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Original Article

HPLC fingerprint and simultaneous quantitative analysis of phyllanthin and hypophyllanthin for identification and authentication of *Phyllanthus niruri* from related species

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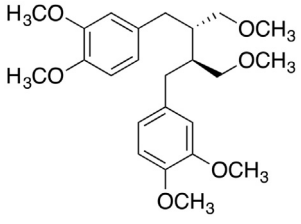
ABSTRACT

A precise and accurate method for the identification and authentication of *Phyllanthus niruri* L. from *P. debilis* Klein ex Willd. and *P. urinaria* L., Phyllanthaceae, was developed using high-performance liquid chromatography. Chromatographic fingerprint analysis was combined with simultaneous quantification of phyllanthin and hypophyllanthin for the developed method. Phyllanthin and hypophyllanthin were successfully separated and quantified under this proposed method. The highest amount of phyllanthin and hypophyllanthin was found in *P. niruri* compared to *P. debilis* and *P. urinaria*. Fingerprint chromatogram of the three *Phyllanthus* species showed distinct profiles that these may be used to identify and authenticate each *Phyllanthus* species, which improved by marker compounds present in each species. The combination of chromatographic fingerprint analysis and discriminant analysis was successfully discriminated all three species, including *P. niruri* adulterated with *P. debilis* or *P. urinaria*. The method can be used for the identification and authentication of *P. niruri* from related species, such as *P. debilis* and *P. urinaria*.

Introduction

The genus of *Phyllanthus* belongs to the family of Phyllanthaceae comprising of more than 600 species and one of them is *Phyllanthus niruri* L., which mostly found in the tropical and subtropical regions. *P. niruri* has a long history in some traditional medicinal system, such as Indonesian Jamu, Indian Ayurveda and Traditional Chinese Medicine, to prevent or treat various diseases (Bagalkotkar et al., 2006). In Indonesia, *P. niruri* is known as meniran and usually grown like a weed on agricultural and wastelands. Considering its efficacy to treat many diseases and maintaining health, *P. niruri* is considered as one of the most important medicinal plants in Jamu. *P. niruri* has been used to treat health problems such as diarrhea, jaundice, wounds, diabetes, diuretic, stomachache and hepatitis (Joseph and Raj, 2011; Patel et al., 2011). *P. niruri* denotes many pharmacological activities such as antioxidant (Lim and Murtijaya, 2007), antiviral (Tan et al., 2013), antidiabetic (Adeneye, 2012), anti-inflammatory (Kassuya et al., 2005) and hepatoprotective (Pramyothin et al., 2007). It is also used to improve the immune system (immunostimulant) (Taiwo et al., 2009). *P. niruri* has numerous secondary metabolites such as lignans, alkaloids, flavonoids, hydrolyzable tannins (ellagitannins), triterpenoids and polyphenolic compounds (Patel et al., 2011). The primary bioactive compounds present in *P. niruri* are lignans, such as phyllanthin (**1**), hypophyllanthin (**2**), phyltetralin, nirtetralin and niranthin (Murugaiyah and Chan, 2007; Shanker et al., 2011). Compounds **1** and **2** are two lignans commonly used as marker compounds in the quality control of *P. niruri* as described in Indian Pharmacopoeia (2010).



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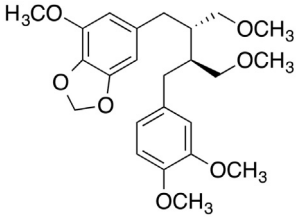

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Since the efficacy of a medicinal plant depends on more than one single chemical compound that work synergistically, there

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are two approaches in the quality control of herbal medicine, *i.e.*, multicomponent-based and pattern-based approaches from the chromatograms, spectrum or other graphs from chemical instrumentation. The pattern-based approach, which is also known as the fingerprint analysis, can reveal all detectable compounds in a medicinal plant, and therefore, it could be applied in the detection of adulteration of raw materials of herbal medicinal products (Mok and Chau, 2005; Ma et al., 2011). On the other hand, quantitative analysis of multicomponents can reveal the concentration of bioactive compounds in a medicinal plant. Therefore, the combination of chromatographic fingerprint and quantitative analysis of multicomponents often used for the complementary properties between these approaches makes it more comprehensive for identification, discrimination and authentication of medicinal herbs. This combination has been used, for example, in the quality evaluation of *xiaoyanlidan* tablets (Tang et al., 2014), authentication of *Curcuma xanthorrhiza* from *C. longa* (Rafi et al., 2015) and chemical characterization of two morphologically related *Espeletia*, Asteraceae, species (Padilla-Gonzalez et al., 2016).

