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Short communication

Analysis of neolignans compounds of *Piper regnellii* (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck by HPLC

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Abstract

A high performance liquid chromatographic (HPLC) method was developed and validated for quantitative determination of neolignans in extracts of *Piper regnellii* var. *pallescens*. The analysis were carried out on a Metasil ODS column (150 mm × 4.6 mm, 5 μm) at 30 °C, using as mobile phase acetonitrile–water (60:40, v/v) containing 2% acetic acid. The flow rate was 1.0 ml/min and the detection was at 280 nm. The validation using conocarpan as standard demonstrated that the method presents linearity (linear correlation coefficient = 0.9991), precision (relative standard deviation <5%) and accuracy (mean recovery = 104.55%) in the concentration range 31.25–500 μg/ml. The limit of detection (LOD) was 1.68 μg/ml and the limit of quantitation was 5.60 μg/ml. This method allowed the identification and quantification of conocarpan, eupomatenoid-5 and eupomatenoid-6 in the hydroethanolic extracts obtained from the leaves, stems and roots by maceration process. All the extracts showed the same chromatographic profile, being that the extract of the roots presented the highest concentration of neolignans.

Keywords: *Piper regnellii*; Neolignans; HPLC; Validation

1. Introduction

Piper regnellii (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck, popularly known as "pariparoba", is a species belonging to Piperaceae family used in folk medicine, being the leaves and roots used in form of crude extracts, infusions or poultices in the treatment of wounds, swellings and skin irritations [1].

Benevides et al. [2] related the first phytochemical investigation carried out on the specie *P. regnellii* on the chemistry of lignans/neolignans. Among the isolated compounds from the ethyl acetate extracts of the roots of *P. regnellii*, are the neolignans conocarpan, eupomatenoid-3, eupomatenoid-5 and eupomatenoid-6 (Fig. 1).

Benzofuran neolignans represent a sub-class with a variety of biological activities including anti-PAF, antifungal and insecticidal activity. Several compounds of this class have been isolated from Piperaceae species and in case of *P. regnellii*, phytochemical studies of its roots showed the accumulation of several phenylpropanoids and benzofuran neolignans including conocarpan as the major compound [3].

Pessini et al. [1] isolated and identified the neolignans conocarpan, eupomatenoid-3, eupomatenoid-5 and eupomatenoid-6 from the hydroethanolic extract of the leaves of *P. regnellii*. Moreover, the antimicrobial activity of the compounds that demonstrated potential activity was evaluated, with exception of the compound eupomatenoid-3, that was inactive against bacteria and yeasts. The compounds eupomatenoid-5 and eupomatenoid-6 were active only against bacteria.

The analysis of neolignans of *P. regnellii* by HPLC is not much explored. There is a report of Benevides et al. [2] that uses the HPLC method to demonstrate that the profile of neolignans is similar in roots, stems and leaves of *P. regnellii*. Moreover, Sartorelli et al. [3] carried out analysis by HPLC to verify the separation of benzofuran neolignans from the ethyl acetate extract of the leaves of *P. regnellii*.

HPLC method is gaining increasing importance for qualitative and quantitative analysis of plant extracts, being useful for quality control of phytochemicals.

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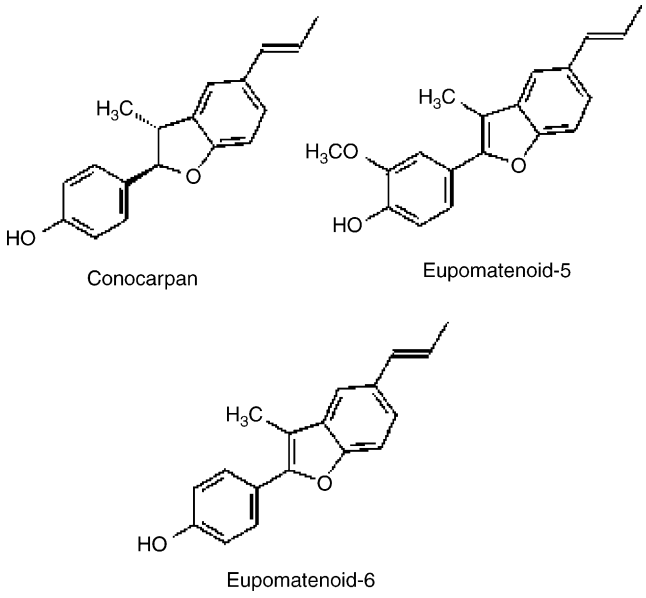

Fig. 1. Structures of the neolignans isolated from *P. regnellii*.

