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Short communication

Validation of a LC method for the analysis of phenolic compounds from aqueous extract of *Phyllanthus niruri* aerial parts

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Abstract

A reversed-phase high-performance liquid chromatographic separation and quantitative method using a phosphoric acid-acetonitrile gradient was developed to analyze phenolic compounds present in aqueous extract from the aerial parts of *Phyllanthus niruri*. The chromatographic method was validated for linearity, precision and accuracy for both reference substance (gallic acid) and for three well resolved peaks from *P. niruri* aqueous extract. Both calibration curves were linear with correlation coefficients higher than 0.999. The reproductibility for the three peaks ranged from 2.3% to 4.6% and the accuracy for gallic acid in the aqueous extract was 103%. The method allowed the complete resolution of three peaks, one of them was identified by diode array detection as gallic acid. The analysis of the botanic morphological elements of the aerial parts from *P. niruri* showed that the leaves have a higher amount of phenolic compounds than the branches. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Phyllanthus niruri*; HPLC assay; Phenolic compounds

1. Introduction

Phyllanthus niruri L. (Euphorbiaceae) is a medicinal plant widely distributed, and is largely used in folk medicine to treat kidney stones and viral hepatitis [1,2]. Pharmacological experiments confirm its therapeutic efficacy and safety [1,3–5]. Intensive phytochemical examinations of this plant have been carried out. Constituents such as alkaloids, flavonoids, lignans, tannins, phenols and terpenes have been identified. However, the composition of the aqueous extract, as used for medicinal purposes, has not been adequately studied. Although the specific compounds have not been precisely defined, some research results credit the therapeutic action on urinary tract stones to the phenols [1,6]. Valid quality control methods need to be developed in order to comply with regulatory requirements if this plant is to be used by the pharmaceutical industry [7,8].

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Several high performance liquid chromatographic (HPLC) methods have been reported to quantify phenolic compounds in complex biological matrices such as herbal raw materials, extracts and food products [9–13]. However, currently there is no method with adequate resolution to quantify substances present in the aqueous extractives of *P. niruri*. Therefore the aim of this work is to validate a HPLC method applied to the quality control of *P. niruri* both as raw material and as a technological intermediate product.

2. Experimental

2.1. Plant material

P. niruri aerial parts were delivered by the 'Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas' from the Campinas State University, SP, Brazil. The plant was dried at 40 °C for a week in an air oven. The separated leaves and branches were reduced in a knife mill (Retsch SK1).

2.2. HPLC analysis

The analyses were carried out using a Shimadzu liquid chromatograph equipped with a pump (LC-10 AD), a gradient controller (FCV-10 AL), an autosampler (SIL-10 A) and a UV/vis detector (SPD-10 A), controlled by CLASS LC-10 software. The column was a RP-18 LiChrospher 250 × 4 mm i.d., 5 μm particle diameter (Merck, Darmstadt, Germany). A pre-column Shimadzu (10 × 4 mm i.d.) packed with Bondapak C18 125 Å (Waters, Milford, USA) was employed. A Waters diode array detector was used to check the peak purity of each compound. The absorbance was measured from 200 to 800 nm every 1 s with 4.8 nm resolution.

2.2.1. Chemicals and reagents

Acetonitrile (HPLC grade, Merck, Darmstadt, Germany), phosphoric acid (Merck, Darmstadt, Germany) and ultrapure water from Milli-Q system (Millipore, Bedford, USA) with conductivity of 18 MΩ were used for the mobile phase preparation. Gallic acid (Reag. Ph. Eur., Merck, Darmstadt, Germany) was used as external standard.

2.2.2. Chromatographic conditions

The chromatographic separation was carried out using a mobile phase with phosphoric acid 1% (w/w) as solvent A and acetonitrile: phosphoric acid 1% (w/w) (50:50 (v/v)) as solvent B at a flow-rate of 0.6 ml min^{-1}. The gradient program was as follows: 22–24% B (7 min), 24–40% B (10 min), 40–100% B (8 min), 100–22 B% (15 min). The injection volume was 20 μl. The peaks were detected at 275 nm. Chromatographic peaks were identified by comparison of the retention time with standard gallic acid and by diode array spectra.