There are two closely related species that have similar morphology and grow sympatrically with *P. niruri*, *i.e.*, *P. debilis* and *P. urinaria*. These may lead to misidentification and quite vulnerable to adulteration of *P. niruri* from *P. debilis* and *P. urinaria*. Therefore, a simple and reliable chemical analysis method should be developed for the identification and authentication of *P. niruri* using the secondary metabolite profiles. Several analytical methods using HPLC have been developed for quantitative determination of the two lignans of these species, phyllanthin (**1**) and hypophyllanthin (**2**) (Murugaiyah and Chan, 2007; Shanker et al., 2011), as well as for chromatographic fingerprint analysis (Martins et al., 2011) in some *Phyllanthus* species. HPLC with ultraviolet (UV) or photodiode array (PDA) detection is widely applied for those two analytical methods attributed to its convenience and efficiency.

Previously reported analytical methods for *P. niruri* were only developed for quantitative analysis for its marker compound or chromatographic fingerprint analysis alone. There is no reported work combining the chromatographic fingerprint and simultaneous quantitative analysis of **1** and **2** for the identification and authentication of *P. niruri* from *P. debilis* and *P. urinaria*. In this study, we developed a simple and reliable method for the identification and authentication of *P. niruri* by combining the two approaches (simultaneous quantification and HPLC fingerprint analysis) with one multivariate analysis, *i.e.*, discriminant analysis. The developed method can be used for the identification and authentication of *P. niruri* from related species, such as *P. debilis* and *P. urinaria*.

Materials and methods

Plant material

Twenty samples consisting of six samples of *Phyllanthus niruri* L., eight samples of *P. debilis* Klein ex Willd. and six samples of *P. urinaria* L., Phyllanthaceae, were collected in the year 2017 from eight different locations in West Java province, Indonesia (Box 1). All samples were identified in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Science. All samples were dried at 40 °C, pulverized, and sieved (60-mesh) before analysis. Three simulated samples of *P. niruri* adulterated with 25, 50 and 75% of *P. debilis* and *P. urinaria* were also used.

Box 1: Origin and code of the *Phyllanthus* species samples.

No.	Origin (sub-district, regency)	Sample code		
		P. niruri	*P. debilis*	*P. urinaria*
1	Dramaga-1, Bogor	PN-1	PD-1	PU-1
2	Dramaga-2, Bogor	PN-2	PD-2	PU-2
3	Central Bogor, Bogor	PN-3	PD-3	PU-3
4	Southern Bogor, Bogor	PN-4	PD-4	PU-4
5	Rajapolah, Tasikmalaya	PN-5	PD-5	PU-5
6	Purwadadi, Subang	PN-6	PD-6	PU-6
7	Pondok Gede, Bekasi	—	PD-7	—
8	Ciomas, Bogor	—	PD-8	—

Chemicals and reagents

Phyllanthin (**1**) and hypophyllanthin (**2**) were purchased from ChromaDex Inc. (Santa Ana, CA, USA) with about 98.9% purity. All solvents used for the analysis were obtained from Merck (Darmstadt, Germany), *i.e.*, methanol (HPLC grade), ultrapure water from purification system of Direct-Q® 5 UV-R, and formic acid (purity 98–100%). All standards and samples were filtered through 0.2 μm nylon membrane filter obtained from Whatman (Kent, UK). The HPLC mobile phase was prepared fresh daily, filtered through 0.2 μm polyvinylidene difluoride (PVDF) membrane filter obtained from Waters Corp. (Milford, Massachusetts, USA) and degassed before analysis.

Apparatus and chromatographic conditions

The HPLC system used was LC-20A series (Shimadzu, Kyoto, Japan) equipped with a UV detector. The chromatographic separation was performed on a Luna C18 (2) column (150 mm × 4.6 mm, 5 μm) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of methanol (solvent A) and aqueous solution of formic acid 0.5% (v/v, pH 2.3) (solvent B) at a flow rate of 1 ml/min. The gradient elution program was set as follow: 0–3 min, 23% A; 3–9 min, 23–35% A; 9–16 min, 35–42% A; 16–17 min, 42–70% A; 17–29 min, 70% A; 29–30 min, 70–100% A; 30–40 min, 100% A. This was followed by a 15 min equilibrium period prior to the next injection. The chromatogram was monitored at a wavelength of 210 nm during experiment. The column temperature was maintained at 34 °C and the injection volume was 20 μl.

