DIETARY SUPPLEMENTS

High-Performance Thin-Layer Chromatography Densitometric Method for Simultaneous Quantitation of Phyllanthin, Hypophyllanthin, Gallic Acid, and Ellagic Acid in *Phyllanthus amarus*

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Whole plant of *Phyllanthus amarus* Linn. is a reputed drug of the Indian systems of medicine that is used as hepatoprotective agent. A simple high-performance thin-layer chromatography (HPTLC) densitometric method has been developed for the simultaneous quantitation of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid in the whole plant of *P. amarus*. They were found at levels of 0.37, 1.16, 0.36, and 0.17% (w/w), respectively. The method was validated for precision, repeatability, and accuracy. Instrumental precision was found to be 0.54, 0.93, 0.08, and 0.78% (coefficient of variation, CV); repeatability of the method was 1.01, 0.79, 0.98, and 1.06% (CV) for phyllanthin, hypophyllanthin, gallic acid, and ellagic acid, respectively. Accuracy of the method was determined by a recovery study conducted at 3 different levels, and the average recovery was found to be 99.09% for phyllanthin, 99.27% for hypophyllanthin, 98.69% for gallic acid, and 100.49% for ellagic acid. The proposed HPTLC method was found to be simple, precise, specific, sensitive, and accurate and can be used for routine quality control of raw material of *P. amarus* and formulations containing *P. amarus*. It also has the applicability in quantitating any of these marker compounds in other drugs.

Phyllanthus amarus Linn. (Euphorbiaceae) is an annual herb, growing as a weed throughout India, commonly known as Jamgli amli, Jaramla, or Bhuiamla. Traditionally it is useful in treatment of dropsy, jaundice, diarrhea, dysentery, intermittent fever, diseases of the urinogenital system, scabies, ulcers, and wounds (1). Some of the major chemical constituents of *P. amarus* include phyllanthin, hypophyllanthin, hydrolyzable tannins, phyllanthusiin D, amariin, amarulone (2), amarinic acid, gallic acid, ellagic acid (3), and phyllnirurin (4, 5). *P. amarus* whole plant powder administered at a dosage of 0.66 g/kg in rat showed hepatoprotective activity against CCl_4 induced liver damage (6). *P. amarus* crude extract produced in vitro inactivation of hepatitis B surface antigen (HBsAg; 7). The beneficial use in the treatment of acute and chronic hepatitis B patients was also proved at the cellular level using an aqueous extract of *P. amarus* on human hepatocellular carcinoma-derived cells at 1 mg/mL concentration in a single dose, where inhibition of the secretion of HBsAg for a period of 48 h was observed (8, 9). Phyllanthin and hypophyllanthin have been reported to exhibit antihepatotoxic activity in primary cultured rat hepatocytes (10). Aqueous extract of the leaves of *P. amarus* lowered blood glucose level in normal and alloxan induced diabetic rabbits (11). A clinical trial conducted on mild hypertensive patients indicated that *P. amarus* is a potential diuretic hypotensive and hypoglycemic drug for humans (12). Phenolic compounds of natural origin, including gallic acid and ellagic acid, have been shown to have significant biological activities, including bactericidal activity, inhibition of HIV replication, free radical scavenging activity, cytotoxic activity, gastric protective action, and antiinflammatory activity (13).

In the past 2 decades high-performance thin-layer chromatography (HPTLC) emerged as an efficient tool for the phytochemical evaluation of herbal drugs (14–16). Considering the therapeutic importance of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid, we developed a simple HPTLC densitometric method for the simultaneous quantitation of these marker compounds in the whole plant of *P. amarus*.

Experimental

Materials and Reagents

Whole plant of *P. amarus* was collected from 3 different locations in India. The samples were authenticated, and voucher specimens were deposited in our Pharmacognosy and Phytochemistry Department. The samples were stored at 25°C in air-tight containers and ground to 40 mesh when required.

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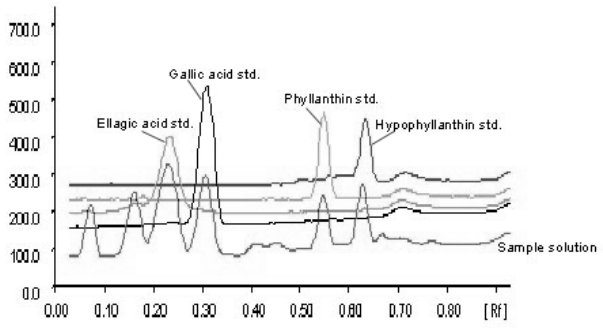

Figure 1. TLC densitograms of sample solution of *Phyllanthus amarus* whole plant, gallic acid standard, ellagic acid standard, phyllanthin standard, and hypophyllanthin standard.

