been measured.

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Abbreviation: **HPTLC**, high-performance TLC

2 Experimental

2.1 Plant materials

Tested *Phyllanthus* species *viz P. amarus*, *P. maderaspatensis*, *P. urinaria*, and *P. virgatus* were grown in the experimental farm of CIMAP, Lucknow, India. The nursery sowing was done on 3rd July 2006; crop was transplanted in the field on 7th August, 2006. Matured crops of the *Phyllanthus* species were harvested on 16th September, 2006. All the standard agricultural practices were followed during the crop period. Herbarium specimen and seeds of the above test plant species are available in the National Gene Bank of Medicinal and Aromatic Plants at CIMAP, Lucknow.

2.2 Chemicals

All the solvents used were either of analytical or HPLC grade and purchased from E. Merck (Mumbai, India). Before use, the solvents were filtered through a 0.45 μm Millipore membrane (Millipore, Billerica, MA) after sonication for 15 min. Precoated TLC silica gel $60F_{254}$, HPTLC silica gel $60F_{254}$, and chiral TLC glass plates (10×10 cm^2; 20×10 cm^2; E. Merck, Darmastadt, Germany) were used for the optimization of analytical methods. The standard compounds phyllanthin (**A**), hypophyllanthin (**B**), niranthin (**C**), and nirtetralin (**D**) were isolated (all purity >99%) and characterized by spectral analysis (Table 1) in our laboratory.

2.3 Extraction procedure of lignans from *P. amarus*

Air-dried and finely powdered aerial parts of the plant (4.0 kg) were exhaustively extracted at room temperature ($25 \pm 3^{\circ}$C) using ethanol (10 L × 3) by percolation method and the ethanolic extract was concentrated *in vacuo* to give a residue (804 g). Water (200) was added and it was then partitioned with *n*-hexane, chloroform, and *n*-butanol. A part (190 g) of the *n*-hexane soluble fraction (290 g) was subjected to column chromatography on silica gel, 1.5 kg (60–120 mesh). Elution was carried out in varying percentage of ethyl acetate in hexane. Fractions 312–318 and 337–341 eluted with *n*-hexane/ethyl acetate (90:10 v/v) afforded compounds **C** (300 mg) and **D** (200 mg), respectively. Fractions 373–439 of *n*-hexane/ethyl acetate (90:10 v/v and 85:15 v/v) yielded compound **B** (1.60 g), whereas fractions 454–529 of *n*-hexane/ethyl acetate (80:20 v/v) resulted in the compound **A** (6.10 g). Compounds **A** and **B** were purified by the crystallization of chloroform–methanol mixture. Compounds **C** and **D** resisted crystallization and were purified by preparative HPLC: Shimadzu LC-8A; PDA detector, column: 25 cm × 21.2 mm id (Supelcosil™ L-18, 12 μm), mobile solvent: MeOH–water (70:30), flow rate: 15 mL/min,

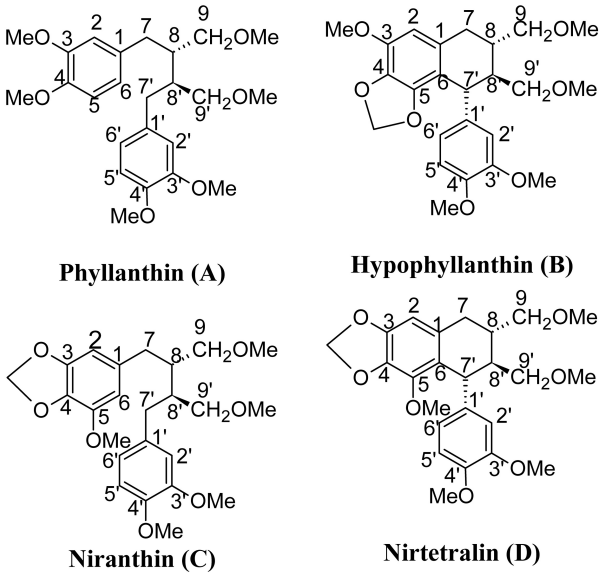


Figure 1. Structures of lignans isolated from *P. amarus*.

detection 220 nm; compound **C** (R_t = 20.18) and compound **D** (R_t = 18.97). Structures of compounds **A**–**D** (Fig. 1) were elucidated by cocomparison of their spectral data to that of reported one [21, 28, 29]. Proton and carbon NMR (Bruker Avance 300 MHz SP Spectrometer) data are presented in Table 1.