Preparation of samples and standard solutions

Powdered samples of 200 mg each were accurately weighed and sonicated with 10 ml methanol for 60 min at room temperature. The samples were then filtered through a 0.2 μm nylon membrane filter, and methanol was added to a final volume of 10 ml before injected into the HPLC system. A mixed of standard stock solution of PTN and HPN was prepared in methanol at a concentration of 1000 μg/ml. An appropriate amount of standard stock solutions was diluted with methanol to obtain six concentrations in the range of 1.05–83.87 μg/ml for **1** and 2.10–83.87 μg/ml for **2**. The calibration curve was obtained by plotting the peak area (y) against concentration (x) of each compound and were fitted to a linear regression equation of $y = ax + b$.

Method validation

Quantitative analysis of phyllanthin (**1**) and hypophyllanthin (**2**) was validated following the International Conference on Harmonization (ICH) guidelines (ICH, 2005) in terms of linearity of the calibration curves, accuracy, precision, stability, limit of detection (LOD) and limit of quantification (LOQ). The linearity of the calibration curve of each analyte was evaluated by plotting the peak areas (y) versus the concentrations (x, μg/ml). Recovery test was used to determine the accuracy of the analytical method. The accuracy (recovery test) was performed using standard addition

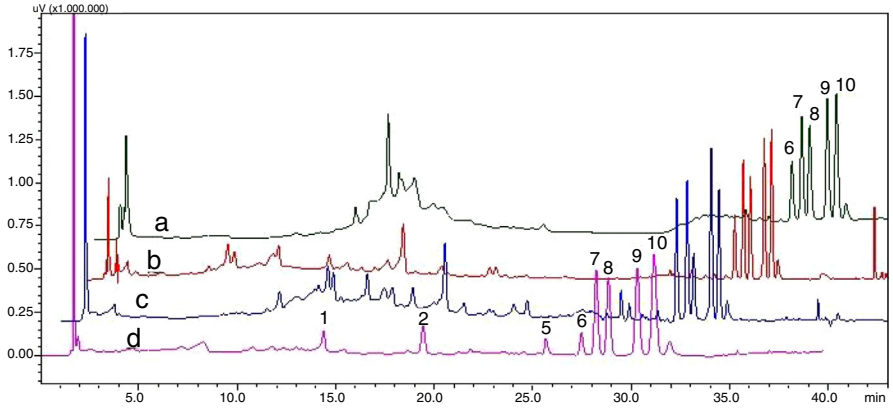

Figure 1. HPLC chromatograms of PN-1 sample using various solvent compositions: (a) methanol–water, (b) methanol–0.5% acetic acid, (c) methanol–0.5% phosphoric acid and (d) methanol–0.5% formic acid.

