CHAPTER V
A VALIDATED TLC-IMAGE ANALYSIS METHOD FOR DETECTING AND QUANTIFYING BIOACTIVE PHYLLANTHIN IN *PHYLLANTHUS AMARUS* AND COMMERCIAL HERBAL DRUGS

Because of increasing patient demand for the treatment of liver disease through herbal medicine, many *Phyllanthus amarus* raw materials and commercial herbal drugs are sold in herbal markets. However, the content of bioactive phyllanthin in these products is unknown. To control the quality of the plant materials and commercial herbal drugs, a simple, low-cost, and rapid method for screening and quantitating phyllanthin is necessary. The analytical methods used to quantitate phyllanthin in *P. amarus* are high-performance thin-layer chromatography (HPTLC) (Tripathi, *et al.* 2006, Annamalai and Lakshmi 2009, Nayak 2011) and high-performance liquid chromatography (HPLC) (Annamalai and Lakshmi 2009, Alvari, *et al.* 2011), which require expensive analytical instruments and user expertise. Herein, we try to develop a TLC-image analysis method using a computer software technology and applied it to the simple, inexpensive, and convenient quantitation of bioactive compounds from herbal and crude drugs.

5.1 Materials and Methods

5.1.1 Plant materials

P. amarus plant materials were collected from 15 different locations in Thailand (P1 - P15, **Table 7**). The plants were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi at the College of Public Health Sciences, Chulalongkorn University. Twelve commercial herbal drugs (C1 - C12) were purchased from herbal markets, drugstores, and via the Internet. The chemicals were of analytical grade (LAB-SCAN, Dublin, Ireland). Pure compound, phyllanthin, isolated and identified from our previous work in chapter IV was used as standard compound.

Table 7 Details for the *P. amarus* 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

5.1.2 Standard and samples preparation

The phyllanthin (PH) standard was prepared as stock solution in methanol (5 mg/mL). For plant materials, we used P1-P15, which include the aerial parts of *P. amarus*; these materials were shade dried, ground into powder, and passed through a sieve (20 mesh). Ten grams of plant material was extracted using methanol (500 mL) in a Soxhlet apparatus for 24 hr. The crude extract was concentrated by rotary evaporation under reduced pressure at 40°C, diluted with methanol (15 mg/mL), sonicated, and filtrated through a DURAPORE® 0.45 µm membrane filter (Millipore, Massachusetts, USA). For the commercial herbal drugs (C1 - C12), 10 g of the herbal product was extracted and prepared using the same protocol as that used for the plant materials.

5.1.3 Chromatographic condition

TLC analysis was performed using TLC silica gel $60F_{254}$ aluminium plates (20 cm×10 cm, Merck, Darmstadt, Germany). Five microliters of the phyllanthin standard and crude extract solution were applied as a 6×1 mm band onto a TLC plate using a CAMAG Linomat 5 (Camag, Muttenz, Switzerland). The distance between each band was 9.4 cm. The plate was developed to 10 cm in a TLC chamber that was saturated with hexane-ethyl acetate-methanol-formic acid (7:3:0.2:0.3, v/v/v/v) for 30 min prior to the experiment.

5.1.4 Analytical methods

5.1.4.1 TLC-densitometric method

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

5.1.4.2 TLC-image analysis method

An image for the TLC chromatogram under 254 nm UV light was acquired using a digital camera. The plate color image was saved using the tag image file format (TIFF). The image file was opened using Scion Image for Windows, version Alpha 4.0.3.2 (Scion Corp., Maryland, 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 that corresponded to phyllanthin in each sample to measure the area under the peak (unit^2).

5.1.5 Method validation

The analytical method was validated for linearity, limit of detection, limit of quantitation, accuracy, and precision. For the calibration curve, standard phyllanthin solutions at concentrations of 0.2, 0.4, 1, 1.5, and 2 mg/mL were prepared in methanol. 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 previously described 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 on the basis of the standard deviation and slope. The slope was estimated using the analyte calibration curve. The standard deviation was estimated using a variety of linear regression data. The LOD and LOQ were expressed as $3.3\delta/S$ and $10\delta/S$, where δ the standard deviation and S is the slope of the calibration curve.

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

We determined the repeatability (intra-day) by measuring the areas 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) by comparing the assays from six consecutive days. The relative standard deviation (%RSD) values were calculated.