2.4 Standard stock solution and sample preparation

Standard stock solutions containing 1.0 mg/mL of pure compounds **A**–**D** were prepared in methanol and filtered through 0.45 μm filters for calibration studies. Plant extracts were prepared from dried and powdered aerial parts (100 mg) of each *Phyllanthus* species. The powder was extracted with methanol (3 × 10 mL; 10 h for extraction), and combined extracts were filtered, dried under vacuum and made up to 1 mL with methanol and filtered through 0.45 μm filter, prior to the HPTLC analysis.

2.5 Apparatus

Automatic TLC Sampler (ATS-4), Vario system, Immersion device III, TLC plate heater, TLC scanner WinCats-III, Reprostar 3 (All CAMAG, Muttenz, Switzerland) were used during the work.

2.6 Chromatographic experiments

Precoated TLC silica gel $60F_{254}$ (Merck Cat no. 1.05729.001), HPTLC silica gel $60F_{254}$ (Merck Cat no. 1.05628.0001), and TLC chiral glass plates (Merck Cat no. 12381) were prewashed by methanol and activated at

Table 1. NMR data of isolated lignans used as reference compounds (**A**–**D**)

	^{1}H-NMR [δ (ppm), multiplicity *J* (Hz)]				^{13}C-NMR [δ (ppm)]			
Position	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
1	–	–	–	–	134.30	132.32	133.74	132.45
2	6.60 (d, 1.8)	6.34 (s)	6.16 (brd s)	6.39 (s)	113.34	107.48	109.27	103.18
3	–	–	–	–	149.49	142.59	143.76	147.87
4	–	–	–	–	147.87	133.88	136.00	135.89
5	6.74 (d, 7.8)	–	–	–	112.13	147.53	147.91	142.37
6	6.63 (dd, 7.8, 1.8)	–	6.12 (brd s)	–	121.60	115.55	121.68	125.15
7	2.63 (m)	2.76–2.82 (m)	2.44–2.53 (m)	2.50–2.70 (m)	35.42	33.57	35.09	33.67
7′	2.63 (m)	4.11 (d, 7.5)	2.44–2.53 (m)	4.29 (d, 6)	35.42	42.17	35.55	41.68
8	2.03 (m)	1.96 (m)	1.89–1.93 (m)	1.90 (m)	41.28	37.31	40.92	37.56
8′	2.03 (m)	1.92 (m)	1.89–1.93 (m)	1.81 (m)	41.28	45.72	40.97	45.62
9	3.23 (m)	3.20–3.46 (m)	3.20 (m)	3.20–3.30 (m)	73.29	76.06	73.15	76.61
9′	3.23 (m)	3.20–3.46 (m)	3.34 (m)	3.20–3.30 (m)	73.29	72.75	73.06	74.32
1′	–	–	–	–	134.30	138.67	134.42	140.25
2′	6.60 (d, 1.8)	6.68 (d, 3)	6.52 (brd s)	6.68 (d, 1.5)	113.34	112.95	113.36	112.93
3′	–	–	–	–	149.49	149.28	149.55	149.17
4′	–	–	–	–	147.87	147.88	149.24	147.54
5′	6.74 (d, 7.8)	6.76 (d, 8)	6.72 (d, 8)	6.70 (d, 8)	112.13	111.73	112.43	111.71
6′	6.63 (dd, 7.8, 1.8)	6.65 (dd, 8, 3)	6.54 (d, 8)	6.55 (dd, 6, 1.5)	121.60	120.94	121.68	120.42
3OMe	3.79 (s)	3.88 (s)	–	–	56.41	56.96	–	–
4OMe	3.83 (s)	–	–	–	56.24	–	–	–
5OMe	–	–	3.71 (s)	3.46 (s)	–	–	56.31	59.36
3′OMe	3.80 (s)	3.82 (s)	3.66 (s)	3.80 (s)	56.41	56.31	55.77	56.35
4′OMe	3.84 (s)	3.86 (s)	3.72 (s)	3.80 (s)	56.24	56.40	55.49	56.41
9OMe	3.32 (s)	3.34 (s)	3.24 (s)	3.27 (s)	58.92	59.14	57.94	59.10
9′OMe	3.32 (s)	3.33 (s)	3.24 (s)	3.32 (s)	58.92	59.14	57.94	59.13
$-O-CH_2-O-$	–	5.64 (d, 1.2) 5.74 (d, 1.2)	5.80 (s)	5.8 (d, 3.0)	–	101.35	101.23	100.92

