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Analysis of lignans from *Phyllanthus niruri* L. in plasma using a simple HPLC method with fluorescence detection and its application in a pharmacokinetic study

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Abstract

A simple analytical method using HPLC with fluorescence detection was developed for the simultaneous determination of four lignans, phyllanthin (**1**), hypophyllanthin (**2**), phyltetralin (**3**) and niranthin (**4**) from *Phyllanthus niruri* L. in plasma. The method recorded limits of detection for **1**, **2**, **3** and **4** as 1.22, 6.02, 0.61 and 1.22 ng/ml, respectively, at a signal-to-noise ratio of 5:1 whereas their limits of quantification were 4.88, 24.41, 4.88 and 9.76 ng/ml, respectively, at a signal-to-noise ratio of 12:1. These values were comparable to those of other sensitive methods such as gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography–MS (HPLC–MS) and HPLC–electrochemical detection (HPLC–ECD) for the analysis of plasma lignans. A further advantage over known methods was its simple protocol for sample preparation. The within-day and between-day accuracies for the analysis of the four lignans were between 87.69 and 110.07% with precision values below 10.51%. Their mean recoveries from extraction were between 91.39 and 114.67%. The method was successfully applied in the pharmacokinetic study of lignans in rats. Following intravenous administration, the lignans were eliminated slowly from the body with a mean clearance of 0.04, 0.01, 0.03 and 0.02 l/kg h and a mean half-life of 3.56, 3.87, 3.35 and 4.40 h for **1**, **2**, **3** and **4**, respectively. Their peak plasma concentration upon oral administration was 0.18, 0.56, 0.12 and 0.62 μg/ml, respectively, after 1 h. However, their absorption was incomplete with a calculated absolute oral bioavailability of 0.62, 1.52, 4.01 and 2.66% for **1**, **2**, **3** and **4**, respectively.
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Keywords: *Phyllanthus niruri* L.; Euphorbiaceae; Lignans; HPLC–fluorescence detection; Pharmacokinetic study; Absolute oral bioavailability

1. Introduction

Phyllanthus niruri L. (Euphorbiaceae), known locally as "dukong anak", is a small herb, found in most tropical and subtropical regions. It has been traditionally used as expectorant, anti-febrile, anti-diarrhoea, a diuretic and a remedy for colic and kidney problems [1]. The plant contains a series of lignans [2–8], alkaloids [9–11], terpenoids [12] and tannins [13,14], which have been reported to be hepatoprotective [15], anti-inflammatory [16] and endothelin-1 antagonist [17]. They also inhibited HIV reverse transcriptase [18–20], viral hepatitis [21] and increased vinblastine cytotoxicity towards multidrug-resistant cancer cells [22]. Our previous study has shown that the lignans of *P. niruri* were able to reverse the plasma uric acid to normal in hyperuricemic animals [23].

Despite numerous reports on the biological activities of lignans in the *Phyllanthus* genus, there is still a lack of information on their bioavailability and pharmacokinetics. To date, there is only one report on the pharmacokinetic of retrojusticidin B from *P. myritifolius*, where its plasma level was measured by a photodiode array (PDA) spectrophotometer [24]. In contrast, there are several reports of other plants on lignans analysis in the biological samples using gas chromatography (GC), high-performance liquid chromatography–ultraviolet detection (HPLC–UV), HPLC–electrochemical detection (HPLC–ECD), HPLC–mass spectrometry (HPLC–MS) and GC–MS [24–33].

Though HPLC–PDA and HPLC–UV have been used routinely, they are relatively insensitive especially for the detection of very low level of chemical constituents found in the plasma. On the other hand, even though GC–MS and HPLC–MS

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methods are highly sensitive and reliable, their methodologies are laborious and expensive. Such instruments are also not easily accessible [31,32]. These methods may often require sample clean up or pre-treatment such as hydrolysis, pre-purification and derivatization. HPLC–ECD has limited use in plant lignan analysis because relatively few lignans have free phenolic hydroxyl groups as they are either methylated or replaced as methylendioxy groups [32]. Thus, there is a need for the development of a rapid, simple and reliable method for the analysis of lignans in plasma. The present study has developed a simple and sensitive HPLC–fluorescence detection method for the simultaneous plasma determination of four lignans, phyllanthin (**1**), hypophyllanthin (**2**), phyltetralin (**3**) and niranthin (**4**) from *P. niruri* L. The method was then applied for the pharmacokinetic study of the lignans in rats.