5.1.6 Phyllanthin quantitation in plant materials and commercial herbal drugs

Phyllanthin contents in *P. amarus* 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 TLC plates and was analyzed using the methods described for the chromatographic conditions. The contents of phyllanthin in the plant materials and commercial herbal drugs were analyzed using both methods in triplicate. The results were expressed as the mean phyllanthin content (%w/w).

5.1.7 Statistical analysis

The TLC-densitometry and TLC-image phyllanthin quantitation results were expressed as the mean±S.D. and compared using a paired *t*-test at the 95% confidence level using the SPSS 17.0 for Windows software package (SPSS, USA).

5.2 Results and Discussions

5.2.1 Analysis of phyllanthin contents in plant materials

Phyllanthin is the primary bioactive compound in whole plant extracts from *P. amarus* and was used as a marker for quantitating plant materials. The chromatographic condition for quantitating phyllanthin was examined using commonly used silica gel $60F_{254}$ TLC plates. Hexane-EtOAc-MeOH-formic acid (7:3:0.2:0.3, v/v/v/v) was used as the mobile phase. The phyllanthin bands of the samples were verified through comparison with the phyllanthin standard at the R_f value 0.42±0.02 (**Figure 36a**). The standard phyllanthin TLC chromatogram was interpreted through TLC-image analysis (**Figure 36b**) and through the TLC-densitometric method (**Figure 36c**).

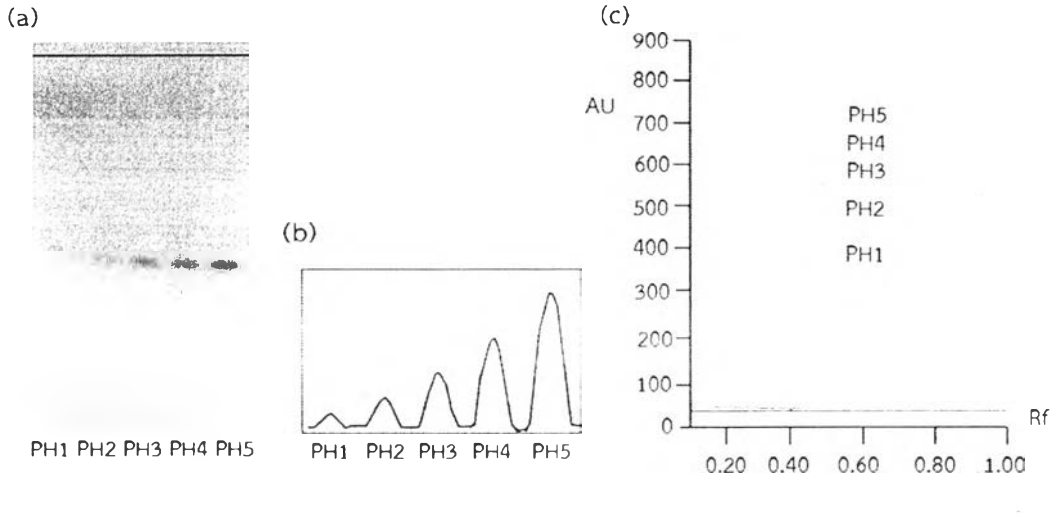

Figure 36 The TLC pattern (a) for the phyllanthin standard at concentrations of 1, 2, 5, 7.5, and 10 µg/spot (PH1-PH5). The TLC chromatogram was generated through (b) TLC-image analysis and (c) the TLC-densitometric method.

The TLC-image analysis and TLC-densitometry analytical methods were validated for linearity, limit of detection, limit of quantitation, precision, and accuracy. Calibration curves were generated for the peak area and phyllanthin standard contents (µg/spot) (**Figure 37**). The linear correlation data showed that the 1, 2, 5, 7.5, and 10 µg/spot concentrations related well to the data generated using both the TLC-image and TLC-densitometric methods (correlation coefficient;

$R^2 \geq 0.995$). For the TLC-image analysis, the linear equation was $y = 13.323x + 4.7801$ with $R^2 = 0.9967$ (**Figure 37a**). The calibration curve for the TLC-densitometric method produced the linear equation $y = 1946.7x + 3716.6$ with $R^2 = 0.9964$ (**Figure 37b**).

