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Original Article

A validated TLC-image analysis method for detecting and quantifying bioactive phyllanthin in *Phyllanthus amarus* and commercial herbal drugs

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Abstract

Phyllanthus amarus Schum. and Thonn. has long been used for the treatment of liver diseases. The hepatoprotective compound presented in *P. amarus* was phyllanthin. In this study, the fast determination and quantitation of bioactive phyllanthin in *P. amarus* and its commercial herbal drugs were developed using a simple thin-layer chromatographic (TLC)-image analysis method. Chromatographic separation of phyllanthin was carried out and the intensity of phyllanthin was examined by TLC-image analysis. The linear equation line showed good relationship for the calibration curve in the various concentrations of phyllanthin. The limits of detection and limits of quantitation were 0.16 and 0.49 µg/spot, respectively. The contents of phyllanthin from fifteen plant materials collected from different locations and twelve commercial herbal drugs determined using the TLC-image analysis method were not significantly different from those determined using the TLC-densitometric method. These results suggest that the proposed TLC-image analysis method can be used as an effective method for quantitating phyllanthin in *P. amarus* plants and commercial products.

Keywords: *Phyllanthus amarus*, Phyllanthin, Validation, TLC-image analysis, TLC-densitometry

1. Introduction

Phyllanthus amarus Schum. and Thonn. (Euphorbiaceae) is a small herb found in tropical and subtropical countries worldwide, including the United States, Brazil, India, and Thailand (Patel *et al.*, 2011). Reports on *P. amarus* indicate that it exhibits various pharmacological properties, such as anti-inflammatory (Mahat and Patil, 2007), antiplasmodial (Ajala *et al.*, 2011), antiviral (Notka *et al.*, 2003), and hepatoprotective activities (Pramyothin *et al.*, 2007). The whole plant has been widely used in traditional medicine to treat liver diseases, and aqueous extracts from aerial parts of the plant have shown hepatoprotective effects against ethanol- (EtOH) (Pramyothin *et al.*, 2007), paracetamol- (Wongnawa *et al.*, 2005), and carbon tetrachloride (CCl_4) induced liver toxicity in animal models (Yadav *et al.*, 2008). Methanolic and ethanolic extracts of the leaves have shown not only liver protection against EtOH in adult male rats (Faremi *et al.*, 2008) but also antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* (Okolo *et al.*, 2012). In addition, the ethanolic extract of the whole plant has shown a synergistic hepatoprotective effect through significant changes in various liver parameters for male mice when it is

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used together with silymarin, a hepatoprotective drug (Faremi *et al.*, 2008).

In previous phytochemical studies, hydrolyzable tannins such as phyllanthusiin D, ellagitannin 1, and corilagin have been isolated (Foo and Wong, 1992). Alkaloids such as securinine, isobubbialine, and epibubbialine (Houghton *et al.*, 1996), flavonoids such as kaempferol, rutin, and quercetin (Londhe *et al.*, 2008), and lignans such as phyllanthin, hypophyllanthin, niranthin, phyltetralin, and nirtetralin (Maciel *et al.*, 2007) have also been characterized. Among these chemical components, a major lignan, phyllanthin (Figure 1), has been reported to be responsible for the hepatoprotective effects against EtOH-induced liver disease in primary rat hepatocyte culture (Chirdchupunseree and Pramyothin, 2010) and against CCl_4-induced hepatic damage by enhancing antioxidant activity (Krithika *et al.*, 2011).