2.2.3. Samples preparation

2.2.3.1. Extract preparation and calibration curve. The aqueous extract from *P. niruri* 7.5% (w/v) was obtained by decoction of the aerial parts for 15 min. For the calibration curve, a stock solution (4.0 ml of aqueous extract in 100.0 ml water) was diluted with acetonitrile: water (20:80, v/v) yielding concentrations of 1.6, 3.2, 4.8, 6.4 and 8.0 μl ml^{-1}.

2.2.3.2. Gallic acid calibration curve. Gallic acid was dissolved in acetonitrile:water (20:80, v/v) to produce concentrations of 0.2, 0.4, 0.8, 1.2 and 1.6 μg ml^{-1}. The samples were filtered through 0.45 μm membrane (Millipore, Bedford, USA) prior to injection. Each analysis was repeated three times and the calibration curves were fitted by linear regression.

2.3. Validation parameters

2.3.1. Linearity

The linearity of the HPLC calibration curves was determined for both the gallic acid standard preparation and the three peaks of the extract from *P. niruri* over a 3 days period. The slope and the other statistical parameters of the calibration curves were calculated by linear regression.

2.3.2. Reproductibility and intermediate precision

The reproductibility was evaluated on the same day, for each sample, while the inter-day precision was assessed for 3 consecutive days. The data were expressed as the relative standard deviation (R.S.D.%).

2.3.3. Accuracy

The accuracy was evaluated through recovery studies by adding known amounts of the gallic acid solution to the extract with a concentration of 4.8 μl ml^{-1}. Controls from all samples were prepared and analyzed. The recovery experiment was performed at three concentrations of standard gallic acid (low, medium and high) [14].

2.3.4. Detection limit

The detection limit was determined from the calibration curve of the standard gallic acid [14].

2.4. Analysis of leaves and branches of P. niruri

The aqueous extracts of the leaves and branches were prepared by decocotion as described above.

3. Results and discussion

A variety of solvent systems based on acetonitrile, methanol and phosphoric acid were tested to improve the separation of phenolic compounds in the aqueous extract of *P. niruri*. A gradient system was chosen because of the matrix complexity. The mobile phase acetonitrile:water was found to be adequate, although phosphoric acid was essential for a good resolution of the chromatogram.

The coelution of substances absorbed by the column packing is a frequent problem in the HPLC analysis of complex mixtures, such as vegetable raw material. This is due to the negative influence of the saturation of the chromatographic column by nonpolar substances [15]. In order to clean the column and detector and reduce this negative influence, a gradient method was designed using a significantly high organic concentration of the mobile phase prior to return to initial conditions.

The chromatographic profile (Fig. 1) of the extract from *P. niruri* showed three resolved peaks ($Rs_{1-2} = 1.61$; $Rs_{2-1} = 1.61$; $Rs_{3-2} = 2.66$). Peak 1 with a retention time of 6.6 min was identified through comparison of diode array (DAD) spectra and co-cromatography as gallic acid. The DAD spectrum of peak 2 (Fig. 2), with a retention time of 20.2 min, can be assigned to a flavone [16] and peak 3, with a retention time of 21.5 min as a gallic acid derivative.

The chromatographic method was also evaluated for linearity, precision and accuracy. The linearity of the method was determined through the gallic acid calibration curve and the calibration curve of the corresponding peak of the extract from *P. niruri* on three different days (Table 1). When gallic acid was used as a reference substance, the method showed a highly significant