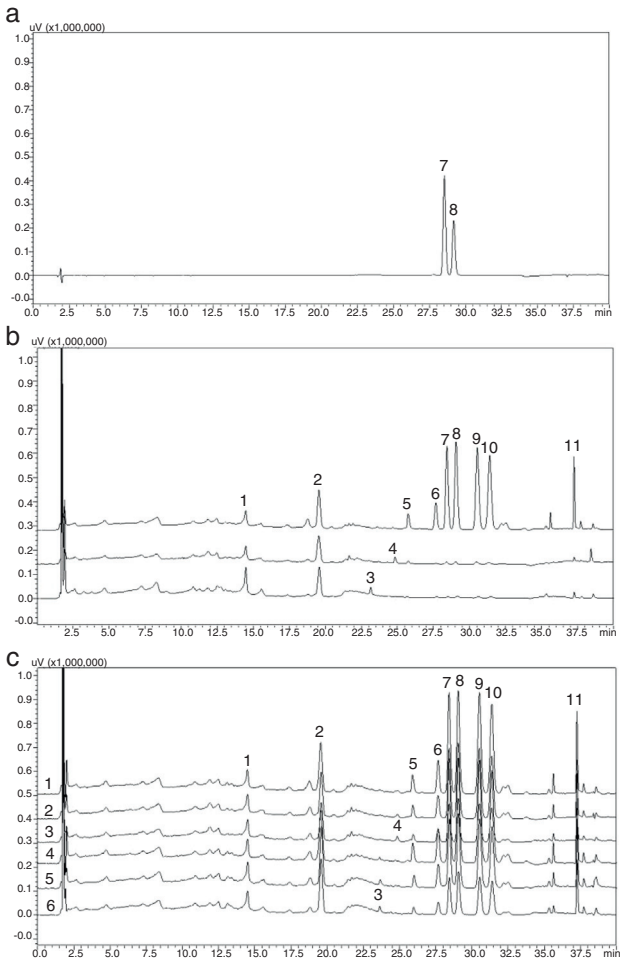

Figure 2. HPLC chromatograms of (a) mixed standard solutions of phyllanthin (**1**) and hypophyllanthin (**2**), (b) individual *Phyllanthus* sample and (c) adulterated samples. Composition: (b1) PN-5, 100%; (b2) PU-5, 100%; (b3) PD-5, 100%; (c1) PN-5:PU-5, 75:25%; (c2) PN-5:PU-5, 50:50%; (c3) PN-5:PU-5, 25:75%; (c4) PN-5:PD-5, 75:25%; (c5) PN-5:PD-5, 50:50%; (c6) PN-5:PD-5, 25:75%.

method. The known amount of the mixed standard solutions with the high, intermediate and low level were spiked into the sample (PN-1) in triplicates of each level. The precision of the method was evaluated by intra- and inter-day repeatability of six individual samples (PN-1) each day within three consecutive days. Stability of analytes was assessed by analyzing the same sample solution (PN-1) at 0, 4, 8, 12, 24 and 48 h after preparation. Validation of the

Table 1
Resolution of all common peaks present in *Phyllanthus niruri*, *P. debilis* and *P. urinaria* samples.

Peak number	Resolution		
	P. niruri	*P. debilis*	*P. urinaria*
1	–	–	–
2	15.141	14.914	14.213
3	–	13.473	–
4	–	–	16.024
5	17.919	9.514	3.753
6	6.035	5.216	5.836
7	2.177	2.207	1.946
8	1.721	1.807	1.748
9	3.636	3.786	3.786
10	1.954	1.997	2.009
11	18.659	19.495	19.699

HPLC fingerprint analysis was performed by evaluating intra- and inter-day precision and stability which expressed as relative standard deviation (RSD) of the relative retention time (RRT) and relative peak area (RPA) of the characteristic peaks to the reference peak (peak number 9).

Statistical analysis

Discriminant analysis was performed in XLSTAT ver 2012 software (Addinsoft, New York, USA) for the identification and authentication of *P. niruri* from *P. debilis* and *P. urinaria*.

Results and discussion

Optimization of HPLC condition

To obtain the most chemical information and good separation for chromatographic fingerprint and quantitative analyses, we optimized the mobile phase composition, gradient elution procedure and detection wavelength. We used mixed standard solution and PN-1 sample in this step to obtain the optimum condition. The mobile phase containing methanol–water gave better separation for phyllanthin (**1**) and hypophyllanthin (**2**) compared with acetonitrile–water. In order to give a better resolution, formic acid, phosphoric acid and acetic acid were added to the aqueous phase of a binary mixture of methanol–water. As a result, mobile phase consisting of methanol–0.5% formic acid in water (v/v, pH 2.3) was found to be optimum for chromatographic fingerprint and quantitative analyses of **1** and **2** (Fig. 1).

The resolution of phyllanthin (**1**) (peak 7) and hypophyllanthin (**2**) (peak 8) in the mixed standard solution was >1.5 (acceptance

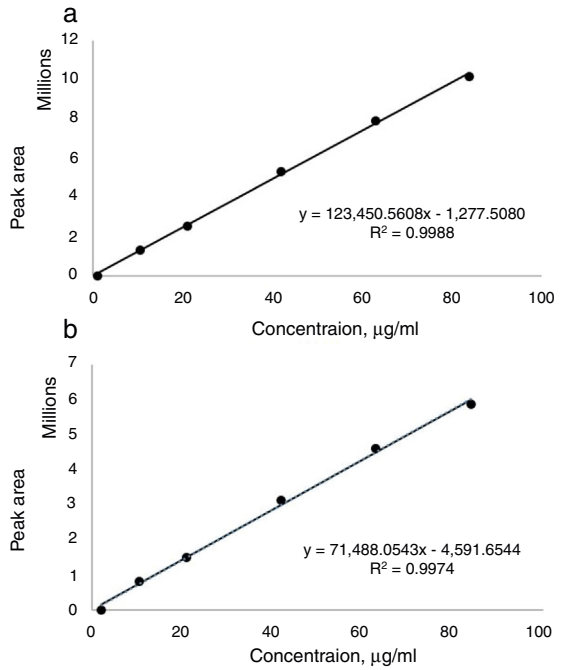

Figure 3. Plot of calibration curves of standard (a) phyllanthin (**1**) and (b) hypophyllanthin (**2**).