Because of increasing patient demand for the treatment of liver disease using herbal medicine, many raw plant materials and commercial herbal drugs derived from *P. amarus* have been highly distributed in herbal markets; however, the content of bioactive phyllanthin in these products has not necessarily been quality-controlled. Therefore, a simple, low-cost, and rapid method for screening and quantitating phyllanthin is strongly desired. The known analytical methods for the identification of phyllanthin are high-performance thin-layer chromatography (HPTLC) (Nayak *et al.*, 2010; Tripathi *et al.*, 2006; Annamalai and Lakshmi, 2009) and high-performance liquid chromatography (HPLC) (Annamalai and Lakshmi, 2009; Alvari *et al.*, 2011), which require user expertise and expensive instrumentation. TLC-image analysis method using computer software technology has been in consideration as a simple, inexpensive, and convenient quantitation method with good accuracy and precision for bioactive components in herbal and crude drugs. This methodology successfully applied to quantitate many bioactive compounds such as curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin, in turmeric (*Curcuma longa*) (Sotanaphun *et al.*, 2008). However, there have been no reports on application to the detection and quantification of phyllanthin in *P. amarus* plant materials and in commercial herbal drugs. Therefore, the aim of the study was to develop a rapid TLC-image analysis method for simple quantification of phyllanthin in *P. amarus* plant materials that can be used to determine the phyllanthin content in commercial herbal drugs. The proposed method was validated in accordance with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the Association of Official Agricultural Chemists (AOAC) guidelines.

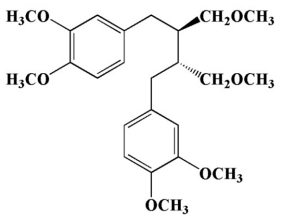

Figure 1. Structure of phyllanthin.

2. Materials and Methods

2.1 Materials

A phyllanthin standard was isolated from *P. amarus* collected in the Nakhon Sawan province of Thailand. The plant was authenticated by Assoc. Prof. Dr. Chaiyo Chaichantipyuth, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The plant materials (P1-P15) in this study were collected at 15 different locations in Thailand (Table 1) and authenticated by Prof. Dr. Nijsiri Ruangrungsi at the College

Table 1. Details for plant materials used herein.

Sample	Region	Location in Thailand (Province)	Voucher specimen
P1	North	Chiang Mai	PK141011
P2		Chiang Rai	PK071011
P3	Northeast	Buriram	PK161011
P4		Nong Khai	PK230911
P5		Udonthani	PK181011
P6	Central	Bangkok	PK210911
P7		Bangkok	PK111011
P8		Bangkok	PK191011
P9		Lop Buri	PK021011
P10		Nakhon Nayok	PK250911
P11		Nakhon Pathom	PK280911
P12		Ratchaburi	PK221011
P13		Rayong	PK300911
P14		Samut Songkhram	PK091011
P15	South	Chumphon	PK041011

of Public Health Sciences, Chulalongkorn University. Twelve commercial herbal drugs (C1-C12) were purchased from herbal markets, drugstores, and via the Internet. Analytical-grade chemicals (LAB-SCAN, Dublin, Ireland) were used.

2.2 Standards and sample preparation

The shade-dried plant materials (2.2 kg) were ground into powders and extracted with ethyl acetate (EtOAc) (5×4 L) at room temperature for 72 h. The EtOAc solution was evaporated at 40°C under reduced pressure. The residue (31.4 g) was separated by column chromatography (SiO_2) with 0-40% EtOAc in hexane to give 5 fractions (E1-E5). The more-polar E3-E5 fractions (E35) were combined and purified by column chromatography (SiO_2) with hexane-acetone-EtOAc (7:2:1, v/v/v) to give 10 fractions E35F1-E35F10, among which E35F3 - E35F6 were combined and separated by column chromatography (Sephadex LH - 20) with 50-100% methanol (MeOH) in water to give a crystalline product. Recrystallization from MeOH provided phyllanthin (461 mg, yield = 0.2095%) as white needles, melting point 96-97°C. The phyllanthin standard was prepared as a stock solution in MeOH (5 mg/mL).

For sample preparation, each plant material (10 g) and herbal product (10 g) was ground into powder and extracted using MeOH (500 mL) in a Soxhlet apparatus for 24 h. The crude extract was evaporated at 40°C to dryness under reduced pressure, diluted with MeOH (15 mg/mL), sonicated, and filtered through a DURAPORE® 0.45 μm membrane filter (Millipore, MA, USA).