All chemicals used were of analytical grade.

Phyllanthin (purity 98%, w/w), hypophyllanthin (purity 98%, w/w), and ellagic acid (purity 97%, w/w) were purchased from Natural Remedies Pvt. Ltd., Bangalore, India. Gallic acid (purity 99%, w/w) was a gift sample from Tetrahedron Ltd., Chennai, India.

Apparatus

(**a**) *Spotting device.*—Linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland).

(**b**) *Syringe.*—100 µL (Hamilton, Bonaduz, Switzerland).

(**c**) *TLC chamber*.—Glass twin-trough chamber (20 × 10 × 4 cm; Camag).

(**d**) *Densitometer*.—TLC Scanner 3 linked to winCATS software (Camag).

(**e**) *HPTLC plates*.—20 × 10 cm, 0.2 mm layer thickness, precoated with silica gel 60 F_{254}, Cat. No. 1.05548, E. Merck KgaA, Darmstadt, Germany.

Preparation of Standard Solutions

(**a**) *Standard solutions of phyllanthin*.—A stock solution of phyllanthin was prepared by dissolving 8 mg of accurately weighed phyllanthin in methanol in a 25 mL volumetric flask. From this stock solution, standard solutions of 32 to 128 mg/mL were prepared by transferring aliquots (1 to 4 mL) of stock solution into 10 mL volumetric flasks and adjusting the volume with methanol.

(**b**) *Standard solutions of hypophyllanthin*.—A stock solution of hypophyllanthin was prepared by dissolving 8 mg of accurately weighed hypophyllanthin in methanol in a 25 mL volumetric flask. From this stock solution, standard solutions of 32 to 192 mg/mL were prepared by transferring aliquots (1 to 6 mL) of stock solution to 10 mL volumetric flasks and adjusting the volume with methanol.

(**c**) *Standard solutions of gallic acid.*—Gallic acid standard (10 mg) was dissolved in 100 mL methanol in a volumetric flask. From this stock solution, standard solutions of 15–75 mg/mL were prepared by transferring aliquots (1.5 to 7.5 mL) of stock solution to 10 mL volumetric flasks and adjusting the volume with methanol.

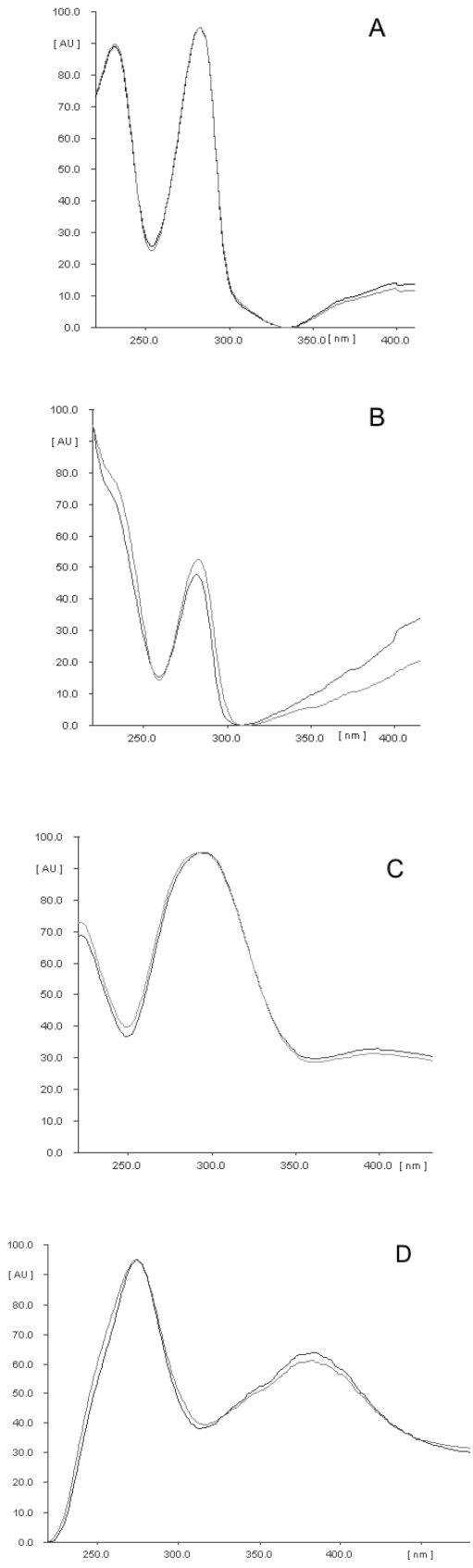

Figure 2. Overlay of UV absorption spectra of the marker compounds in the sample track with respective standards. A = Phyllanthin, B = hypophyllanthin, C = gallic acid, and D = ellagic acid.

