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Quantitative Determination of Phyllanthin and Hypophyllanthin in *Phyllanthus* Species by High-performance Thin Layer Chromatography

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Abstract: A simple, precise and rapid high-performance thin-layer chromatographic method has been developed for the estimation of phyllanthin (**1**) and hypophyllanthin (**2**), the important lignans of *Phyllanthus* species, especially *Phyllanthus amarus*. Separation of **1** and **2** was carried out on silica gel 60 F_{254} layers eluted with hexane:acetone:ethyl acetate (74:12:8), and the analytes were visualised through colour development with vanillin in concentrated sulphuric acid and ethanol. Scanning and quantification of spots was performed at 580 nm. Recoveries of **1** and **2** were 98.7 and 97.3%, respectively. The method was validated and the peak purities and limits of detection and quantification were determined. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: HPTLC; HPLC; quantitative determination; lignans; phyllanthin; hypophyllanthin; *Phyllanthus* species.

INTRODUCTION

The genus *Phyllanthus* (Euphorbiaceae) contains 550–750 species in 10–11 subgenera that are distributed in all tropical regions of the world from Africa to Asia, South America and the West Indies. *Phyllanthus amarus* is the most widespread species and is typically to be found along roads and valleys, and on riverbanks and near lakes in tropical areas. Other species found in India are *P. fraternus*, *P. urinaria*, *P. virgatus*, *P. maderaspatensis* and *P. debilis*. The genus *Phyllanthus* has a long history of use in the treatment of diabetes, intestinal parasites and liver, kidney and bladder problems (Kirtikar and Basu, 1933; Nadkarni, 1976). *P. amarus* is highly valued in the treatment of liver ailments and kidney stones and has been shown to posses anti-hepatitis B virus surface antigen activity in both *in vivo* and *in vitro* studies (Calixto *et al.*, 1998; Khatoon *et al.*, 2006).

The major lignans of the genus, namely, phyllanthin (**1**) and hypophyllanthin (**2**), have been shown to be anti-hepatotoxic against carbon tetrachloride- and galactosamine-induced hepatotoxicity in primary cultured rat hepatocytes (Syamsunder *et al.*, 1985). Thus, an appropriate analytical procedure for the quantitative determination of these lignans in different *Phyllanthus* species is of considerable importance. Several analytical procedures involving HPLC (Sharma *et al.*, 1993; Wang and Lee, 2005) and HPTLC (Deb and Mandal, 1996; Sane *et al.*, 1997) have been described.

Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity (Pothier, 1996; Gupta *et al.*, 1996, 1998, 1999a,b; Gupta and Verma, 1996; Saxena *et al.*, 2000; Srivastava *et al.*, 2000). However, the HPTLC methods for the quantification of **1** and **2** that are currently available have not been validated and also suffer from poor resolution of **2** from other lignans. In the present paper we report an HPTLC method that provides good resolution of the peaks associated with **1** and **2** from those of closely related compounds in different *Phyllanthus* species. Various validation aspects of the analysis, namely peak purity, recovery and the limits of detection and quantification etc., have been measured.

EXPERIMENTAL

Materials. Reagents and prepared TLC plates were purchased from Merck (Darmstadt, Germany). Phyllanthin (**1**) and hypophyllanthin (**2**) were isolated in our laboratory and their identities confirmed by comparison with the authentic standards kindly provided by Professor Wagner, Center of Pharma-Research, Pharmaceutical Biology, University of Munich, Munich, Germany. *Phyllanthus amarus* (CIMAP-1421), *P. urinaria* (CIMAP-1424), *P. fraternus* (CIMAP-1426), *P. maderaspatensis* (CIMAP-1425), *P. debilis* (CIMAP-1428) and *P. virgatus* (CIMAP-1427) were grown at the experimental farm at CIMAP, Lucknow, India, during April 2005 and voucher specimens have been deposited in the gene bank of the institute. Mature leaves (90 days after transplanting) of

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the different species were collected as pooled samples from several plants of the same species (with the same accession number).

Extraction procedure. Air-dried leaves (1 g) of *P. amarus* were separately extracted with either hexane, chloroform, ethyl acetate or methanol. In each case, the extraction was carried out three times with 10 mL of solvent for 10 h at room temperature (25 ± 5°C), and the solvent was removed from the combined extract under reduced pressure to yield the respective crude residue. In order to determine the appropriate extraction solvent, each of the crude residues was dissolved separately in 1 mL of methanol and the contents of the two lignans in each sample was determined by HPLC. The maximum content of lignans **1** and **2** was obtained by extracting with methanol (Table 1).