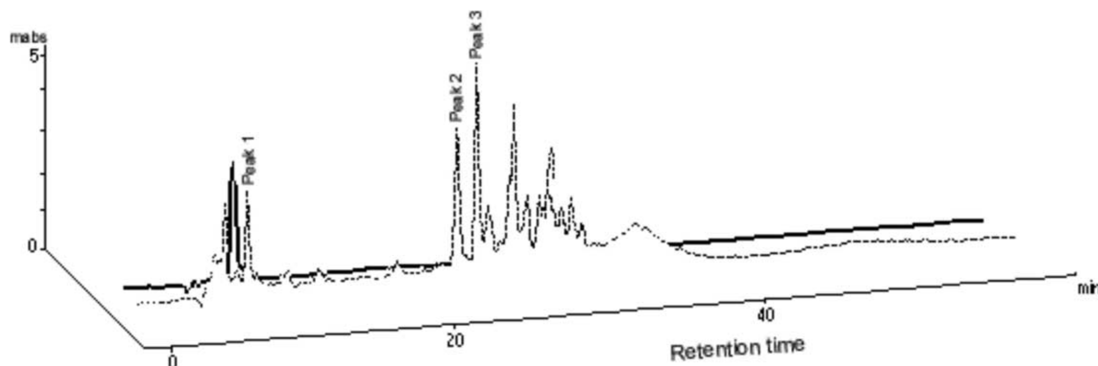

Fig. 1. HPLChromatogram of *P. niruri* extract (---) and galic acid (–).

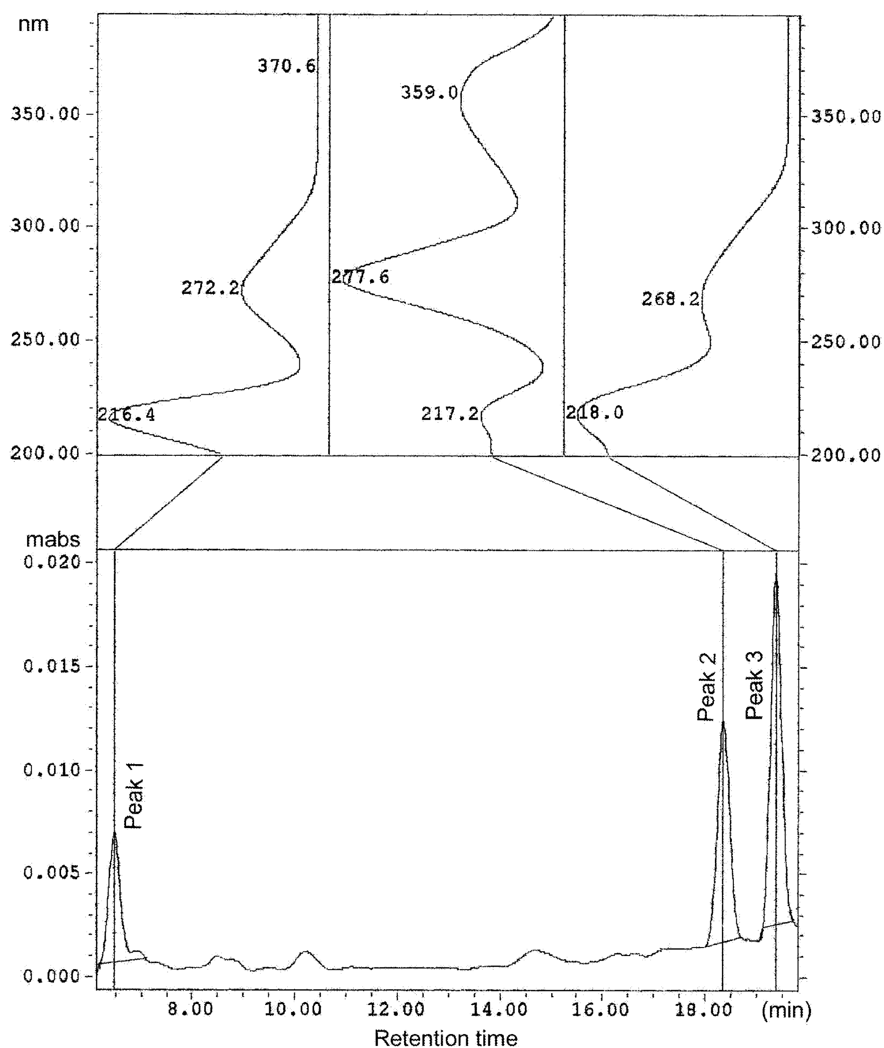

Fig. 2. DAD spectra of three studied HPLC peaks from *P. niruri* extract.

correlation coefficient (0.9991) with no significant deviation from linearity and very low detection limit (0.13 μg ml^{-1}). The R.S.D. of the slope was of 3.2%.

The regression curves for the peaks of the extract from *P. niruri* revealed a correlation coefficient greater than 0.999 (R.S.D. > 0.1%) and no significant deviation from linearity. The R.S.D. of the slopes of the curves from the extract were 2.7, 5.9 and 4.4% for peak 1–3, respectively. These results indicate the method has a high linearity.