2. Experimental

2.1. Chemicals and reagents

The HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Sodium heparin was purchased from Acros Organic (NJ, USA). Deionized water for HPLC was prepared in-house using a Maxima ultra pure water purifier (Elga, England) system.

2.2. Preparation of lignan enriched P. niruri extract and isolation of lignans

The dried powdered leaves of *P. niruri* were Soxhlet-extracted repeatedly with fresh methanol for 5 days. The pooled extracts upon removal of the solvent by evaporation under partial vacuum yielded a greenish residue that was subsequently passed onto a column of styrene-divinylbenzene resins (Diaion HP 20, Mitsubishi, Japan), and was next eluted by a stepwise percentage increase of MeOH in water (0, 30, 50, 80 and 100%, each 2.5–4.0 l). The eluates collected from 80% of MeOH in water and 100% of MeOH were pooled and evaporated to dryness under partial vacuum to produce the lignan enriched *P. niruri* extract. Four lignans, phyllanthin (**1**), hypophyllanthin (**2**), phyltetralin (**3**) and niranthin (**4**) (Fig. 1) were isolated from this extract following the protocol described previously [23] and were used as external standards for method validation and quantification of the plasma samples. The purity of the compounds was determined using a Waters Delta Prep HPLC system equipped with a Waters 2996 photodiode array detector and an Empower 2 workstation (Waters, Milford, MA, USA).

2.3. Instrumentation

The HPLC system consisted of an Agilent 1100 Series HPLC system and was equipped with a solvent delivery pump connected to a Rheodyne 7725i (Cotati, CA, USA). A fluorescence detector was set at an excitation wavelength of 280 nm and an emission wavelength of 344 nm. An Inertsil ODS-3 column (4.6 i.d. × 250 mm, GL Sciences Inc., Tokyo, Japan) was used with the following analytical conditions: a mobile phase of acetonitrile-water (55:45), a flow-rate of 1.0 ml/min at room temperature (25 °C). Samples were quantified following an external standard method and their peak heights were determined by a ChemStation A.08.03 software (Agilent Technologies, Waldbronn, Germany) for data acquisition.