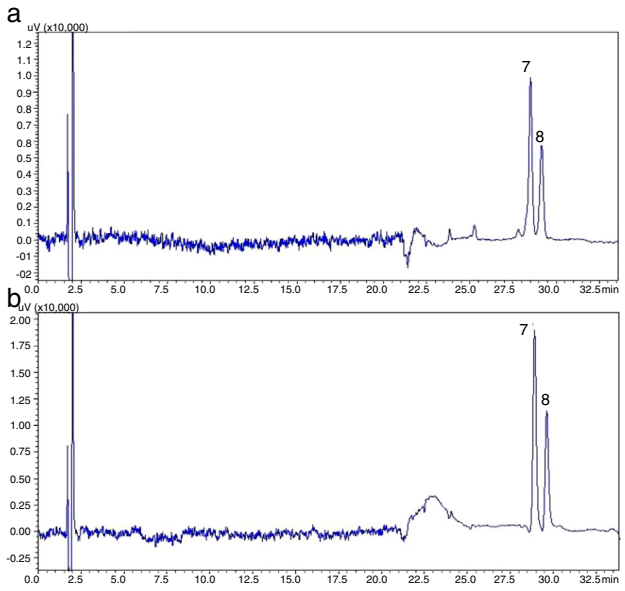

Figure 4. HPLC chromatograms of standard (a) phyllanthin (**1**) 1.05 µg/ml and (b) hypophyllanthin (**2**) 2.10 µg/ml for evaluation of LOQ.

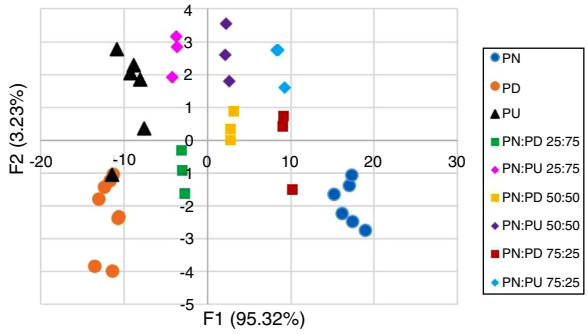

Figure 5. DA plots of individual samples (*Phyllanthus niruri*, *P. debilis* and *P. urinaria*) and adulterated samples (*P. niruri* with *P. debilis* and *P. urinaria*).

criteria for resolution >1.3) with a total analysis time of 40 min (Fig. 2a). The resolution of all common peaks in *P. niruri*, *P. debilis* and *P. urinaria* samples was also >1.5 (Table 1). Chromatograms of **1** and **2** in *P. niruri* sample and the mixed standard solution at detection wavelength between 210 and 370 nm were observed. Detection wavelength of 210 nm was selected for the chromatographic fingerprint and quantitative analyses because it provided higher signal intensities of target compounds and all detectable separated compounds.

Validation of the developed method

The linear regression equation was expressed as follow: $y = 123\,450.5608x - 1277.5080$, $r^2 = 0.9988$ (phyllanthin (**1**) linear range was 1.05–83.87 µg/ml) and $y = 71\,488.0543x + 4591.6544$, $r^2 = 0.9974$ (for hypophyllanthin (**2**), linear range was 2.10–83.87 µg/ml) (Fig. 3). All calibration curves showed a good linear regression within the test ranges (acceptance criteria: $r^2 \geq 0.995$). Data of calibration curves of **1** and **2** are shown in Table 2. The LOD and LOQ of the two analytes were determined at a signal to noise ratio (S/N) of 3 and 10, respectively (Fig. 4). The LOQ of **1** and **2** were 1.05 and 2.10 µg/ml, respectively. The chromatograms used for LOQ evaluation are shown in Fig. 5. The LOD of **1** and **2** were 0.31 and 0.63 µg/ml, respectively.

The recovery of **1** and **2** were found to be 98.78–100.20 and 97.72–99.97%, respectively, and in accordance with acceptance criteria of recovery about 80–120%. These results indicated that the assessed method was accurate and reliable. The RSD values of intra- and inter-day repeatability were less than 1.83 and 1.59%, respectively, and below the acceptance criteria value for repeatability (RSD ≤ 2%). These results showed excellent repeatability of the analytical method. The RSD values of stability were <1.02% with the acceptance criteria of RSD for stability ≤2%) which indicated that the analytes were stable within 48 h. The results of accuracy, precision and stability are summarized in Table 3.