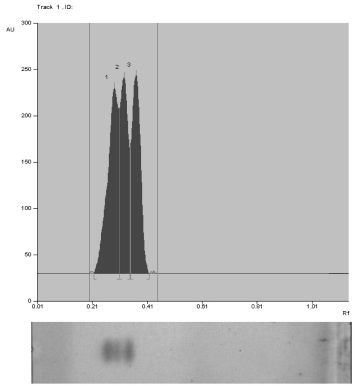
60°C for 5 min, prior to chromatography. Standard and sample solutions were spotted in the form of bands of width 5 mm at 12 mm from both the lower and left edge, and 15 mm space between the two bands, with a microliter syringe using an automatic TLC Sampler, under continuous nitrogen current. A constant application rate of 5 μL/s was employed. Linear ascending development was carried out in twin trough glass chambers 10×10 cm^2/20×20 cm^2 saturated with the mobile phase. The mobile phase selection was carried out using the Vario System wherein different compositions consisting of different ratios of solvents of varying polarity with all three stationary phases were tried. Finally, a mobile phase consisting of *n*-hexane/acetone/1,4-dioxane (9:1:0.5 by volume) and chiral plate as stationary phase was optimized for quantitative chromatography. A poor resolution of isomeric compounds **A**–**D** was observed both on TLC as well as HPTLC plates (Fig. 2a and b). A satisfactory separation of the four targeted lignans (Fig. 2c) was achieved by using a chiral plate. The saturation time of the TLC chamber in the mobile phase was optimized to 5 min for a better resolution of the four tested lignans. Detection of the spots was carried out by dipping the chromatographic plates in a freshly prepared vanillin/sulfuric acid/ethanol (1 g:5 mL:95 mL) reagent using an Immersion device and subsequent heating at 110°C for 15 min on the TLC plate heater. TLC Scanner-III fitted with winCATS 1.3.3 software was applied for quantitative evaluation. TLC plates were scanned at different wavelengths to select the scanning wavelength 620 nm for all the test compounds **A**–**D**, a wavelength chosen to be appropriate for the simultaneous quantitation of all the four test compounds **A**–**D** after derivatization. The densitometry scanning was performed in the absorbance/reflectance mode at 620 nm, slit width 6.00×0.40 mm^2, scanning speed 20 mm/s, and data resolution 10 μm/step. For the recording of characteristic spectra of derivatized band of compounds **A**–**D** in the range of 400–800 nm, a tungsten lamp was used. Reprostar 3 with cabinet cover and mounted digital camera was used for imaging and archiving the thin layer chromatograms. Concentrations of the compounds chromatographed were determined from the intensity of the reflected light. Evaluation was *via* peak areas with linear regression.

3 Results and discussion

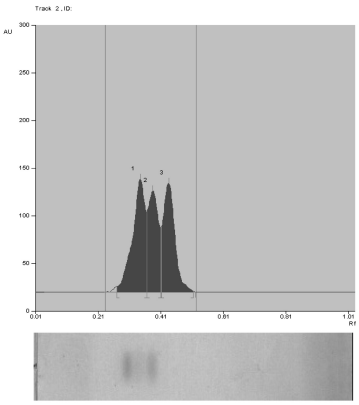
3.1 Separation of four lignans of *P. amarus* by classical TLC, HPTLC, and chiral TLC