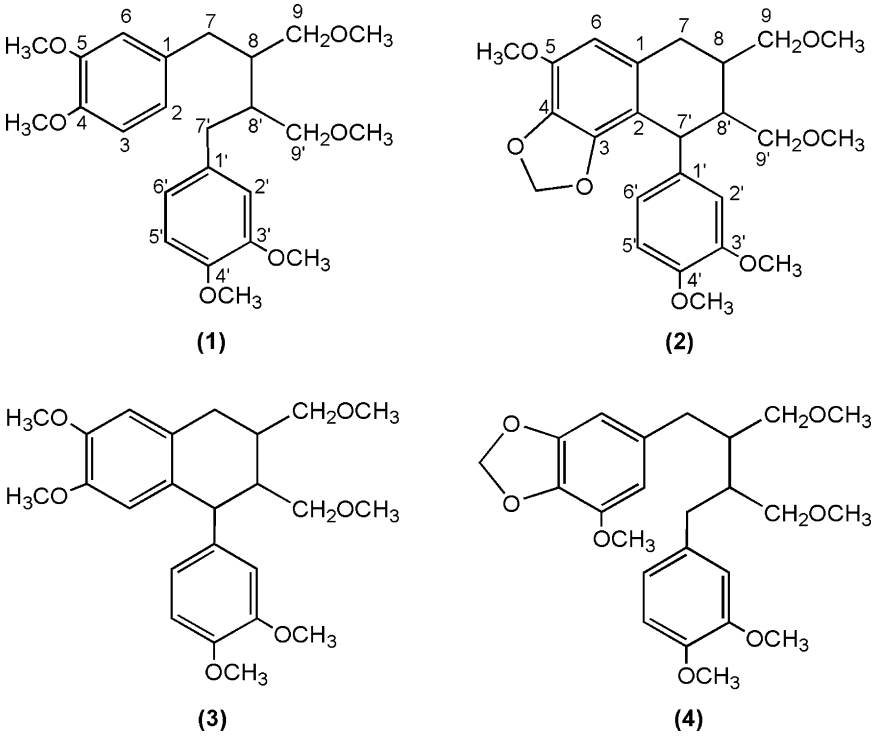

Fig. 1. Chemical structures of lignans isolated from *P. niruri*.

2.4. Sample preparation

A 100 μl aliquot of plasma sample was measured accurately in a microcentrifuge tube and deproteinized by adding 100 μl of acetonitrile. The mixture was vortex-mixed for 1 min using a Maxi Mix II Vortex Mixer (Thermolyne, IA, USA) and then centrifuged at 7711 × *g* (Hanil Science Industrial, Inchun, Korea) for 15 min. The supernatant was transferred into a new microcentrifuge tube and a 20 μl volume was injected onto the column.

2.5. Limit of detection (LOD), limit of quantification (LOQ) and linearity

Stock solutions (50 μg/ml) of **1**–**4** were prepared in pooled blank rat plasma. LOD and LOQ were determined by injections of successive two-fold dilution of the stock solutions in blank rat plasma. The LOD was defined as the lowest concentration that the analytical system can reliably differentiate from the background level, whilst the LOQ was defined as the lowest quantifiable concentration that can be measured with a stated level of confidence [34]. The calibration curve of a lignan standard was constructed as plots of peak height against the corresponding concentration at a range of 4.88–2500 ng/ml for **1** and **3**, 9.76–5000 ng/ml for **4** and 24.41–12,500 ng/ml for **2**. The linearity of the curve was evaluated by linear regression analysis.

2.6. Method validation

A 10 μg each of **1** and **3**, a 20 μg of **4** and a 50 μg of **2** were dissolved in 1 ml of methanol, to form a stock solution of mixed standards. From the stock solution, a series of working standard solutions were prepared by dilution with pooled blank rat plasma. Working solutions for **1** and **3** were each prepared as 4.88, 9.77, 39.06, 156.25, 625 and 2500 ng/ml, for **2** as 24.41, 48.83, 195.32, 781.25, 3125 and 12,500 ng/ml and for **4** as 9.76, 19.53, 78.13, 312.50, 1250 and 5000 ng/ml. These diluted solutions were used to determine the recovery, accuracy and precision of within-day and between-day.

A separate standard curve of the lignans in plasma was constructed on each day of analysis. The within-day accuracy and precision were determined for each compound at five concentrations with six replicates each carried out in a single day, whilst the between-day values were carried out over 6 consecutive days. Accuracy was expressed as a percentage error, whereas precision was expressed as a coefficient of variation. The recovery of the direct extraction method using acetonitrile was calculated by comparing the peak height of the lignan after extraction with that of its standard solution (in mobile phase) at similar concentration.

2.7. Animals

Male *Sprague–Dawley* rats of 12–16 weeks old, weighing 280–315 g were obtained from the animal house of the School of Pharmaceutical Sciences, Universiti Sains Malaysia. The animals were maintained on a 12-h light:12-h dark cycle, at room temperature of 25 °C and were allowed free access to standard food pellets (Gold Coin, Penang, Malaysia) and tap water. The study protocol was approved by the Animal Ethics Committee, Universiti Sains Malaysia, Penang, Malaysia.