Peaks existed in samples with narrow peak shapes (tailing factor < 1.2), good resolutions (resolution > 1.3) and high intensities compared to the other peaks were assigned as characteristic peaks. There were nine characteristic peaks (peaks 1, 2, 5, 6, 7, 8, 9, 10 and 11) found in the fingerprint chromatogram of *P. niruri* (Fig. 2b1).

Table 2
Analytical data of calibration curves of phyllanthin (**1**) and hypophyllanthin (**2**).

Phyllanthin (**1**)		Hypophyllanthin (**2**)	
Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
1.05	8971	2.10	11 022
10.48	1 339 581	10.48	809 575
20.97	2 538 863	20.97	1 511 167
41.93	5 326 690	41.93	3 127 475
62.90	7 912 859	62.90	4 593 076
83.87	10 172 635	83.87	5 863 455

Table 3
Analytical data of recovery, precision and stability for quantitative determination of lignans in PN-1samples.

Analyte	Recovery ($n = 3$)		Precision (RSD, %)		Stability ($n = 6$)
	Average (%)	RSD (%)	Intra-day ($n = 6$)	Inter-day ($n = 3$)	RSD (%)
(1)	99.81	0.24	Day 1 = 0.52	1.42	0.82
	100.20	0.49	Day 2 = 1.41		
	98.78	0.55	Day 3 = 0.49		
(2)	99.72	0.17	Day 1 = 0.71	1.58	1.01
	99.97	0.40	Day 2 = 1.82		
	97.72	0.59	Day 3 = 0.34		

Phyllanthin (**1**) and hypophyllanthin (**2**).

Table 4
Analytical data of precision and stability of nine characteristic peaks in PN-1 sample.

Peak number	RSD of RRT (%)			RSD of RPA (%)		
	Intra-day precision ($n = 6$)	Inter-day precision ($n = 3$)	Stability ($n = 6$)	Intra-day precision ($n = 6$)	Inter-day precision ($n = 3$)	Stability ($n = 6$)
1	0.01	0.03	0.00	0.79	1.52	1.60
2	0.02	0.06	0.08	0.51	0.62	0.93
5	0.13	0.37	0.52	1.01	0.71	1.53
6	0.01	0.02	0.00	0.06	0.40	0.12
7	0.01	0.01	0.00	0.12	0.44	0.30
8	0.01	0.01	0.00	0.23	0.65	0.56
9[a]	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.18	0.24	0.08
11	0.03	0.05	0.08	0.60	1.12	1.21

[a] Reference peak.

Peak 9 was chosen as the reference peak since it had a large peak area. The RSD of RRT and RPA of the characteristic peaks for intra- and inter-day precision and stability test were less than 0.53 and 1.61%, respectively. The acceptance criteria for precision is ≤2%. The analytical results of the precision and stability for chromatographic fingerprint are shown in Table 4. These results indicated that the developed method was reliable for simultaneous determination of **1** and **2** and HPLC fingerprint analysis of *P. niruri*.

*Quantitative determination of phyllanthin (**1**) and hypophyllanthin (**2**)*

The proposed HPLC method was successfully applied to simultaneously determined **1** and **2** in the three *Phyllanthus* species used in this work. About 20 samples comprising of six samples of *P. niruri*, eight samples of *P. debilis* and six samples of *P. urinaria* were analyzed. The analysis of each sample was performed in triplicates. The contents of **1** and **2** in the six batches of *P. niruri* were in the range 0.73–3.05 and 2.58–6.27 μg/mg, respectively. The large variation of **1** and **2** contents in samples from different areas may be due to many factors, such as geographical conditions, environmental growth conditions and the age of the plants. Meanwhile, the contents of **1** and **2** in *P. debilis* and *P. urinaria* are mostly not detected except in the samples of PD-2 and PU-5. Overall, the contents of **1** and **2** in *P. niruri* are significantly higher than in *P. debilis* and *P. urinaria* (Fig. 5). These results showed that the proposed method could be used to identify and discriminate *P. niruri* from *P. debilis* and *P. urinaria* based on the contents of **1** and **2** in the samples.