Analytical procedure. Air-dried leaves (1 g) of different *Phyllanthus* species were extracted separately at room temperature (25 ± 5°C) with methanol (3 × 10 mL; 10 h for each extraction), and the combined extracts were filtered, dried under vacuum and made up to 1 mL with methanol prior to HPTLC analysis.

HPTLC analysis. Chromatography was performed using commercially-prepared, pre-activated (110°C) silica gel 60 F_{254} TLC plates (either 20 × 5 cm or 20 × 20 cm). A Linomat IV (Camag, Muttenz, Switzerland) automatic TLC applicator was used to apply samples and standards onto the TLC plate under a flow of nitrogen gas. The application parameters were identical for all the analysis performed and the delivery speed of the syringe was 10 s/μL. Each TLC plate was developed to a height of about 10 cm with a mobile phase of hexane:acetone:ethyl acetate (74:12:8, v/v/v) under laboratory conditions (25–30°C and 40–50% relative humidity).

Table 1 Effect of solvent on the extraction of phyllanthin (1) and hypophyllanthin (2) from the leaves of *Phyllanthus amarus*

	Amount of compound determined[a] (percentage of dry weight)	
Extraction solvent	**1**	**2**
Hexane	0.08	0.02
Chloroform	0.42	0.22
Ethyl acetate	0.40	0.20
Methanol	0.53	0.32

[a] Mean values (n = 3).

Developed plates were dried in a stream of air and then immersed in a freshly prepared mixture of vanillin (1 g) in 100 mL of concentrated sulphuric acid:ethanol (5:95, v/v). After drying, the plates were heated at 110°C for 25 min to develop the colour of the spots. For quantitative determination, spots corresponding to **1** and **2** were scanned using a Camag TLC Scanner 3 at 580 nm (wavelength chosen to be appropriate for both **1** and **2** after staining) with a slit size of 6 × 0.4 mm.

Calibration. Stock solutions (1 mg/mL) of standard compounds **1** and **2** were prepared individually in methanol and different concentrations were spotted onto TLC plates in order to prepare the calibration graphs.

HPLC analysis. HPLC analysis was performed on a Shimadzu (Tokyo, Japan) model LC-10 A instrument equipped with a Shimadzu SPD-M10 A_{VP} photodiode array detector (PAD) in order to determine peak purity and similarity test of lignans. HPLC grade solvents (Merck, Darmstadt, Germany) were pre-filtered using a Millipore (Billerica, Massachusetts, USA) system and analysis was performed on a Waters (Milford, Massachusetts, USA) C_{18} Spherisorb S10 ODS_2 (250 × 4.6 mm i.d.; 10 μm) column. The mobile phase was methanol:water (70:30) at a flow rate of 0.7 mL/min; the detection wavelength was 220 nm, which was close to the absorption maxima for both compounds. The injection volume for standards and samples was 10 μL, and **1** and **2** were eluted at retention times of 20.13 and 17.70 min, respectively. Validation data for the HPLC method were: phyllanthin, precision CV 1.6%; recovery 100.4%; linearity, correlation coefficient 1.000; hypophyllanthin, precision CV 2.4%; recovery, 98.8%; linearity, correlation coefficient 0.9999.

RESULTS AND DISCUSSION

The HPTLC method reported by Deb and Mandal (1996) suffers from poor resolution of phyllanthin (**1**) and hypophyllanthin (**2**) from other closely related compounds. Whilst the mobile phase reported by Sane *et al.* (1997) gave resolved peaks of **1** and **2**, the content of the latter (0.858%) was reportedly higher than that of the former (0.709%). During our HPLC study of the variation of phyllanthin and hypophyllanthin in samples of *Phyllanthus* species collected from different geographical regions, none of the samples was found to contain higher concentrations of **2** compared with **1**. This led us to examine the reported TLC method more carefully. It was observed that the reported higher concentration of **2** was due to other lignans present at the same R_f as that of hypophyllanthin. Therefore, a number of mobile phases of different compositions were tried for TLC. The phase finally chosen, namely,