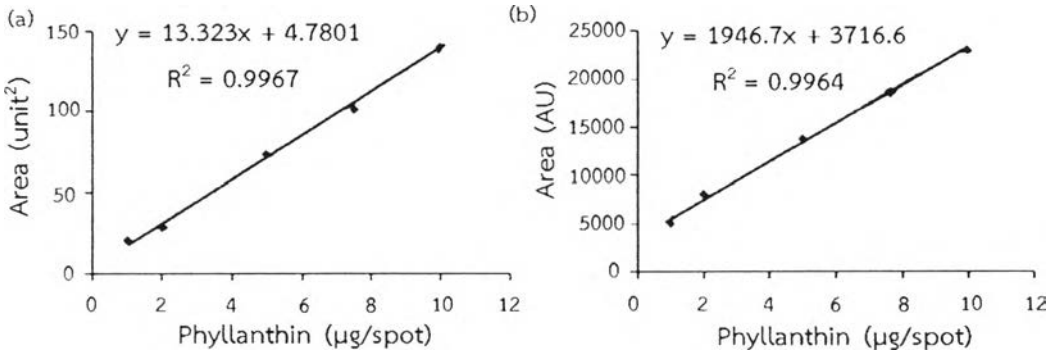

Figure 37 Standard curve between the peak area and phyllanthin concentrations from (a) TLC-image analysis (unit^2) and (b) TLC-densitometry (AU) (n = 6).

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

The accuracy was assessed using the percent recovery values. The TLC-image analysis percent recovery ranged from 97.36 to 101.04%, and the TLC-densitometric method percent recovery ranged from 96.24 to 99.14% (**Table 8**). The results show that both methods are reasonably accurate.

Table 8 Accuracy for the *P. amarus* plant materials analyzed through TLC-image analysis and the TLC-densitometric method (n = 3).

Phyllanthin added (µg/spot)	TLC-image analysis		TLC-densitometry	
	Phyllanthin detected (µg/spot)	%Recovery	Phyllanthin detected (µg/spot)	%Recovery
0	1.522	-	1.464	-
1.25	2.739	97.36	2.667	96.24
2.5	3.982	98.40	3.670	98.56
5	6.574	101.04	7.318	99.14

Repeatability was determined through six experiments in one day and six experiments on consecutive days (for the intermediate precision), which are reported as %RSD (**Table 9**). The repeatability %RSD value was determined through the TLC-image analysis and the TLC-densitometric method; the intra-day precision ranged from 1.07 to 1.52% and 0.63 to 1.40%, and the intermediate precision ranged from 1.66 to 1.88% and 0.75 to 1.26% for the TLC-image analysis and the TLC-densitometric methods, respectively. According to the AOAC, the results demonstrate acceptable repeatability and intermediate precision because the %RSD values do not exceed 2%.

Table 9 Intra-day and inter-day precision for the *P. amarus* plant materials determined through TLC-image analysis and the TLC-densitometric method.

Phyllanthin concentration	TLC-image analysis*		TLC-densitometry*	
(µg/spot)	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

*Relative standard deviation (%RSD), %RSD ≤ 2 (n = 6)

The validated TLC-image analysis was sufficiently accurate and precise to quantitate phyllanthin content; this method could therefore be used for routine phyllanthin content analysis in *P. amarus* plant materials.

Herein, the phyllanthin contents in *P. amarus* plant materials (P1-P15) from different locations were determined through TLC-image analysis and the TLC-densitometric methods (**Table 10**).

Table 10 Phyllanthin contents in *P. amarus* plant materials determined through TLC-image analysis and the TLC-densitometric method.

Samples	Extract yield (%)	Phyllanthin contents* (%w/w)	
		TLC-image analysis	TLC-densitometry
P1	4.338	0.013 ± 0.0023	0.016 ± 0.0012
P2	6.272	0.004 ± 0.0006	0.005 ± 0.0006
P3	8.745	0.011 ± 0.0006	0.014 ± 0.0006
P4	5.317	0.008 ± 0.0015	0.009 ± 0.0006
P5	5.193	0.007 ± 0.0010	0.011 ± 0.0006
P6	4.869	0.011 ± 0.0012	0.015 ± 0.0010
P7	9.866	0.013 ± 0.0012	0.015 ± 0.0010
P8	13.138	0.018 ± 0.0021	0.016 ± 0.0010
P9	9.032	0.013 ± 0.0026	0.014 ± 0.0006
P10	9.086	0.012 ± 0.0015	0.015 ± 0.0006
P11	7.775	0.012 ± 0.0030	0.015 ± 0.0006
P12	17.515	0.005 ± 0.0010	0.008 ± 0.0010
P13	5.296	0.011 ± 0.0023	0.013 ± 0.0000
P14	8.668	0.012 ± 0.0021	0.015 ± 0.0012
P15	8.137	0.012 ± 0.0021	0.015 ± 0.0010