2.3 Chromatographic conditions

TLC analysis was performed using TLC silica gel 60 F_{254} aluminum plates (20 cm × 10 cm, Merck, Darmstadt, Germany). Five microliters of the phyllanthin standard and the extract solution were applied as a 6 mm band onto a TLC plate using a CAMAG Linomat 5 (Camag, Muttenz, Switzerland). The distance between each band was 9.4 mm. The plate was developed to 10 cm in a TLC chamber that was saturated with hexane-EtOAc-MeOH-formic acid (7:3:0.2:0.3, v/v/v/v) for 30 min prior to the experiment.

2.3.1 TLC-densitometric method

The TLC plates developed under the chromatographic conditions at room temperature were scanned using a CAMAG TLC Scanner III (S/N 170302) in absorbance mode at 282 nm (Khan *et al.*, 2010) with the winCATS software. The slit dimensions were 4.00 mm × 0.30 mm, and the scanning speed was 20 mm/s.

2.3.2 TLC-image analysis method

An image for the TLC chromatogram under 254 nm UV light was acquired using a digital camera. The image file was opened using Scion Image for Windows, version Alpha 4.0.3.2 (Scion Corp., MD, USA). The original color image was converted to grayscale. The smoothing function was applied until the overlapped bands were clear. A profile plot for the chromatogram was generated using Load Macros to open the GelPlot2 file. The wand tool was used to select the peak corresponding to phyllanthin in each sample for the measurement of the peak area (unit^2).

2.4 Method validation

For the calibration curve, standard phyllanthin solutions at concentrations of 0.2, 0.4, 1, 1.5, and 2 mg/mL were prepared in MeOH. Five microliter aliquots from each standard phyllanthin solution were spotted onto a TLC plate to generate phyllanthin concentrations of 1, 2, 5, 7.5, and 10 μg/spot, which were developed under the aforementioned chromatographic conditions. The calibration curve was constructed using the peak area and standard concentration in μg/spot.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined from the standard deviation and slope. The slope was estimated using the analyte calibration curve. The standard deviation was estimated using various linear regression data. The LOD and LOQ were expressed as 3SD/S and 10SD/S, where SD and S indicate the standard deviation and the slope of the calibration curve, respectively.

The accuracy analysis was determined using a standard addition method. A sample was spiked with three standard phyllanthin concentrations of 1.25, 2.5, and 5 μg/spot. The percent recovery was calculated. Each concentration was measured in triplicate.

The repeatability (intra-day) was determined by measuring the area under the peaks at the five phyllanthin concentrations (1, 2, 5, 7.5 and 10 μg/spot) in six experiments within one day, and the intermediate precision (inter-day) was determined by comparing the assays from six consecutive days. The relative standard deviation (% RSD) values were calculated.

2.5 Quantitation of phyllanthin content in plant materials and commercial herbal drugs

The phyllanthin content of plant materials and commercial products were determined using the TLC-densitometry and TLC-image analysis methods. Five microliters of a prepared sample was spotted onto a TLC plate. The results were analyzed in triplicate using the previously described methods for the chromatographic conditions; the results were expressed as the mean of phyllanthin content (% w/w).

2.6 Statistical analysis

The TLC-densitometry and TLC-image phyllanthin quantitation results were expressed as the mean ± SD.

3. Results and Discussion

3.1 Identification of the isolated phyllanthin

The identification of isolated standard phyllanthin was undertaken by comparing the spectroscopic data with those previously reported (Hanh *et al.*, 2014).

3.2 The chromatographic condition

The chromatographic condition for quantitating phyllanthin was examined using silica gel $60F_{254}$ TLC plates. A mixed solvent of hexane-EtOAc-MeOH-formic acid (7:3:0.2:0.3, v/v/v/v) demonstrated the best separation of phyllanthin with R_f value 0.42±0.02. The phyllanthin band of the samples was ensured by comparing an R_f value with that of standard phyllanthin (Figure 2a). The TLC chromatogram was determined by the TLC-image analysis (Figure 2b) and the TLC-densitometric method (Figure 2c).