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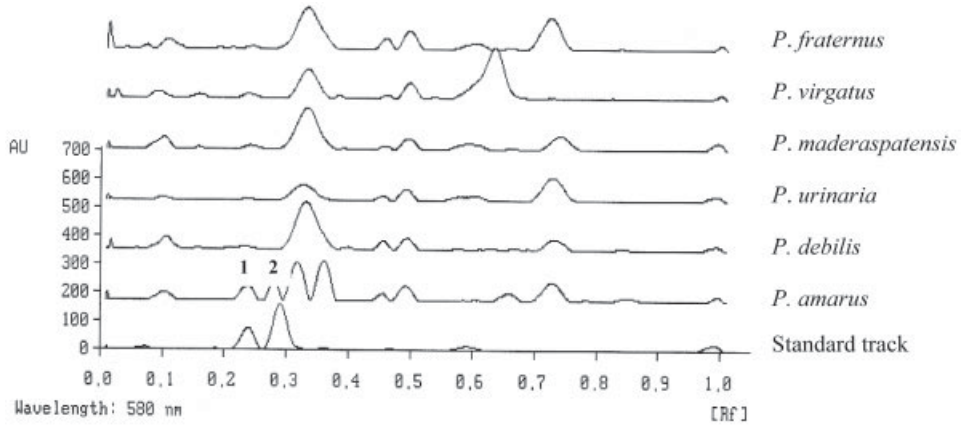

Figure 1 The HPTLC separation of phyllanthin (**1**) and hypophyllanthin (**2**) in extracts of *Phyllanthus* species and in the standard track.

Table 2 Peak purity test for compounds 1 and 2

	r (S, M)[a]		*r* (M, E)[b]	
Compound	Standard track	Sample track	Standard track	Sample track
Phyllanthin (1)	0.999	0.999	0.999	0.999
Hypophyllanthin (2)	0.999	0.999	0.999	0.999

[a] *r* (S, M) = correlation of spectrum at start of peak with spectrum at the centre of peak.
[b] *r* (M, E) = correlation of spectrum at centre of peak with spectrum at end of peak.

Table 3 Calibration data for compounds 1 and 2

	Linear regression	
Compound	Equation	*r*-Value
Phyllanthin (1)	$y = 1457\,x + 69071$	0.995
Hypophyllanthin (2)	$y = 2052.8\,x + 959.86$	0.996

hexane:acetone:ethyl acetate (74:12:8, v/v/v) gave good resolution of phyllanthin (**1**) and hypophyllanthin (**2**) from other closely related lignans (Fig. 1). The reagent used for colour development gave intense blue colours with **1** and **2** at R_f values of 0.24 and 0.29, respectively. Scanning of the TLC plates was performed at 580 nm in the absorption-reflection mode. The selected mobile phase produced symmetrical peaks with good resolution (Fig. 1) for lignans **1** and **2**. Peak purity tests were carried out by comparing the spectra of the two lignans in both standard and sample tracks (Table 2). The identity of the peaks was checked in the samples by addition of the respective standard. The calibration curves were found to be linear in the working range of 2–20 μg (Table 3). The recovery percentages of **1** and **2** were 98 and 97% (Table 4), respectively, as calculated by addition of known amounts of pure compounds to the plant extract. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated for **1** and **2** on the basis of three- and 10-times the noise level, respectively. The LOD values were found to be 0.18 and 0.20 μg/mL, respectively, whereas the LOQ values were 0.59 and 0.66 μg/mL, respectively. Results of five determinations obtained by HPTLC analysis were comparable with those obtained by HPLC (Table 5).

The HPTLC chromatograms of extracts of the species *P. fraternus*, *P. virgatus*, *P. maderaspatensis*, *P. urinaria*, *P. debilis* and *P. amarus* are shown in Fig. 1. Only *P. amarus* was found to be a good source of both phyllanthin (**1**) and hypophyllanthin (**2**). Low concentrations of **1** were detected in samples of *P. fraternus*, *P. maderaspatensis* and *P. virgatus*. With the exception of *P. amarus*, compound **2** was not detected in any of the species analysed.

The HPTLC method reported here is suitable for the rapid screening of germplasm of *Phyllanthus* species for the determination of chemical profiles and quantification of the major lignans. The assay can also be applied without any special pre-treatment of the sample, and a large number of samples can be analysed in a single run without compromising accuracy.

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Table 4 Percentage recovery, R_f value and LOD/ LOQ of compounds 1 and 2 by HPTLC

Compound	Recovery (%) ± standard deviation	R_f	LOD (μg/mL)	LOQ (μg/mL)
Phyllanthin (**1**)	98.70 ± 0.44	0.24	0.18	0.59
Hypophyllanthin (**2**)	97.33 ± 1.01	0.29	0.20	0.66

Table 5 Comparison of results obtained by HPLC and HPTLC

	Content (%)			
	Phyllanthin		Hypophyllanthin	
Species	HPLC	HPTLC	HPLC	HPTLC
P. amarus	0.53	0.52	0.32	0.31
P. fraternus	0.02	0.02	nd[a]	nd
P. urinaria	nd	nd	nd	nd
P. maderaspatensis	0.01	0.01	nd	nd
P. virgatus	0.02	0.02	nd	nd
P. debilis	nd	nd	nd	nd

[a] nd = not detected.

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