*Values are the mean ± S.D. (n = 3)

We did not observe additional interference bands in the TLC chromatogram at the phyllanthin R_f value (0.42±0.02) for the selected samples analyzed (P8 and P2) (**Figure 38a**). 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 (**Figure 38b**). The phyllanthin content measured using the TLC-densitometric method was 0.005-0.016%w/w. Samples P1 and P8 contained the same (greatest) content of phyllanthin; the phyllanthin content was the lowest in sample P2 (**Figure 38c**).

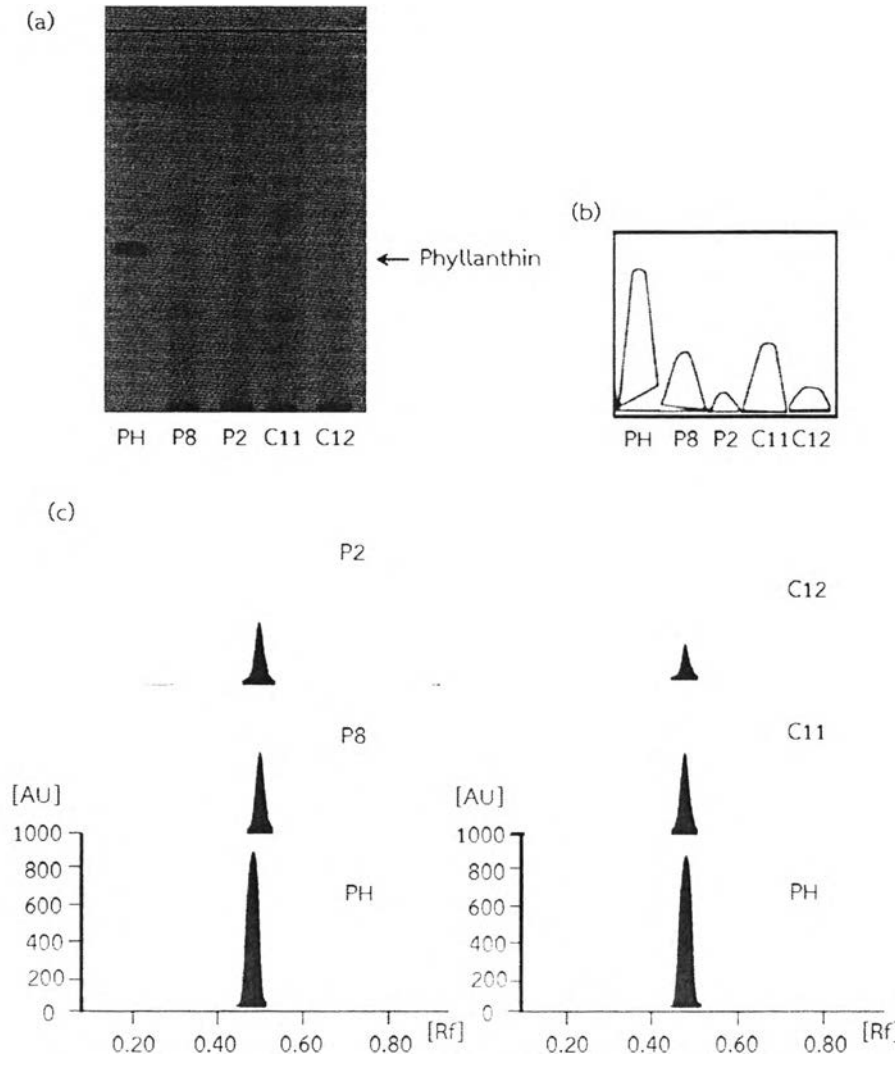

Figure 38 The TLC patterns (a) for the phyllanthin standard (PH), *P. amarus* plant materials (P8 and P2), and commercial herbal drugs (C11 and C12). The TLC chromatogram was generated through (b) TLC-image analysis and (c) the TLC-densitometric method.