Earlier reported HPTLC methods [13, 14, 16] gave a poor resolution of all the tested lignans **A**–**D**. A satisfactory

a. TLC Silica Gel 60F_{254} Plate



b. HPTLC Silica Gel 60F_{254} Plate



c. Chiral TLC Plate

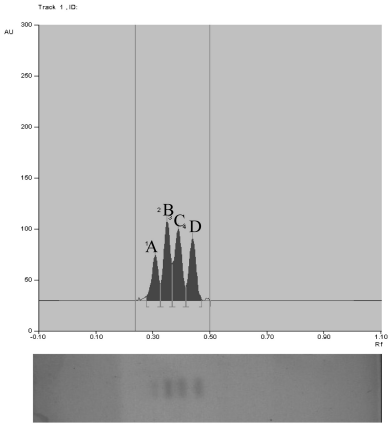


Figure 2. Separation of artificial mixture of four lignans (**A**) phyllanthin, (**B**) hypophyllanthin, (**C**) niranthin, and (**D**) nirtetralin on various stationary phases.

separation of the four major lignans **A**–**D** could not be achieved by classical TLC as well as HPTLC plates using *n*-hexane, cyclohexane, *n*-heptane, toluene, *etc*. as the basic components and ethyl acetate, acetone, and methanol in the capacity of the modifier of polarity and acetic and formic acids in the capacity of a sorbent modifier. The assimilation of peaks of **A**–**B** and **B**–**C** with TLC while **B**–**C** and **C**–**D** with HPTLC, was observed when the four

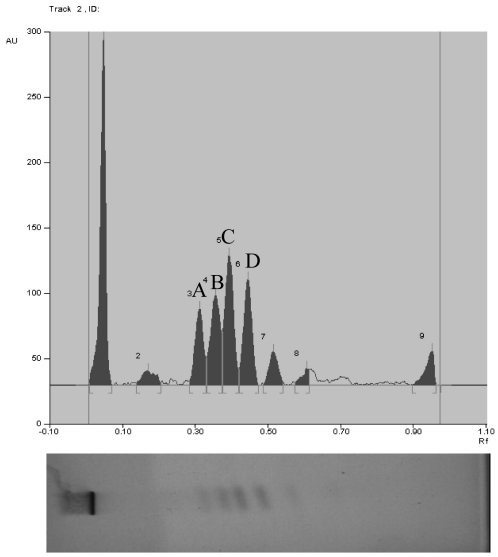


Figure 3. Separation of four lignans **A**–**D** in *P. amarus* extract on a chiral plate.

chemical markers (**A**–**D**) with a mobile phase consisting of *n*-hexane/acetone/1,4-dioxane (9:1:0.5 by volume) (Fig. 2a and b) were tried to separate. The same mobile phase composition gave a better resolution of all the four markers **A**–**D** (Fig. 2c) on a chiral plate in the studied concentration range. The suitability of chiral stationary phase and the mobile phase composition as mentioned above was further confirmed in the separation of lignans in *P. amarus* extract (Fig. 3). The identity of bands of **A**–**D** in the *Phyllanthus* extracts was confirmed by overlaying their absorption spectra with those of the standards (Fig. 4) as well as coincidence of R_f values (Fig. 5). A well-resolved separation of tested lignans was also obtained in the plant extract.

3.2 Method validation

3.2.1 Linearity and quantification

Working stock solutions were prepared by dilution to give solutions containing reference lignans **A**–**D** in the concentrations of 50, 62.5, 83.3, 125, and 250 μg/mL. Two microliters of each standard solution was spotted on the chiral TLC plate to obtain final concentrations of 100, 135, 166, 250, and 500 ng/spot. Each concentration was spotted thrice on the chiral TLC plate. The calibration curves were linear in the concentration range of 100–500 ng/spot with correlation coefficients (r^2) = 0.998, 0.997, 0.997, and 0.998 for **A**, **B**, **C**, and **D**, respectively. The regression data obtained showed a good linear relationship (Table 2).

Two microliters of each sample solution from different locations were taken and each one of them was applied on the chiral plate in triplicate with a similar band pat-