3.3 Method validation

The TLC-image analysis and TLC-densitometry analytical methods were validated for linearity, limit of sensitivities, precision, accuracy, and recovery. Calibration curves were generated for the peak area and phyllanthin standard content (µg/spot) (Figure 3). The linear correlation data showed that the 1, 2, 5, 7.5, and 10 µg/spot concentrations correlated well with the data using both the TLC-image and TLC-densitometric methods (correlation coefficient; $R^2 \geq 0.995$). With respect to the TLC-image analysis, the linear equation was $y = 13.323x + 4.7801$, with $R^2 = 0.9967$ (Figure 3a). The calibration curve for the TLC-densitometric method produced a linear equation of $y = 1946.7x + 3716.6$, with $R^2 = 0.9964$ (Figure 3b).

The LOD and LOQ values were 0.16 and 0.49 µg/spot, respectively, as determined using the TLC-image analysis, whereas these values were 0.48 and 1.49 µg/spot when the TLC-densitometric method was applied. To determine the accuracy, three standard phyllanthin concentrations (1.25, 2.5, and 5 µg/spot) were spiked into a sample collected in Chiang Rai province (P2).

The accuracy was assessed using the percent recovery values. Percent recoveries by TLC-image analysis and the TLC-densitometric method ranged from 97.36 to 101.04% and from 96.24 to 99.14%, respectively (Table 2). The results show that both methods are reasonably accurate.

Repeatability was determined from six experiments in one day (for intra-day precision) and from six experiments on consecutive days (for the inter-day precision), which are

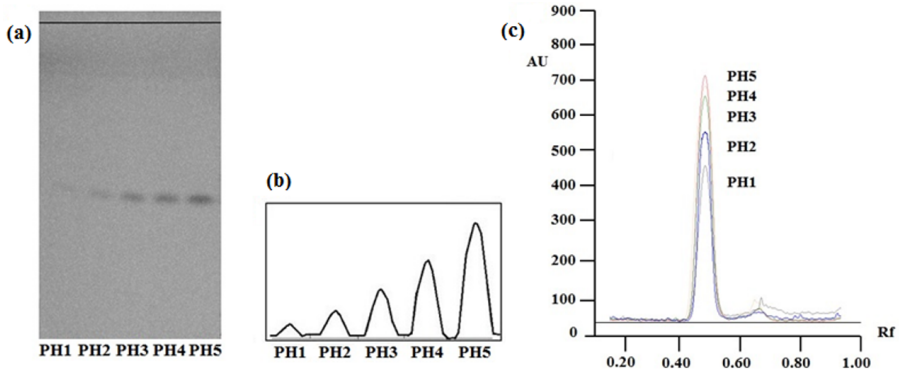

Figure 2. The TLC pattern (a) for the phyllanthin standard at concentrations of 1, 2, 5, 7.5, and 10 µg/spot (PH1 - PH5). The TLC chromatogram was generated through (b) the TLC-image analysis and (c) the TLC-densitometric method.

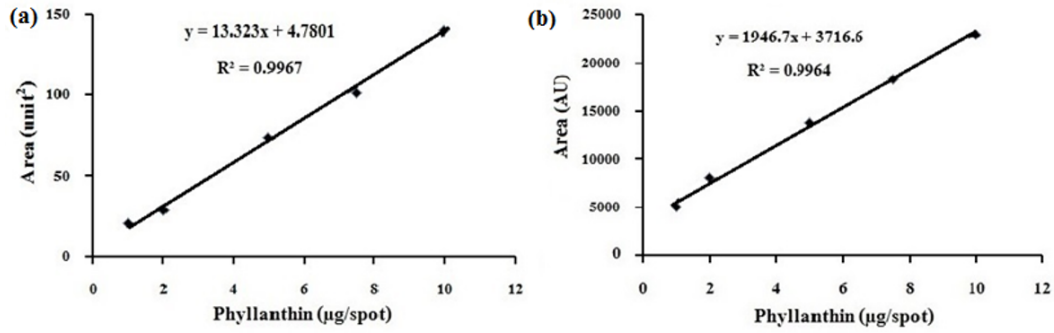

Figure 3. Standard curve for the peak area and phyllanthin concentrations from (a) TLC-image analysis (unit^2) and (b) TLC-densitometry (AU) (n = 6).