However, validated quality control methods need to be developed since the validation of analytical procedures is an important part of the registration application for a new drug. Besides the regulatory requirements, the performance and reliability of the control test procedure are essential to the quality control of drugs. Therefore, validation should be regarded as part of an integrated concept to ensure the quality, safety and efficacy of pharmaceuticals [4,5].

In this work, extraction, determination of the chromatographic conditions by HPLC method for analysis of neolignans in extracts of *P. regnellii*, validation of the method evaluation was developed.

2. Experimental

2.1. Plant material

The leaves, stems and roots of *P. regnellii* var. *pallescens*, were collected in September 2004 in the Medicinal Plants Garden "Prof[a]. Irenice Silva" of the State University of Maringá Campus, Maringá, PR, Brazil. The plant material was identified by Marília Borgo of the Botanical Departament of the Federal University of the Paraná. A voucher specimen (number HUM 11411) is deposited at the Herbarium of the State University of Maringá.

The samples of leaves, stems and roots of *P. regnellii* were dried at 35 °C in an air oven and were ground in a knife mill before extraction.

2.2. Extract preparation

Dried leaves, stems and roots of *P. regnellii* (10 g) were extracted with ethanol:water (9:1, v/v, 100 ml) by maceration method at room temperature for 5 days at dark room. The extracts were filtered, evaporated under vaccum at 40 °C and lyophilized.

2.3. HPLC analysis

2.3.1. Reagents and chemicals

Acetonitrile (HPLC grade from OmniSolv EM Science, Gibbstown, NJ), ultrapure water (Milli-Q system, Millipore, Bedford, USA) and acetic acid (analytical grade, Merck, Darmstadt, Germany) were used for the mobile phase preparation. Methanol (HPLC grade from OmniSolv EM Science, Gibbstown, NJ) was used for samples preparation. The neolignans conocarpan, eupomatenoid-5 and eupomatenoid-6 were isolated from the specie *P. regnellii* [1]. The conocarpan was used as external standard. Eupomatenoid-5 and eupomatenoid-6 were only used as reference to the corresponding peak in the sample extracts.

2.3.2. Sample preparation

Stock solutions of conocarpan, eupomatenoid-5 and eupomatenoid-6 and extracts of the leaves, stems and roots from *P. regnellii* were prepared in methanol at a concentration of 1000 μg/ml. The solutions were filtered through 0.45 μm membrane filter (Millipore, São Paulo, Brazil).

2.3.3. Instrumentation and chromatographic conditions

The analyses were carried out using a Shimadzu LC-10 liquid chromatograph equipped with quaternary pump (LC-10 AD), manual injection valve (Rheodyne) with loop of 20 μl, degasser (DEU-14), thermostatted column compartment (CTO-10Avp) and a UV–vis detector (SPD-10A), controlled by CLASS LC-10 Software.

In the chromatographic analysis was used Metasil ODS column, 5 μm, 150 mm × 4.6 mm, maintained at 30 °C. The separation was carried out in isocratic system, using as mobile phase a mixture of acetonitrile–water (60:40, v/v) containing 2% acetic acid, with flow rate of 1.0 ml/min. The detection was carried out at 280 nm and the running time was 25 min. The sample injection volume was 20 μl. Three determinations were carried out for each sample.

The statistical analysis of the data were performed by Statistica 6.0 software (Statsoft Inc., Tulsa, OK, USA).

2.4. Validation parameters

2.4.1. Linearity

The linearity of the calibration curve for the conocarpan was determined by the external standard method. Stock standard solution at a concentration of 1000 μg/ml was diluted in methanol yielding concentrations of 31.25 μg/ml, 62.5 μg/ml, 125 μg/ml, 250 μg/ml and 500 μg/ml. Three determinations were carried out for each solution. The calibration curves were obtained by plotting the peak area of the conocarpan versus the concentration of the standard solutions. The statistical parameters of the calibration curve as slope, intercept and correlation coefficient were calculated by linear regression analysis.

2.4.2. Precision

The repetibility of the method was evaluated on the same day while the intermediate precision was determined for 2 non-

consecutive days. The standard solution was analysed at three concentration levels (31.25 μg/ml, 125 μg/ml and 500 μg/ml). Three determinations were carried out for each solution. The precision was expressed as relative standard deviation (R.S.D.%) of the concentrations of conocarpan.