Table 1. Method validation parameters for the quantitation of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid by the proposed HPTLC densitometric method

Parameters	Phyllanthin	Hypophyllanthin	Gallic acid	Ellagic acid
Instrumental precision (CV, %, *n* = 7)	0.54	0.93	0.08	0.78
Repeatability of standards (CV, %, *n* = 6)	1.01	0.79	0.98	1.06
Repeatability of sample (CV, %, *n* = 6)	0.26	0.38	0.50	0.16
Limit of detection, ng	40	80	50	40
Limit of quantitation, ng	80	160	150	80
Specificity	Specific	Specific	Specific	Specific
Linearity (correlation coefficient)	0.999	0.999	0.997	0.999
Range, ng/spot	320–1280	320–1920	150–750	80–240

(**d**) *Standard solutions of ellagic acid*.—A stock solution of ellagic acid was prepared by dissolving 2 mg of accurately weighed ellagic acid in methanol in a 25 mL volumetric flask. From this stock solution, standard solutions of 8 to 24 mg/mL were prepared by transferring aliquots (1 to 3 mL) of stock solution to 10 mL volumetric flasks and adjusting the volume with methanol.

Preparation of Sample Solutions

Dried powder of whole plant of *P. amarus* (2.5 g) was extracted exhaustively with 70% ethanol (3 × 50 mL) under reflux for 1 h on a water bath. The extract was filtered and concentrated to dryness under vacuum. Methanol was added to the residue and filtered. The methanol-soluble portion was made up to 25 mL in a volumetric flask.

Procedure

(**a**) *Calibration curves*.—Quantities (10 mL) of each of the standard solutions of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid were applied in triplicate on an HPTLC plate. The plates were developed with toluene–ethyl acetate–formic acid (6 + 2 + 1, v/v) mobile phase at 25 ± 2°C and 40% relative humidity for a distance of 8 cm. After development, the plate was dried in air and scanned at 280 nm because the maxium absorbance wavelengths of all the 4 marker compounds falls in the range of 278 to 284 nm. The peak areas were recorded, and calibration curves were prepared by plotting peak areas vs concentration.

(**b**) *Simultaneous quantitation of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid*.—Sample solution (10 μL) was applied in triplicate on a precoated silica gel 60 F_{254} HPTLC plate. The plate was developed and scanned at 280 nm to obtain peak areas. Ultraviolet (UV) absorption spectra were recorded; to check the identity of the bands, the spectrum of each standard was overlayed with the corresponding band in the sample track. The purity of the bands in the sample extract was checked by overlaying the absorption spectra at start, middle, and end position of the bands. The amounts of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid in the sample were calculated using the respective calibration curves.

(**c**) *Validation of the method*.—International Conference on Harmonization (ICH) guidelines (CPMP/ICH/381/95; CPMP/ICH/281/95) were followed for the validation of the analytical procedure. The method was validated for precision, repeatability, and accuracy. Instrumental precision was checked by repeated scanning of phyllanthin (800 ng), hypophyllanthin (960 ng), gallic acid (450 ng), and ellagic acid (160 ng) 7 times and was expressed as coefficient of variation (%CV). The repeatability of the method was confirmed by analyzing an 800 ng/spot of standard solution of phyllanthin, 960 ng/spot of standard solution of hypophyllanthin, 450 ng/spot of standard solution of gallic acid, and 160 ng/spot of standard solution of ellagic acid after application on the HPTLC plate (*n* = 6) and was expressed as %CV. Variability of the method was studied by analyzing aliquots of standard solution of phyllanthin (480, 640, and

Table 2. Intraday and interday precision study

Marker compound	Concentration, ng/spot	Intraday precision[a]	Interday precision[a]
Phyllanthin	480	1.54	1.23
	640	1.06	1.58
	800	1.80	1.29
Hypophyllanthin	640	0.90	0.98
	960	1.71	1.74
	1280	1.32	1.03
Gallic acid	150	1.60	1.81
	300	1.12	1.05
	450	1.40	1.75
Ellagic acid	120	0.92	1.18
	160	1.50	1.31
	200	0.77	1.06

[a] CV %; *n* = 3.