The results showed variations in the phyllanthin content in plant materials collected from various locations in Thailand since the chemical constituent contents in herbs might be validation of the harvest season, the plant origin, the environment, and the herbal preparation method (Kunle, *et al.* 2012).

5.2.2 Analysis of phyllanthin contents in commercial products

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) (**Figure 39**) that claimed to include *P. amarus*.

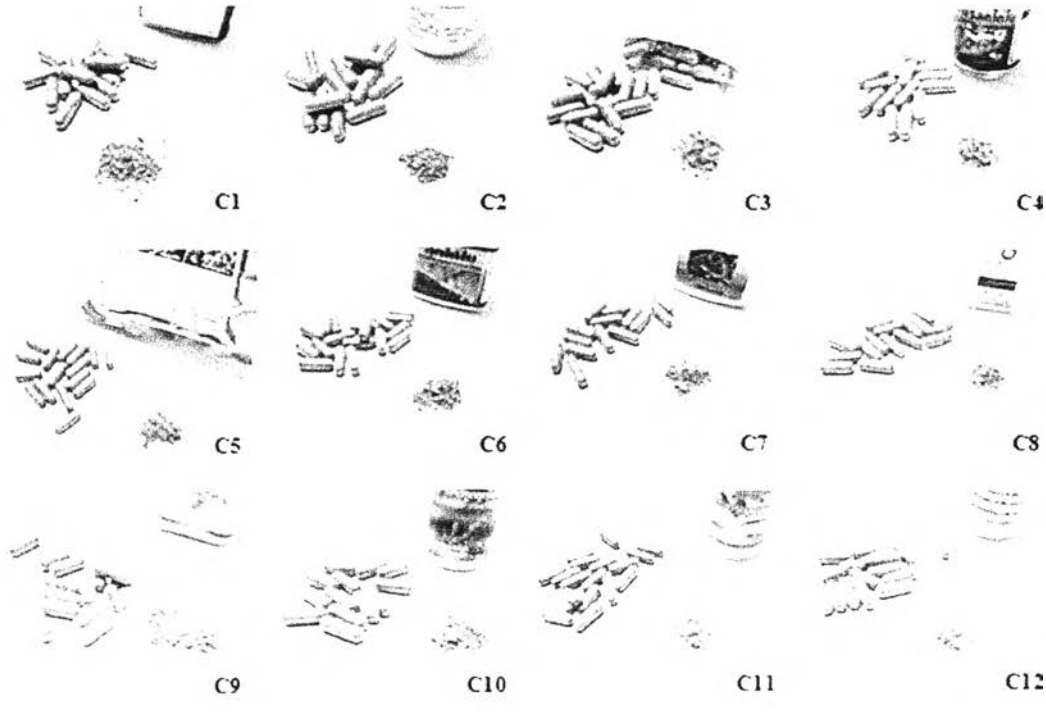

Figure 39 *P. amarus* commercial herbal drug samples (C1 – C12).

The crude extract percent yields and phyllanthin contents in *P. amarus* from 12 different commercial herbal drugs were measured (**Table 11**).

Table 11 Phyllanthin contents in *P. amarus* commercial herbal drugs determined through TLC-image analysis and the TLC-densitometric method.

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	0.010 ± 0.0006	0.007 ± 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	0.005 ± 0.0006
C8	2.861	0.010 ± 0.0025	0.009 ± 0.0006
C9	5.531	0.014 ± 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)

When the TLC-image analysis method was used, the phyllanthin content was highest in sample C11; however, sample C12 exhibited the lowest of that (**Figure 38b**). When the TLC-densitometric method was used, the phyllanthin content in commercial herbal drug sample C11 was the highest, whereas; the lowest contents were detected in samples C7 and C12 (**Figure 38c**). 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.

A paired *t*-test indicates that the phyllanthin contents in commercial herbal drugs did not significantly differ ($p<0.05$) when measured using the TLC-densitometry and TLC-image 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.

5.3 Conclusion

In conclusion, the TLC-image analysis method validated herein can be utilized to quantitate phyllanthin contents in *P amarus* plant materials. The TLC-image analysis method is rapid, reliable, and cost-effective for such quantitation. This method will be a valuable tool for standardizing traditional drugs derived from *P. amarus* and for assessing the quality of *P. amarus* plant materials and commercial herbal drugs.