2.8. Pharmacokinetic study of lignans

Six animals were used in the study conducted according to a two-way crossover study design. The animals were fasted overnight with free access to water prior to the experimentation. Food was only allowed after the sampling of blood at 4 h. On the first occasion, three rats (group 1) were randomized to receive 5 mg/kg of the lignan enriched *P. niruri* extract (containing 0.89, 0.20, 0.08 and 0.40 mg/kg of **1**, **2**, **3** and **4**, respectively) administered intravenously via the tail vein, while the other three rats (group 2) were given orally 50 mg/kg of the extract (containing 7.94, 1.76, 0.60 and 3.45 mg/kg of **1**, **2**, **3** and **4**, respectively). After a washout period of 2 weeks, the animals from group 1 received 50 mg/kg of the lignan enriched *P. niruri* extract orally, whilst those from group 2 were given 5 mg/kg intravenously. For both routes of administration, the lignan enriched extract was prepared in an aqueous solution of Tween 20. The volume for intravenous injection was 1 ml/kg whereas for oral route, it was 5 ml/kg of body weight. The rats were placed in animal restraining cages during blood collection and blood samples of 0.5 ml were withdrawn from the tail vein at 0, 30 min, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10 and 24 h after intravenous injection or at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 h after oral administration. The blood samples were collected into microcentrifuge tubes containing approximately 10 mg heparin; centrifuged at 4000 × *g* and the resulting plasma samples were kept frozen at −20 °C prior to HPLC analysis.

2.9. Data analysis

The following pharmacokinetic parameters were estimated from the data of intravenous (i.v.) administration: elimination rate constant (K_e), biological half-life ($t_{1/2}$), volume of distribution (V_d), area under plasma concentration–time curve ($AUC_{0\to\infty}$) and clearance (CL). The K_e (h^{-1}) was calculated form the slope of the plasma concentration versus time curve; $t_{1/2}$ (h) was calculated using the relationship, $t_{1/2} = \ln 2/K_e$ while V_d (l/kg) was calculated from the relationship, $V_d = \text{dose}/K_e \times AUC_{0\to\infty}$. The value of $AUC_{0\to\infty}$ (μg h/ml) was determined by adding the area from time zero to the last sampling time ($AUC_{0\to t}$) to the area from last sampling time to infinity ($AUC_{t\to\infty}$). CL (l/kg h) was calculated from the relationship, $CL = \text{dose}/AUC_{0\to\infty}$. Peak concentration ($C_{max}$) and time to reach C_{max} (T_{max}) following oral administration were obtained from the actual data. The absolute bioavailability of the lignans was estimated from the following equation:

$$\text{Absolute bioavailability } (F) = \frac{\text{AUC for oral}}{\text{AUC for i.v.}} \times \frac{\text{i.v. dose}}{\text{Oral dose}} \times 100\%$$

3. Results and discussion

The chromatograms of blank rat plasma, plasma spiked with mixed standards of phyllanthin (**1**), hypophyllanthin (**2**), phyltetralin (**3**) and niranthin (**4**), lignan enriched *P. niruri* extract at 20 μg/ml and plasma sample from a rat after 1 h administration of 5 mg/kg lignan enriched *P. niruri* extract are shown in Fig. 2A–D, respectively. A well-resolved chromatogram of the lignans was obtained following the use of the present HPLC–fluorescence conditions. The total run time was approximately 37 min. The lignans were unambiguously identified as **1**–**4** in the plasma upon comparison of the retention times with those of their respective standards. Their peaks at retention time after 20 min were clearly separated and free of interference from endogenous compounds in the rat plasma. Deproteinization with acetonitrile gave a higher recovery of more than 87% when compared with that for perchloric acid of only 2.2%. The lower recovery by perchloric acid deproteinization could be due to degradation of the compounds by the acid.

The calibration curve over a concentration range from 4.88 to 2500 ng/ml was linear for **1** and **3** with a mean slope (±standard error of mean) of 0.228 ± 0.004 and 0.637 ± 0.022, respectively, and a mean intercept value of −1.233 ± 0.588 and −7.259 ± 3.152, respectively, for **2** was linear from 24.41 to 12,500 ng/ml with a mean slope of 0.028 ± 0.004 and mean intercept value of 2.598 ± 2.937, whereas for **4** was linear from 9.76 to 5000 ng/ml with a mean slope of 0.134 ± 0.002 and mean intercept value of −1.861 ± 0.649. In addition, the correlation coefficient values of all four lignans were greater than 0.999. Because of an anticipated large difference in plasma level of the lignans, a wider analytical range was used in the present study to cover the entire range of expected concentrations.