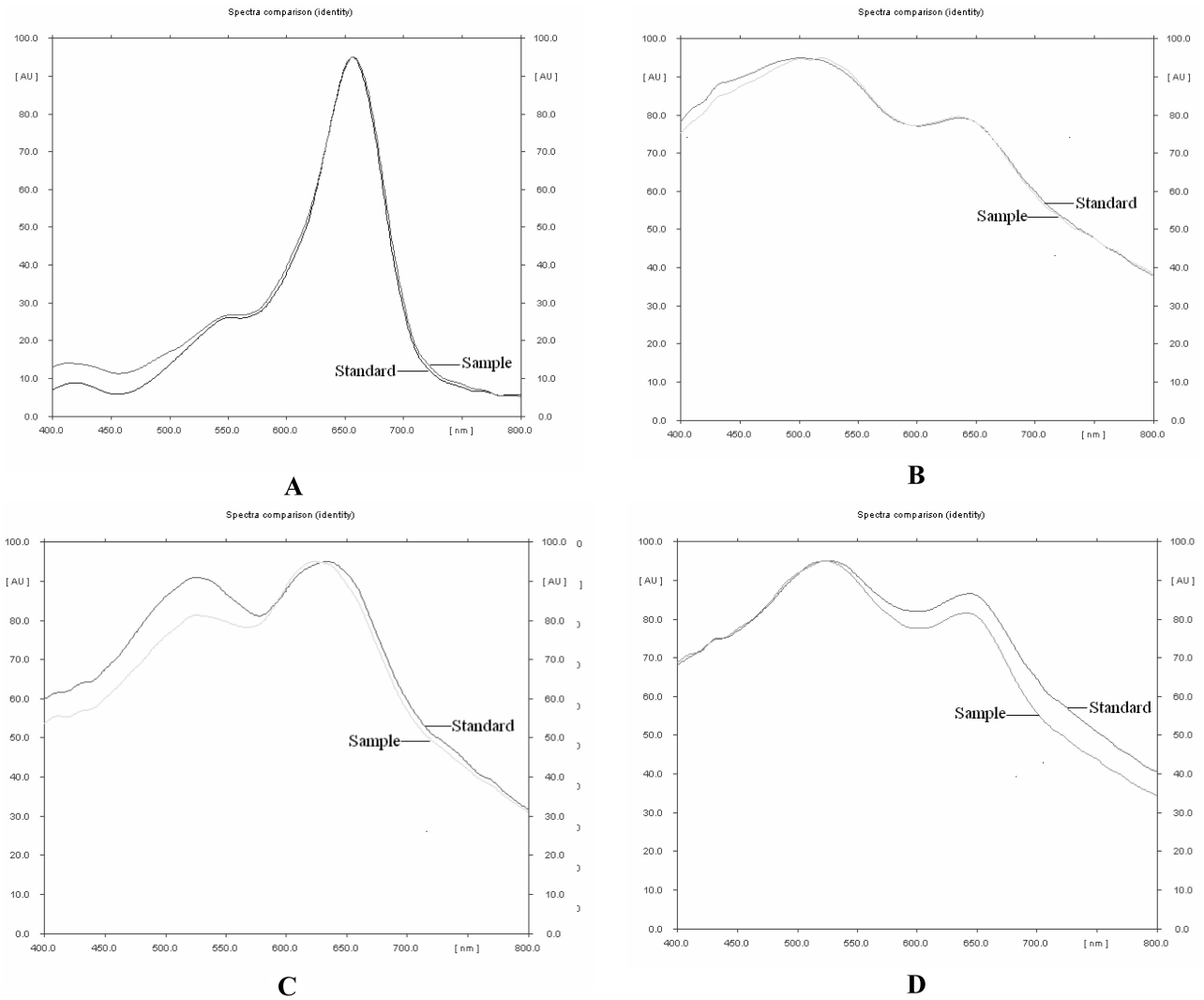


Figure 4. Overlay spectra after derivatization of reference lignans **(A)** phyllanthin, (**B**) hypophylanthin, (**C**) niranthin, and (**D**) nirtetralin along with **A**–**D** in the sample extract of *P. amarus*.

tern. The experimental parameters were identical for all the above analysis. The percent content of analytes in the sample was calculated on the basis of calibration curves.

3.2.2 LOD and LOQ

The LOD and the LOQ were calculated for **A**–**D** on the basis of three and ten times the noise level, respectively. The LOD values were found to be 26.55, 45.22, 53.49, and 56.78 ng/band for compounds **A**, **B**, **C**, and **D**, respectively, whereas LOQ values were 87.61, 135.66, 176.51, and 187.39 ng/band, respectively (Table 2).