Authentication and determination of Phyllanthus niruri *from* P. debilis *and* P. urinaria

The proposed HPLC fingerprint method was successfully applied to the three *Phyllanthus* species obtained from Rajapolah, Tasikmalaya (PN-5, PD-5 and PU-5). There were 11 common peaks found in all chromatograms. There are nine characteristic peaks (peaks 1, 2, 5, 6, 7, 8, 9, 10 and 11) for *P. niruri* (Fig. 2b1), five characteristic peaks (peaks 1, 2, 4, 5 and 11) for *P. urinaria* (Fig. 2b2) and four characteristic peaks (peaks 1, 2, 3 and 11) for *P. debilis* (Fig. 2b3). In order to discriminate *P. niruri*, *P. debilis* and *P. urinaria*, the fingerprint chromatograms of all samples were compared. The result showed that six peaks with relatively high intensities (peaks 5, 6, 7, 8, 9 and 10) were typical peaks for *P. niruri*. These peaks may be used for identification and discrimination of *P. niruri* from *P. debilis* and *P. urinaria* (Fig. 2b). Peaks 3 and 4 were typical peaks for *P. debilis* and *P. urinaria*, respectively. These peaks can be used for authentication of *P. niruri* if there is a contamination from *P. debilis* and *P. urinaria* as can be seen in Fig. 2c when *P. niruri* adulterated with *P. debilis* and *P. urinaria*. The intensity of typical peaks for *P. niruri* was linearly decreased with the increasing adulterant concentrations (*P. debilis* and *P. urinaria*). These results showed that the chromatographic fingerprint might be used for authentication of *P. niruri* from *P. debilis* and *P. urinaria*.

Authentication of *P. niruri* from *P. debilis* and *P. urinaria* could be indicated by visual observation of their fingerprint chromatograms in addition to discriminant analysis (DA) used in this study. DA is one of the supervised pattern recognition analyses and mostly utilized for a discrimination or classification purpose of the object. DA will generate a discriminant function (DF) for each group by searching a linear combination of data that will give separation of two or more observation groups. In this study, DA was used for authentication and discrimination of *P. niruri*, *P. debilis* and *P. urinaria* samples based on the peak areas of 11 common peaks. DA was performed on 38 objects (20 individual and 18 adulterated samples) and 11 variable data matrices.

The individual sample used is six *P. niruri* samples, eight *P. debilis* samples and six *P. urinaria* samples. The adulterated samples used were from six adulterant samples (PD-3, PD-5, PD-6, PU-3, PU-5 and PU-6) with three-level adulteration concentrations (25, 50 and 75%). The result of DA showed that the total variance of two discriminant functions (DF) was 98.55% (DF1 = 95.32% and DF2 = 3.23%). This result showed that the individual samples (*P. niruri*, *P. debilis* and *P. urinaria*) and adulterated samples (*P. niruri* with *P. debilis* and *P. niruri* with *P. urinaria*) could be classified into their own groups as shown in Fig. 5. Leave one out cross-validation was used to validate the DA model and gave about 74% of samples

were correctly classified into their groups. In this case, peak areas of the 11 common peaks are adequate predictors for authentication and discrimination of *P. niruri*, *P. debilis*, *P. urinaria* and *P. niruri* adulterated with *P. debilis* and *P. urinaria*. So, by using a combination between marker peaks in the three samples and DA plot, we could detect an adulteration of *P. niruri* with *P. debilis* and *P. urinaria*.

Conclusions

In this study, a simple, accurate, and reliable method was developed for the identification and authentication of *P. niruri* from *P. debilis* and *P. urinaria* using the established HPLC fingerprint combined with simultaneous quantification of phyllanthin (**1**) and hypophyllanthin (**2**). The results showed that the method was reliable with high accuracy and precision. For the quantitative determination, it was found that **1** and **2** contents in *P. niruri* were much higher than *P. debilis* and *P. urinaria*. HPLC fingerprint analysis combined with DA could be used for the discrimination and authentication of *P. niruri* from *P. debilis* and *P. urinaria*. The developed method, therefore, has a great potential to be widely used for the identification and authentication of *P. niruri* in the quality control process of its raw material.

Authors' contributions

RN contributed in collecting and identifying plant samples, herbarium confection, running the laboratory work, analysis of the data, performing chromatographic analysis and drafting the paper. MR designed the study, supervised the laboratory work and contributed in collecting plant samples, statistical analysis and critical reading of the manuscript. WTW supervised the laboratory work and contributed in critical reading of the manuscript. SS contributed in critical reading and improvement of the manuscript. RH contributed in multivariate analysis. All authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Adeneye, A.A., 2012. The leaf and seed aqueous extract of *Phyllanthus amarus* improves insulin resistance diabetes in experimental animal studies. J. Ethnopharmacol. 144, 705–711.