The LOD values of the lignans were approximately 0.61 ng/ml for **3** (equivalent to 1.47 nM), for **2** was approximately 6.10 ng/ml (equivalent to 14.19 nM) and for **1** and **4** were approximately 1.22 ng/ml (equivalent to 2.91 and 2.84 nM for **1** and **4**, respectively) at a signal-to-noise ratio of 5. The LOQ values for **1** and **3** were 4.88 ng/ml (equivalent to 11.65 and 11.73 nM for **1** and **3**, respectively), for **2** was 24.41 ng/ml (equivalent to 56.77 nM) and for **4** was 9.76 ng/ml (equivalent to 22.70 nM) at a signal-to-noise ratio of 12. Wilkinson et al. [33] reported that the GC–MS methods for the analysis of lignans in biological fluids had limits of detection between 0.04 and 10 nM whereas for HPLC–MS, it was less than 5 nM. Furthermore, Smeds and Hakala [30] reported the limits of detection for the analysis of plasma lignans using an HPLC–MS method were between 0.021 and 0.2 ng/ml. Another sensitive HPLC method using coulometric electrode array detection had detection limits between 1.9 and 3.9 nM for lignan analysis in human urine [35]. In comparison to these sensitive methods often used for analysis of the other lignans in biological fluids, the present HPLC–fluorescence method has comparable detection and quantification limits, together with an additional advantage of simple sample preparation using direct deproteinization.

The recovery, within-day and between-day precision and accuracy for measurements of the lignan concentrations are shown in Table 1. The present method produced a satisfactory recovery of the lignans from 91.39 to 114.67%, thus implying that deproteinization of the plasma with acetonitrile did not result in any substantial loss of the chemical constituents. The coefficient of variation (CV) and percentage error of both within-day and between-day precision and accuracy were between 0.67–10.51% and 87.69–110.07%, respectively, indicating that the method was reliable and reproducible.