Table 2. Accuracy for plant materials analyzed by the TLC-image analysis and TLC-densitometric methods (n = 3).

Phyllanthin added (µg/spot)	TLC-image analysis		TLC-densitometry	
	Phyllanthin detected (µg/spot)	%Recovery	Phyllanthin detected (µg/spot)	%Recovery
1.25	2.739	97.36	3.570	96.73
2.5	3.982	98.40	4.830	98.78
5	6.574	101.04	7.318	99.14

Table 3. Intra-day and inter-day precisions for plant materials determined by the TLC-image analysis and TLC-densitometric methods.

Phyllanthin concentration (µg/spot)	TLC-image analysis*		TLC-densitometry*	
	Intra-day	Inter-day	Intra-day	Inter-day
1	1.26	1.85	0.63	1.26
2	1.52	1.87	0.82	1.17
5	1.07	1.88	0.96	0.75
7.5	1.40	1.79	1.40	0.80
10	1.22	1.66	1.07	1.13

*%RSD $\leq$ 2 (n = 6)

reported as %RSD (Table 3). The %RSD value of phyllanthin was determined by the TLC-image analysis and by the TLC-densitometric method. For the TLC-image analysis and the TLC-densitometric methods, the intra-day precision ranged from 1.07 to 1.52% and from 0.63 to 1.40%, respectively, whereas the inter-day precision varied from 1.66 to 1.88% and from 0.75 to 1.26%, respectively. According to the AOAC, the results demonstrate acceptable repeatability and inter-day precision because the %RSD values do not exceed 2%.

3.4 Quantitation of phyllanthin content in plant materials and commercial herbal drugs

The validated TLC-image analysis was sufficiently accurate and precise to quantitate phyllanthin; this method could therefore be used for routine phyllanthin quantitation in *P. amarus* plant materials. The phyllanthin contents in plant materials (P1 - P15) collected at different locations were determined by the TLC-image analysis and TLC-densitometric methods (Table 4). The correlation between these two methods was good (R^2 = 0.992) (Figure 4a). The standard phyllanthin concentration of 10 µg/spot (PH) and the selected samples analyzed (P8 and P2) were spotted on TLC plates (Figure 5a). The presence of standard phyllanthin in samples was clearly observed in the TLC plate. The content range of phyllanthin measured using the TLC-image method was 0.004-0.018% w/w. The highest and lowest phyllanthin contents were observed in samples P8 and P2, respectively (Figure 5b). The phyllanthin content measured using the TLC-densitometric method was 0.005-0.016% w/w. The highest phyllanthin content was observed in sample P8, whereas the lowest content was contained in sample P2 (Figure 5c). The results show variations in the phyllanthin content in plant materials collected from various locations in Thailand because the chemical constituent contents in herbs can vary with the harvest season, plant origin, environment, and herbal preparation method (Kunle *et al.*, 2012).

The bioactive compound contents may indicate the commercial herbal drug quality. Herein, TLC-image analysis and TLC-densitometric methods were applied to detect and quantitate phyllanthin in commercial herbal drugs (C1-C12) that claim to include *P. amarus*. The extract percent yields and phyllanthin content of 12 different commercial herbal drugs were measured (Table 5) and the correlation between these two results from TLC-image analysis and TLC-densitometric method was good (R^2 = 0.995) (Figure 4b). When the TLC-image analysis method was used, the phyllanthin content was highest in sample C11, whereas it was lowest in sample C12 (Figure 5b). When the TLC-densitometric method was used, the highest content of phyllanthin was also detected in sample C11, whereas the lowest content was detected in sample C12 (Figure 5c). In the case of commercial herbal products that claimed to include *P. amarus*, the results show that such products were derived from *P. amarus* but with different phyllanthin contents. The phyllanthin content in commercial herbal drugs did not significantly differ when measured using the TLC-densitometry and TLC-image

Table 4. Phyllanthin contents of plant materials determined by the TLC-image analysis and the TLC-densitometric methods.