Bagalkotkar, G., Sagineedu, S.R., Saad, M.S., Stanslas, J., 2006. Phytochemical from *Phyllanthus niruri* Linn. and their pharmacological properties: a review. J. Pharm. Pharmacol. 58, 1559–1570.

ICH, 2005. Validation of analytical procedures: text and methodology Q2 (R1). International Conference on Harmonization, Geneva. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf/ (accessed 10 August 2016).

Indian Pharmacopoeia, 2010. The Indian Pharmacopoeia Commission, Ghaziabad, 2488 pp.

Joseph, B., Raj, S.J., 2011. An overview: pharmacognostic properties of *Phyllanthus amarus* Linn. Int. J. Pharmacol. 7, 40–45.

Kassuya, C.A., Leite, D.F., de Melo, L.V., Rehder, V.L.G., Calixto, J.B., 2005. Anti-inflammatory properties of extracts, fractions and lignans isolated from *Phyllanthus amarus*. Planta Med. 71, 721–726.

Lim, Y., Murtijaya, J., 2007. Antioxidant properties of *Phyllanthus amarus* extract as affected by different drying methods. Food Sci. Technol. 40, 1664–1669.

Ma, T., Huang, C., Meng, X., Zhang, Q., Zhang, L., Lv, X., Jin, Y., Xie, J., Li, J., 2011. Fingerprint analysis of Hawk-tea by high-performance liquid chromatography. Food Chem. 129, 551–556.

Martins, L.R.R., Pereira-Filho, E.R., Cass, Q.B., 2011. Chromatographic profiles of *Phyllanthus* aqueous extracts samples: a proposition of classification using chemometric models. Anal. Bioanal. Chem. 400, 469–481.

Mok, D.K.W., Chau, F.T., 2005. Chemical information of Chinese medicines: a challenge to chemist. Chemom. Intell. Lab. Syst. 82, 210–217.

Murugaiyah, V., Chan, K.L., 2007. Determination of four lignans in *Phyllanthus niruri* L. by a simple high-performance liquid chromatography method with fluorescence detection. J. Chromatogr. A 1154, 198–204.

Patel, J.R., Tripathi, P., Sharma, V., Chauhan, N.S., Dixit, V.K., 2011. *Phyllanthus amarus*: ethnomedicinal uses, phytochemistry and pharmacology: a review. J. Ethnopharmacol. 138, 286–313.

Padilla-Gonzalez, G.F., Aldana, J.A., Da Costa, F.B., 2016. Chemical characterization of two morphologically related *Espeletia* (Asteraceae) species and chemometric analysis based on essential oil components. Rev. Bras. Farmacogn. 26, 694–700.

Pramyothin, P., Ngamtin, C., Poungshompoo, S., Chaichantipyuth, C., 2007. Hepatoprotective activity of *Phyllanthus amarus* Schum Thonn extract in ethanol treated rats: *in vitro* and *in vivo* studies. J. Ethnopharmacol. 114, 169–173.

Rafi, M., Wulansari, L., Heryanto, R., Darusman, L.K., Lim, L.W., Takeuchi, T., 2015. Curcuminoid's content and fingerprint analysis for authentication and discrimination of *Curcuma xanthorrhiza* from *Curcuma longa* by high-performance liquid chromatography-diode array detector. Food Anal. Methods 8, 2185–2193.

Shanker, K., Singh, M., Srivastava, V., Verma, R.K., Gupta, A.K., Gupta, M.M., 2011. Simultaneous analysis of six bioactive lignans in *Phyllanthus* species by reversed phase hyphenated high performance liquid chromatographic technique. Acta Chromatogr. 23, 321–337.

Tan, W., Jaganath, I., Manikam, I., 2013. Evaluation of antiviral activities of four local Malaysian *Phyllanthus* species against Herpes simplex viruses and possible antiviral target. Int. J. Med. Sci. 10, 1817–1892.

Tang, D.Q., Li, Z., Jiang, X.L., Li, Y.J., Du, Q., Yang, D.Z., 2014. Fingerprint analysis and multi-ingredient quantitative analysis for quality evaluation of Xiaoyanlidan tablets by ultra high performance liquid chromatography with diode array detection. J. Sep. Sci. 37, 2131–2137.

Taiwo, I.A., Oboh, B.O., Francis-Garuba, P.N., 2009. Haematological properties of aqueous extracts of *Phyllanthus amarus* (Schum and Thonn.) and *Xylopia aethiopica* (Dunal) a rich in albino rats. Stud. Ethno-Med. 3, 99–103.