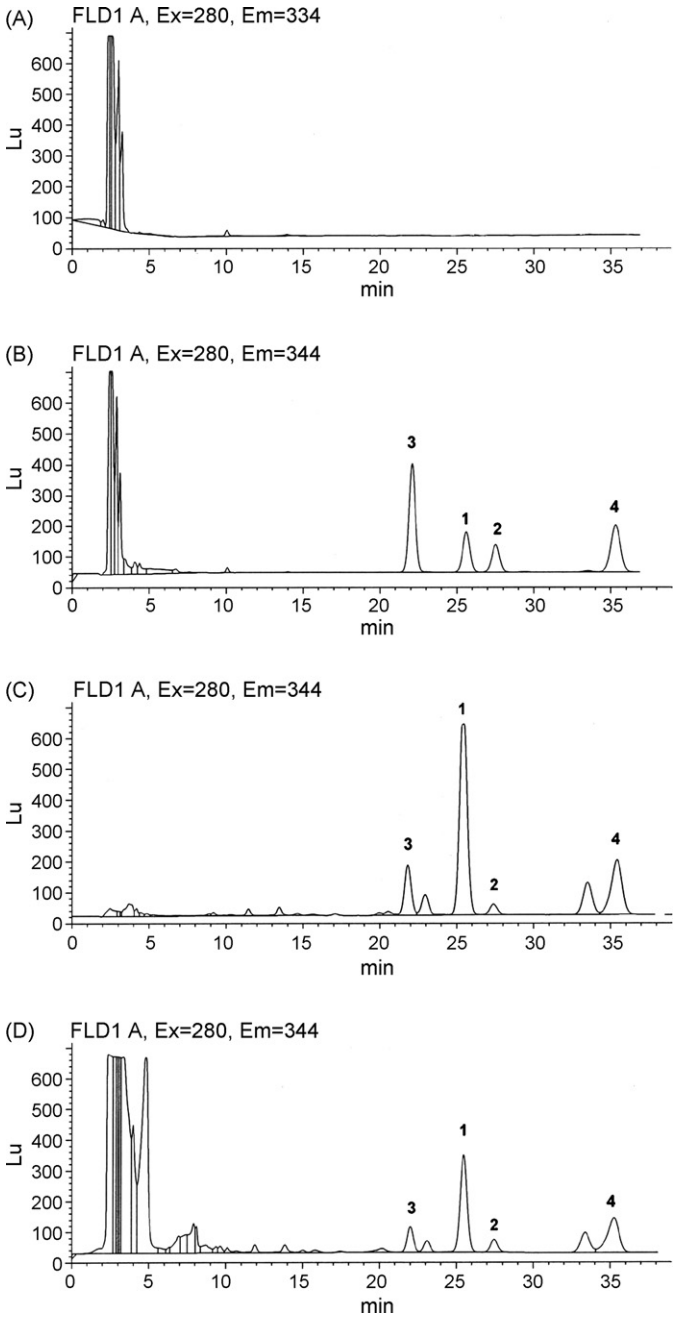

Fig. 2. Chromatograms from the analysis of phyllanthin (**1**), hypophyllanthin (**2**), phyltetralin (**3**) and niranthin (**4**). (A) Blank rat plasma; (B) rat plasma spiked with 625.5, 3125, 625.5 and 1250 ng/ml of **1**, **2**, **3** and **4**, respectively; (C) lignan enriched *P. niruri* extract at 20 μg/ml; (D) a rat plasma at 1 h after intravenous administration of 5 mg/kg lignan enriched *P. niruri* L. extract (containing 0.89, 0.20, 0.08 and 0.40 mg/kg of 1, 2, 3 and 4, respectively). Column, Inertsil ODS-3 (250 mm × 4.6 mm i.d., 5 μM); column temperature, 25 °C; mobile phase, acetonitrile–water (55:45); flow rate, 1.0 ml/min; excitation wavelength, 280 nm; emission wavelength, 344 nm.

Table 1
Recovery, within-day and between-day precision and accuracy values for phyllanthin (**1**), hypophyllanthin (**2**), phyltetralin (**3**) and niranthin (**4**) ($n = 6$)

Concentration (ng/ml)	Recovery ($n = 3$)		Within-day ($n = 6$)		Between-day ($n = 6$)	
	Mean (%)	Mean CV (%)	Accuracy (% of true value)	Precision CV (%)	Accuracy (% of true value)	Precision CV (%)
Phyllanthin (**1**)						
2500	105.47	1.42	100.06	1.85	100.79	1.61
625.50	100.63	1.15	101.12	0.89	99.31	3.77
156.25	94.38	6.13	97.47	2.27	98.41	3.35
39.06	99.01	1.35	102.18	2.25	96.47	2.75
9.77	99.07	7.11	100.80	5.08	100.55	7.64
4.88	93.01	7.57	89.68	5.51	91.59	4.64
Hypophyllanthin (**2**)						
12,500	109.04	1.62	99.81	1.45	101.00	1.72
3125	102.99	0.81	98.05	0.77	97.67	3.90
781.25	106.77	7.98	98.50	2.30	100.60	5.13
195.31	96.48	0.57	99.98	4.48	100.90	5.16
48.83	113.04	9.45	106.71	10.51	110.07	9.45
24.41	104.34	1.58	101.64	5.07	99.22	4.87
Phyltetralin (**3**)						
2500	106.56	1.40	99.90	0.94	100.62	1.53
625.50	102.20	0.87	102.19	0.67	98.80	3.64
156.25	95.46	5.79	100.48	3.13	99.83	2.16
39.06	91.39	0.60	98.05	1.27	94.23	2.80
9.77	107.56	7.58	106.57	4.86	87.82	3.24
4.88	99.89	8.16	87.63	5.00	87.69	6.79
Niranthin (**4**)						
5000	106.07	1.47	101.08	1.55	102.20	3.18
1250	101.15	1.07	99.78	0.89	100.09	3.31
312.50	92.51	3.45	97.95	3.67	98.01	1.95
78.13	97.16	2.33	102.76	3.02	95.95	2.54
19.53	114.67	5.81	100.63	2.37	99.04	10.43
9.76	111.24	5.99	98.47	5.81	94.30	7.62