3.2.3 Specificity

The specificity of the method was ascertained by analyzing standards and sample. The bands for the four tested lignans **A**–**D** in samples were confirmed by comparing the R_f and spectra of the spot with that of standard. The peak purity of individual **A**, **B**, **C**, and **D** was assessed by comparing the spectra at peak start, peak apex, and peak end positions of the band, *i.e.*, r (start, middle) = 0.9991, 0.9993, 0.9981, 0.9993 and r (middle, end) = 0.9998, 0.9989, 0.9989, 0.9993, respectively. Good correlation was also obtained between standards and sample overlain spectra (>0.99) (Table 3).

3.2.4 Recovery

Recovery studies of the analytes in the sample were carried out to assess the accuracy of the method. For this purpose, three different spiking concentrations of the standard stock solutions of compounds **A**–**D** were prepared. Three sets were prepared from a preanalyzed sample, *i.e.*, location 1 (1 mL each of concentration 100 mg/

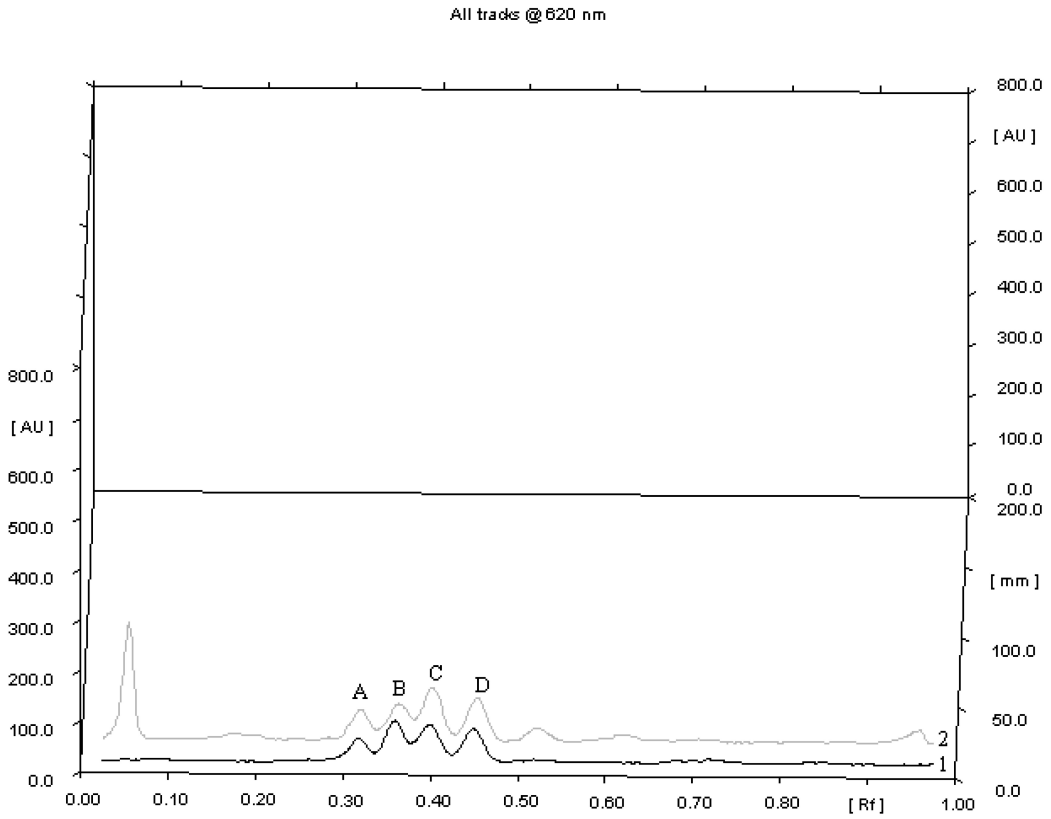


Figure 5. Representative chiral TLC chromatogram showing separation of compounds; track 1: artificial mixture of the four lignans phyllanthin (**A**), hypophyllanthin, (**B**) niranthin (**C**), and nirtetralin (**D**) (1.0 mg/mL); track 2: methanolic extract of *P. amarus* (100 mg/mL).