The reproducibility test demonstrated that the analyzed peaks presented a R.S.D. range from 2.3 to 4.6% (Table 2). This data could be considered

Table 1
Linearity parameters for the HPLC peaks of *P. niruri* extract

Peak	r^2	P-value	a	b
1	0.9999	2.8×10^{-6}	−3543.4	178.1
2	0.9998	1.8×10^{-7}	−4373.2	317.8
3	0.9999	2.4×10^{-7}	−4126.6	374.8

r^2, Regression coefficient; a, slope; b, intercept.

Table 2
Reproducibility and intermediate precision analysis for the reference substance (gallic acid) and *P. niruri* extract

Peak	Concentration (μg ml^{-1})	Retention time (min) $\bar{X}$; R.S.D. (%)	Mean area (mV s^{-1})	Reproducibility R.S.D. (%)	Intermediate precision R.S.D. (%)
Gallic acid	0.82	6.69; 0.95	76274.9	0.05–0.08	0.47
1	0.60	6.61; 0.39	55955.2	3.7–4.6	5.7
2	1.11[a]	20.24; 0.55	104109.6	2.5–3.8	7.2
3	1.33[a]	21.52; 0.43	125190.5	2.3–2.9	6.9

[a] Calculated as gallic acid.

Table 3
Results of recovery studies for gallic acid of the extract from *P. niruri*

Theoretical concentration (μg ml^{-1})	Experimental concentration (μg ml^{-1})	Recovery (%) ($\bar{X}$; CV %)	Total recovery (%) ($\bar{X}$; CV%)
1.09	1.12	102.44; 0.52	
1.56	1.62	103.85; 0.62	103.50; 0.89
2.05	2.14	104.23; 0.27	

satisfactory since the majority of similar research data suggests a R.S.D. up to 6% as acceptable [17]. The inter-day precision for the peaks was lower than 7.2%. R.S.D. values of 15% are considered appropriate when the matrix complexity of the extract is taken into consideration [18]. The Anova showed no statistical difference ($\alpha = 0.001$) between the areas of peaks obtained on different days, confirming acceptable pprecision. Furthermore, the method was evaluated with respect to qualitative aspects, taking into account the precision in retention time and elution purity of the peaks of the extract from *P. niruri*. A high reproducibility in the retention time was obtained with relative standard deviations less than 1% in all cases (Table 2). The elution purity was verified through the spectra taken from the upward slope, the apex and the downward slope of the three peaks of interest. Peak 1 was compared with standard gallic acid and no peak contamination was observed (Fig. 2).

To verify sample stability throughout the analysis time and the behavior of the extract of *P. niruri* under the studied conditions, the same sample was analyzed for 12 h. The results showed the same chromatographic profile during total analysis time and no degradations products were detected. In this experiment the concentrations of peaks presented a R.S.D. of 4.18 for peak 1, 3.65 for peak 2 and 2.19% for peak 3, which confirms reproducibility of the method (Table 2).

Recovery tests were performed to validate the method accuracy. Peak 1, previously identified as gallic acid, was used as a reference for this test. The method produced a medium recovery of 103% with R.S.D. below 2% in all analyzed concentrations (Table 3), confirming adequate accuracy. Furthermore the addition of gallic acid into the aqueous extract from *P. niruri* did not influence the area of other two peaks.

Table 4
Quantification of gallic acid and other major substances in aqueous extracts from the aerial parts of *P. niruri*

Aerial part	Concentration (μg ml^{-1})		
	Gallic acid	Peak 2	Peak 3
Leaves	177	424	534
Branches	14	17.5	13

The investigation of separate aqueous extracts of leaves and branches showed the same chromatographic behavior. Quantitatively the leaves presented a higher concentration of phenolic compounds than the branches (Table 4).

4. Conclusion

The high-performance liquid chromatographic method developed to analyze phenolic compounds in an aqueous extract of *P. niruri* was linear and reproducible for all selected peaks. The method was also accurate for the assay of gallic acid, suggesting its use as a suitable reference substance for quantitative analysis.

The validation procedure confirms that this is an appropriate method for the purpose of quality control when *P. niruri* is used either as raw material or an aqueous extract by the pharmaceutical industry.

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