Figs. 3 and 4 show the mean plasma concentration versus time profiles of the lignans obtained after intravenous and oral administration of 5 and 50 mg/kg of lignan enriched *P. niruri* extract, respectively. It is particularly interesting to note that **3** being present in the extract at a much lower concentration than the other lignans **1**, **2** and **4**, was detected in the plasma at below 100 ng/ml level following oral administration (except for its maximum plasma concentration of 114.72 ng/ml). Using an HPLC–UV method developed previously with a quantification limit of 100 ng/ml, it was not possible to quantify **3** and therefore, an accurate pharmacokinetic analysis could not be done. However, the present HPLC–fluorescence method makes it possible to measure its concentrations in rat plasma even up to 10 h after oral administration. From Figs. 3 and 4, the plasma levels of **1**–**4** given intravenously were relatively much higher than those obtained after oral administration, even though their intravenous doses were approximately 10 times lower, indicating poor and incomplete oral bioavailability of the lignans.

The pharmacokinetic parameters of lignan **1**–**4** in rat plasma after intravenous and oral administration are shown in Table 2. Following intravenous administration, the lignans showed a gradual decline in its plasma concentrations. The estimated vol-

Table 2
Pharmacokinetic parameters of lignans in rat plasma after intravenous and oral administration of lignan enriched *Phyllanthus niruri* L. extract ($n = 6$)

	Phyllanthin (**1**)	Hypophyllanthin (**2**)	Phyltetralin (**3**)	Niranthin (**4**)
Intravenous administration				
$AUC_{0\rightarrow\infty}$ (μg h/ml)	22.44 ± 3.24	15.56 ± 2.25	2.38 ± 0.30	19.14 ± 2.97
K_e (h^{-1})	0.21 ± 0.03	0.21 ± 0.05	0.22 ± 0.02	0.21 ± 0.05
$t_{1/2}$ (h)	3.56 ± 0.47	3.87 ± 0.57	3.35 ± 0.39	4.40 ± 1.05
V_d (l/kg)	0.20 ± 0.03	0.17 ± 0.04	0.14 ± 0.03	0.15 ± 0.04
CL (l/kg h)	0.04 ± 0.01	0.01 ± 0.002	0.03 ± 0.01	0.02 ± 0.01
Oral administration				
$AUC_{0\rightarrow\infty}$ (μg h/ml)	1.25 ± 0.34	2.12 ± 0.44	0.75 ± 0.11	4.39 ± 0.73
C_{max} (μg/ml)	0.18 ± 0.03	0.56 ± 0.13	0.12 ± 0.01	0.62 ± 0.05
T_{max} (h)	1.00 ± 0.12	1.00 ± 0.23	1.00 ± 0.09	1.00 ± 0.09

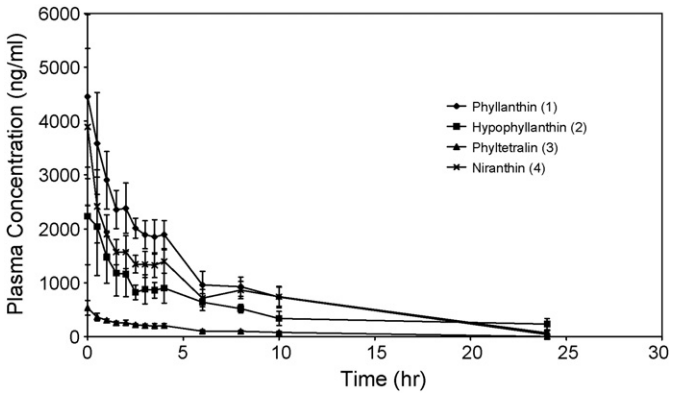

Fig. 3. Mean plasma concentration–time profiles (mean ± S.E.M., $n = 6$) of phyllanthin (**1**), hypophyllanthin (**2**), phyltetralin (**3**) and niranthin (**4**) after intravenous administration of 5 mg/kg of lignan enriched *P. niruri* extract (containing 0.89, 0.20, 0.08 and 0.40 mg/kg of **1**, **2**, **3** and **4**, respectively).