Table 2. Calibration data, R_f and sensitivities for *P. amarus* lignans using chiral TLC plate

Compounds	R_f	Linearity range (μg/mL)	Regression equation	Correlation coefficient (r^2)	RSD (%)	LOD (ng)	LOQ (ng)
Phyllanthin (**A**)	0.30	50–250	$Y = 8.9123X + 68.31$	0.998	0.74	26.55	87.61
Hypophyllanthin (**B**)	0.36	50–250	$Y = 17.347X - 138.79$	0.997	0.79	45.22	135.66
Niranthin (**C**)	0.41	50–250	$Y = 14.866X - 66.605$	0.997	0.89	53.49	176.51
Nirtetralin (**D**)	0.48	50–250	$Y = 15.680X + 5.849$	0.997	0.67	56.78	187.39

X, Amount of compound (ng/band); *Y*, peak area in AU.

Table 3. Peak purity test for lignans **A**–**D**

Compounds	r (s, m)[a]		r (m, e)[b]	
	Standard track	Sample track	Standard track	Sample track
Phyllanthin (**A**)	0.9997	0.9991	0.9999	0.9998
Hypophyllanthin (**B**)	0.9998	0.9993	0.9999	0.9989
Niranthin (**C**)	0.9999	0.9981	0.9999	0.9989
Nirtetralin (**D**)	0.9999	0.9993	0.9999	0.9993

[a] Correlation of spectrum at the start of peak with spectrum at the center of peak.

[b] Correlation of spectrum at the center of peak with spectrum at the end of peak.

mL). Then, these three sets were individually spiked with three different spiking concentrations of the individual standard solutions (in the range of 63, 83, and 125 μg/mL). The chiral TLC procedure of these spiked samples was as described in Section 2.7. The average recovery for compounds **A**–**D** was found to be 99.98, 100.51, 99.22, and 98.74%, respectively, within the acceptable RSD% (Table 4).

3.2.5 Precision

The repeatabilities of measurement ($n = 5$) of peak area for active compounds **A**–**D** were expressed in terms of percent CV (%RSD). The intra and interday variation for the determination of **A**–**D** was carried out at three differ-

Table 4. Recovery study to evaluate accuracy of the method

Compounds	Amount of compounds in sample (μg/mL)	Spiked amount (μg/mL)	Theoretical value (μg/mL)	Experimental value[a)] (μg/mL) (mean SD)	Recovery (%)	Average recovery	RSD (%)
Phyllanthin (**A**)	87	63	150	149.97 ± 1.94	99.98	99.98	0.43
		83	170	169.23 ± 1.60	99.54		
		125	212	212.87 ± 1.34	100.41		
Hypophyllanthin (**B**)	73	63	136	136.09 ± 0.59	100.06	100.51	0.70
		83	156	156.20 ± 0.03	100.13		
		125	198	200.62 ± 0.94	101.32		
Niranthin (**C**)	113	63	173	173.07 ± 0.55	100.04	99.22	0.72
		83	196	193.57 ± 1.16	98.57		
		125	238	235.26 ± 1.83	98.84		
Nirtetralin (**D**)	80	63	143	141.98 ± 0.14	99.29	98.74	0.63
		83	163	161.13 ± 1.20	98.85		
		125	205	201.04 ± 3.54	98.07		

a) $n = 3$.

Table 5. Inter- and intraday precision of lignan assay by chiral TLC

Compound	Concentration (ng/band)	Precision					
		Instrumental		Intraday[a)]		Interday[a)]	
		RSD (%)	Mean RSD (%)	RSD (%)	Mean RSD (%)	RSD (%)	Mean RSD (%)
Phyllanthin (**A**)	100	0.61	0.74	1.22	1.35	1.67	1.65
	250	0.72		1.38		1.65	
	500	0.88		1.44		1.63	
Hypophyllanthin (**B**)	100	0.88	0.92	1.39	1.53	1.83	1.88
	250	0.77		1.58		1.88	
	500	1.12		1.61		1.93	
Niranthin (**C**)	100	0.84	0.89	1.09	1.24	1.77	1.90
	250	0.92		1.33		2.05	
	500	0.92		1.29		1.87	
Nirtetralin (**D**)	100	0.77	0.77	0.87	0.84	1.28	1.61
	250	0.66		0.79		1.88	
	500	0.89		0.86		1.67	

a) $n = 5$.

ent concentration levels: 100, 250, 500 ng/band. The repeatability was found to be 0.74, 0.92, 0.89, and 0.77 for compounds **A**, **B**, **C**, and **D**, respectively. The results depicted in Table 5 showed that no significant intra- and interday variation was observed in the analysis of the compounds **A**–**D**.