Table 3. Recovery study of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid by the proposed HPTLC method

Marker compound	Amount present in sample, μg	Amount added, μg	Amount found, μg[a]	Recovery, %[a]	Average recovery, %
Phyllanthin	370	185.00	540.68 ± 7.33	97.53 ± 1.16	
	370	370.00	728.62 ± 3.51	98.46 ± 0.48	99.09
	370	462.50	838.30 ± 2.93	101.29 ± 1.33	
Hypophyllanthin	1165	582.50	1729.11 ± 3.60	98.93 ± 0.21	
	1165	1165.00	2282.49 ± 3.32	97.95 ± 0.14	99.27
	1165	1456.25	2645.55 ± 5.71	100.92 ± 0.22	
Gallic acid	360	180.00	521.77 ± 1.69	96.62 ± 0.32	
	360	360.00	702.83 ± 1.67	97.59 ± 0.20	98.69
	360	450.00	825.06 ± 5.19	101.86 ± 0.64	
Ellagic acid	170	85.00	249.96 ± 5.61	98.02 ± 2.20	
	170	170.00	346.66 ± 2.14	101.48 ± 0.63	100.49
	170	212.50	390.22 ± 6.00	101.97 ± 1.58	

[a] Mean ± standard deviation (SD; n = 3).

800 ng/spot), hypophyllanthin (640, 960, and 1280 ng/spot), gallic acid (150, 300, and 450 ng/spot), and ellagic acid (120, 160, 200 ng/spot) on the same day (intraday precision) and on different days (interday precision) and the results were expressed as CV.

Accuracy of the method was tested by performing recovery studies at 3 levels (50, 100, and 125% addition). The recovery and average recovery were calculated. For the determination of limit of detection and limit of quantitation, different dilutions of the standard solutions of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid were applied along with methanol as the blank and calculated on the basis of signal-to-noise ratio.

Results and Discussion

Of the various mobile phases tried, toluene–ethyl acetate–formic acid (6 + 2 + 1, v/v) gave the best resolution of phyllanthin (R_f = 0.55), hypophyllanthin (R_f = 0.63), gallic acid (R_f = 0.31), and ellagic acid (R_f = 0.23) from each other and from the other components of the sample extract for the simultaneous quantitation. The identities of the bands of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid in the sample extract were confirmed by overlaying their UV absorption spectra with those of the standards of these compounds using the TLC Scanner 3 (Figures 1 and 2).

The method was validated in terms of precision, repeatability, and accuracy (Tables 1 and 2). The relationship between the concentration of standard solutions and the peak response was linear within the concentration range of 320 ng to 1280 ng/spot with a correlation coefficient of 0.999 for phyllanthin, 320 ng to 1920 ng/spot with a correlation coefficient of 0.999 for hypophyllanthin, 150–750 ng/spot with a correlation coefficient of 0.997 for gallic acid, and 80–240 ng/spot with a correlation coefficient of 0.999 for ellagic acid. The percentage recovery at 3 different levels of phyllanthin was found to be 97.53, 98.46, and 101.29% with an average of 99.09%; hypophyllanthin was found to be 98.93, 97.95, and 100.92% with an average of 99.27%; gallic acid was found to be 96.62, 97.59, and 101.86% with an average of 98.69%; and that of ellagic acid was found to be 98.02, 101.48, and 101.97% with an average of 100.49%. The results are presented in Table 3.

Phyllanthin, hypophyllanthin, gallic acid, and ellagic acid content in the whole plant of *P. amarus* was estimated by the proposed method (Table 4). The method developed was found to be suitable for the quantitation of these marker compounds in the herbal raw materials.

Table 4. Marker compounds quantitated by TLC densitometric method from different samples of *P. amarus*

Marker compound	Content of marker compounds[a], % w/w		
	Sample 1	Sample 2	Sample 3
Phyllanthin	0.370 ± 0.016	0.702 ± 0.003	0.508 ± 0.001
Hypophyllanthin	1.170 ± 0.009	3.106 ± 0.015	1.616 ± 0.016
Gallic acid	0.360 ± 0.006	0.056 ± 0.002	0.041 ± 0.001
Ellagic acid	0.170 ± 0.005	0.111 ± 0.001	0.096 ± 0.001

[a] Mean ± standard deviation (SD; n = 3).

Conclusions

We established an HPTLC densitometric method for the simultaneous quantitation of 4 bioactive compounds, *viz*., phyllanthin, hypophyllanthin, gallic acid, and ellagic acid from *P. amarus* whole plant. The method was found to be simple, precise, specific, sensitive, and accurate and can be used for their quantitation in the plant materials and also in routine quality control of the raw materials as well as formulations containing any or all of these compounds.

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