ume of distribution (V_d) of the lignans was relatively small with a mean value of 0.20, 0.17, 0.14 and 0.15 l/kg for **1**, **2**, **3** and **4**, respectively, suggesting that the lignans were not widely distributed into the tissue compartment. However, these lignans appeared to be cleared slowly from the body as evidenced by their small mean clearance values of 0.04, 0.01, 0.03 and 0.02 l/kg h for **1**, **2**, **3** and **4**, respectively. Their corresponding longer half-lives between 3.35 and 4.40 h may suggest that those of the lipophilic lignans (derived from the hexane fraction of the *P. niruri* extract) able to distribute into the tissue compartment were retained and eliminated slowly, despite having a small volume of distribution.

Following an oral administration, all four lignans showed a rapid rise with a T_{max} of 1 h followed by a gradual decline to 0 after 24 h. Their mean C_{max} values were 0.18, 0.56, 0.12 and 0.62 μg/ml for **1**, **2**, **3** and **4**, respectively. These results were comparable to those obtained for retrojusticidin B, which showed a maximum plasma concentration between 45 and 75 min after oral administration [24]. Being lipophilic, the dissolved lignans could penetrate the gastrointestinal tract more easily achieving their peak plasma concentration after 1 h. However, their absorption after oral administration was incomplete as evidenced by a much smaller AUC, being 18, 7, 3 and 4 times lower for **1**, **2**, **3** and **4**, respectively, compared to those from intravenous administration despite their oral doses being 10 times higher. The calculated absolute bioavailability values for **1**, **2**, **3** and **4** were 0.62, 1.52, 4.01 and 2.66%, respectively. The low oral bioavailability may be contributed by their poor aqueous solubility causing only a small fraction available in dissolved form for absorption after oral administration. These findings merits further investigation for the development of more orally bioavailable lignans.

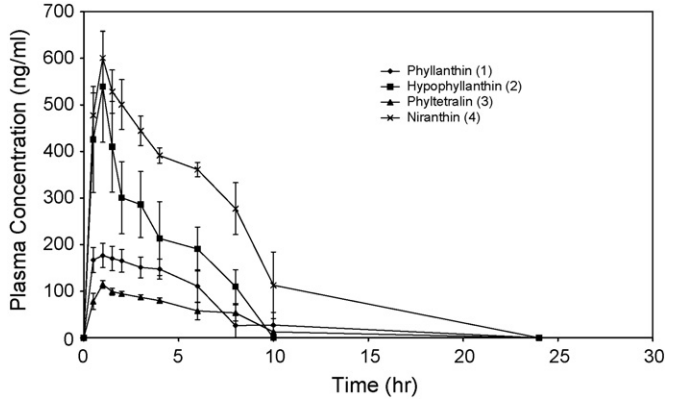

Fig. 4. Mean plasma concentration–time profiles (mean ± S.E.M., $n = 6$) of phyllanthin (**1**), hypophyllanthin (**2**), phyltetralin (**3**) and niranthin (**4**) after oral administration of 50 mg/kg of lignan enriched *P. niruri* extract (containing 7.94, 1.76, 0.60 and 3.45 mg/kg of **1**, **2**, **3** and **4**, respectively).

4. Conclusion

A simple and sensitive HPLC–fluorescence detection method for the simultaneous plasma determination of four lignans from *P. niruri* has been developed. The method was accurate and precise for the quantitative analysis of lignans. Additional advantages over known methods are related to its simple sample preparation, low detection and quantification limits. The method was successfully applied in the pharmacokinetics study of the lignans of *P. niruri* in rats. Following intravenous administration, the lignans appeared to be poorly distributed and cleared slowly from the body whilst following oral administration the lignans were absorbed rapidly. Their absorption was incomplete with calculated absolute oral bioavailability values of 0.62, 1.52, 4.01 and 2.66% for **1**, **2**, **3** and **4**, respectively.

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