3.2.6 Robustness

The robustness of the method was determined by introducing small changes in certain chromatographic parameters. Mobile phase having *n*-hexane/acetone/1,4-dioxane (9:1:0.5 by volume) was tried with a variation of 0.5% v/v in each solvent. The amount of mobile phase, temperature, and the relative humidity was varied in the range of ±5%. The time gap between spotting to chromatography, from chromatography to scanning and derivatization time (plate heating time) was varied from 0, 30, 60 min. The robustness was performed at three levels; 50, 100, and 250 ng/band for compounds **A**–**D**, respectively. At a time only one parameter was varied while the rest were kept constant. The effects on the results, *i.e.*, peak areas were examined. The SD of peak areas was calculated for each parameter and %RSD was found to be less than 2% in each case. The low values of %RSD as shown in Table 6 indicated the robustness of the method. The separation was not effected by changing the scanning wavelength (5 nm).

3.3 Quantitative evaluation of extracts

Three different samples of four *Phyllanthus* species, *i.e.*, *P. amarus*, *P. maderaspatensis*, *P. urinaria*, and *P. virgatus* were analyzed for active lignans. Four well-separated bands of phyllanthin ($R_f = 0.30$), hypophyllanthin ($R_f = 0.36$), nir-

Table 6. Robustness testing to access the stability of the chiral TLC densitometric method ($n = 6$)

Parameters	RSD (%) of peak area			
	Phyllanthin (**A**)	Hypophyllanthin (**B**)	Niranthin (**C**)	Nirtetralin (**D**)
Mobile phase composition	1.77	1.67	1.15	1.18
Amount of mobile phase	1.02	1.18	0.92	1.06
Plate treatment	0.55	0.37	0.29	0.19
Temperature	1.46	2.01	1.98	1.64
Humidity	1.73	1.88	1.92	1.38
Time gap between spotting and plate development	0.46	0.89	0.55	0.74
Derivatization time (plate heating time)	1.69	1.28	1.16	1.27
Time gap between derivatization and scanning	1.47	1.20	1.91	1.82

Table 7. Applicability of the developed method for the determination of lignans in the leaves of different *Phyllanthus* species

Species/sample	Contents (% w/w)			
	Phyllanthin (**A**)	Hypophyllanthin (**B**)	Niranthin (**C**)	Nirtetralin (**D**)
P. amarus				
1	0.517 ± 0.026	0.383 ± 0.019	0.214 ± 0.011	0.306 ± 0.016
2	0.217 ± 0.011	0.183 ± 0.011	0.284 ± 0.012	0.200 ± 0.010
3	0.343 ± 0.017	0.178 ± 0.009	0.116 ± 0.006	0.188 ± 0.009
P. maderaspatensis				
1	0.011 ± 0.002	0.011 ± 0.001	nd	nd
2	0.008 ± 0.002	0.012 ± 0.002	nd	nd
3	0.008 ± 0.002	0.013 ± 0.002	nd	nd
P. urinaria				
1	nd	nd	nd	nd
2	nd	nd	nd	nd
3	nd	nd	nd	nd
P. virgatus				
1	0.018 ± 0.002	0.014 ± 0.002	nd	nd
2	0.011 ± 0.002	0.008 ± 0.002	nd	nd
3	0.017 ± 0.002	0.012 ± 0.002	nd	nd

nd – Not detected.

anthin ($R_f = 0.41$), and nirtetralin ($R_f = 0.48$) were observed in the densitogram of the *Phyllanthus* extract. There was no interference in the analysis of **A**–**D** from the other unknown components present in the extracts. The results are presented in Table 7. The percent content of an individual lignan **A**, **B**, **C**, and **D** was species as well as the source dependent with maximum concentration in *P. amarus*.

4 Concluding remarks

TLC is a globally accepted practical solution to characterize the raw herbs, active constituent enriched extracts, and their formulation. The present chiral TLC densitometric method is precise, specific, accurate, and robust for the simultaneous determination of four important lignans of *Phyllanthus* species. The method was found to be repeatable and specific. Thus, the standardized chiral TLC procedure may be used effectively for the screening analysis or quality evaluation of the plant or its derived products.

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