Samples	Extract yield (%)	Phyllanthin contents* (% w/w)	
		TLC-image analysis	TLC-densitometry
P1	4.338	0.013 ± 0.0023	0.014 ± 0.0012
P2	6.272	0.004 ± 0.0006	0.005 ± 0.0006
P3	8.745	0.011 ± 0.0006	0.012 ± 0.0006
P4	5.317	0.008 ± 0.0015	0.009 ± 0.0006
P5	5.193	0.007 ± 0.0010	0.008 ± 0.0006
P6	4.869	0.011 ± 0.0012	0.012 ± 0.0010
P7	9.866	0.013 ± 0.0012	0.014 ± 0.0010
P8	13.138	0.018 ± 0.0021	0.016 ± 0.0010
P9	9.032	0.013 ± 0.0026	0.013 ± 0.0006
P10	9.086	0.012 ± 0.0015	0.013 ± 0.0006
P11	7.775	0.012 ± 0.0030	0.013 ± 0.0006
P12	17.515	0.005 ± 0.0010	0.006 ± 0.0010
P13	5.296	0.011 ± 0.0023	0.012 ± 0.0000
P14	8.668	0.012 ± 0.0021	0.012 ± 0.0012
P15	8.137	0.012 ± 0.0021	0.013 ± 0.0010

*Values are the mean ± SD (n = 3)

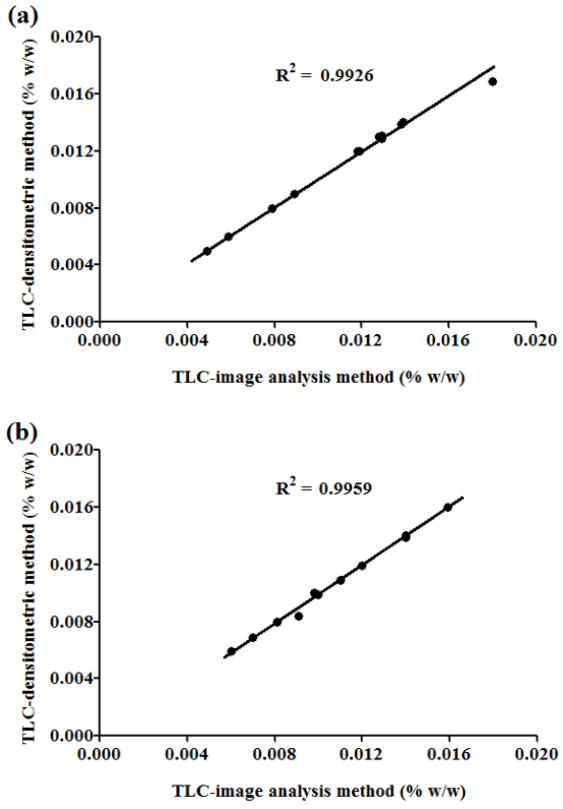

Figure 4. The correlation of phyllanthin contents of (a) plant materials and (b) commercial herbal drugs determined by the TLC-image analysis and the TLC-densitometric methods.

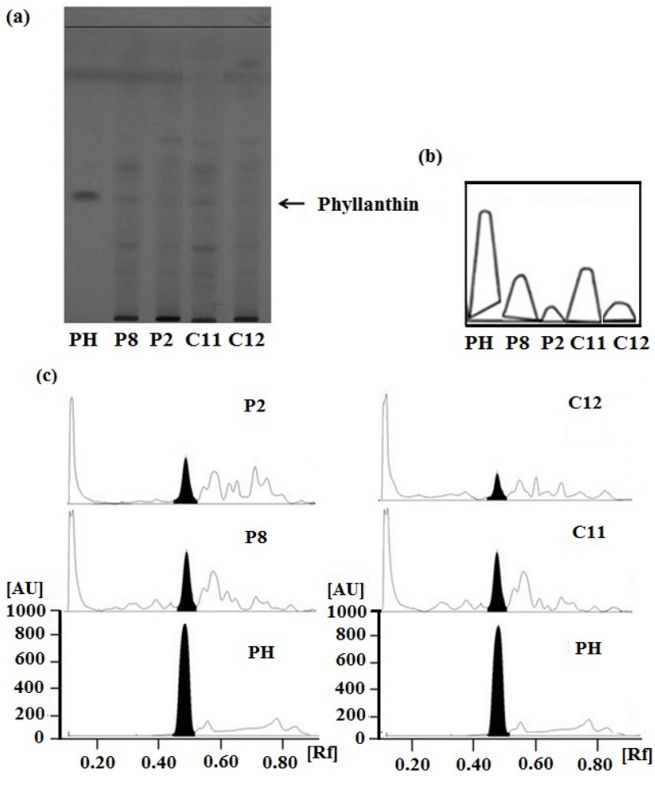

Figure 5. The TLC patterns (a) for the phyllanthin standard at 10 µg/spot (PH), selected plant materials (P2 and P8), and commercial herbal drugs (C11 and C12). The TLC chromatogram was generated through (b) the TLC-image analysis and (c) the TLC-densitometric method.

Table 5. Phyllanthin contents of commercial herbal drugs determined by the TLC-image analysis and TLC-densitometric methods.

Commercial herbal drugs	Extract yield (%)	Phyllanthin contents* (% w/w)	
		TLC-image analysis	TLC-densitometry
C1	5.310	0.014 ± 0.0010	0.014 ± 0.0000
C2	4.328	0.014 ± 0.0010	0.013 ± 0.0006
C3	4.180	0.009 ± 0.0006	0.008 ± 0.0000
C4	2.730	0.008 ± 0.0010	0.008 ± 0.0000
C5	2.446	0.009 ± 0.0006	0.010 ± 0.0010
C6	4.327	0.014 ± 0.0010	0.014 ± 0.0006
C7	2.850	0.007 ± 0.0015	0.006 ± 0.0006
C8	2.861	0.010 ± 0.0025	0.009 ± 0.0006
C9	5.531	0.012 ± 0.0006	0.011 ± 0.0025
C10	3.702	0.011 ± 0.0012	0.010 ± 0.0010
C11	3.563	0.015 ± 0.0012	0.016 ± 0.0006
C12	2.484	0.006 ± 0.0015	0.005 ± 0.0006

*Values are the mean ± S.D. (n = 3)

analysis methods. These results suggest that the proposed TLC-image analysis method using image software may be an alternative to TLC-densitometry to quantitate phyllanthin in *P. amarus* plant materials and to standardize phyllanthin in *P. amarus* commercial products. TLC-image analysis method has been successfully applied for quantification of sibutramine (anorexic drug) in adulterated herbal slimming products (Phattanawasin *et al.*, 2012). Thus, the application of a TLC-image analysis method for the detection and quantification of phyllanthin in *P. amarus* plant materials and in commercial products was demonstrated herein for the first time.

4. Conclusion

This is the first report of the validated TLC-image analysis method that uses image software to the quantitation of a bioactive marker for phyllanthin in different *P. amarus* plant materials and commercial herbal drugs. Statistical analysis indicated that the amount of phyllanthin content determined by the TLC-image analysis and TLC-densitometric method showed no significant. In conclusion, the TLC-image analysis method should be used as a valuable tool for standardizing herbal raw materials and commercial herbal products because of its speed, reliability, and cost-effectiveness.

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