2.4.3. Limit of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curve of the standard conocarpan. LOD was calculated according to the expression $3\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. LOQ was established by using the expression $10\sigma/S$.

2.4.4. Accuracy

The accuracy was evaluated with the recovery test by analysing the mixture prepared by adding of the conocarpan solution at the three concentration levels (31.25 μg/ml, 125 μg/ml and 500 μg/ml) to extract of the leaves of *P. regnellii*, containing known amount of the analyte. Three determinations were carried out for each solution. The percentage recovery was calculated by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by 100.

2.4.5. Stability of the analyte during analysis

The stability was evalueted with standard solutions and sample solutions that were stored at 4 °C and at room temperature during 72 h. The solutions were analyzed every 24 h.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

To develop a HPLC method for the analysis of neolignans in *P. regnellii* extracts, several parameters were optimized to select the proper conditions. An isocratic system was chosen since allowed a good separation of neolignans within a short analysis time. To optimize the mobile phase different compositions of acetonitrile in water containing 2% of acetic acid were tested. The acetonitrile–water 60:40 (v/v) ratio showed to be adequated. The addition of acetic acid decreased the peak tailing of the neolignan eupomatenoid-5, being essencial to improve the resolution of the chromatogram. Flow rates between 0.6 ml/min and 1.0 ml/min were studied. The value of 1.0 ml/min allowed good separation with a analysis time of 25 min. The separation was further improved when column temperature was 30 °C. The maximum absorption of the neolignans was found to be 280 nm, and this wavelength was chosen for the analysis.

The Fig. 2 shows the chromatogram of standard mixture of neolignans. The chromatographic profile showed three well-resolved peaks ($Rs_{1-2} = 15.93$; $Rs_{2-1} = 15.93$; $Rs_{2-3} = 3.54$) when the chromatographic conditions described were employed. Peak 1 with a retention time of 11.23 min was identified as conocarpan. Peak 2 with a retention time of 16.63 min can be assigned to the eupomtenoid-6 and peak 3, with a retention time of 18.07 min as eupomatenoid-5.

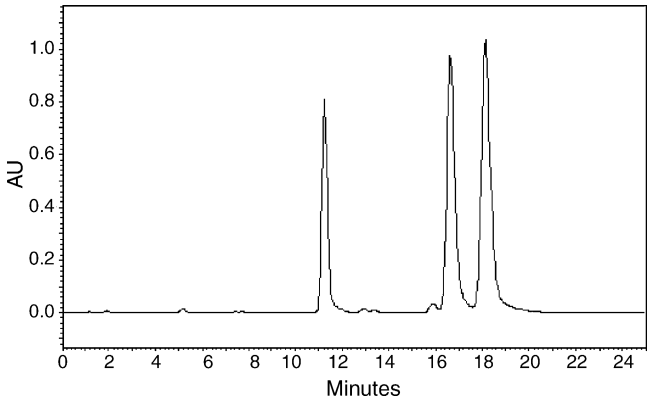

Fig. 2. Chromatogram of the standard mixture of neolignans: (1) conocarpan, (2) eupomatenoid-6 and (3) eupomatenoid-5. *Chromatographic conditions*: Metasil ODS column; mobile phase: acetonitrile/water (60:40, v/v) with 2% acetic acid; flow rate: 1.0 ml/min; temperature: 30 °C; detection: 280 nm.

3.2. Validation

For the validation of the analytical method, the guideline of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use was followed [6]. The conocarpan was used as standard because it can be found as the major compound in *P. regnellii* [3]. Moreover, the conocarpan presents high antimicrobial activity [1].

3.2.1. Linearity

Excellent linearity was observed for conocarpan between peak area and concentration in the range 31.25–500 μg/ml, as confirmed by the correlation coefficient of 0.9991. The validating parameters of the calibration curve, obtained by linear regression analysis, are described in Table 1.

3.2.2. Precision

The method precision was evaluated in terms of repeatability and intermediate precision, by performing three repetitive analyses for each concentration levels (31.25 μg/ml, 125 μg/ml and 500 μg/ml). The repeatability test showed R.S.D. values lower than 2.16% and the intermediate precision, evaluated on 2 non-consecutive days, presented R.S.D. between 2.83% and 4.70% (Table 2). These results could be considered satisfactory since the majority of phytochemicals present R.S.D. values lower than 6% according to literature [7].

Table 1
Linearity parameters for the calibration curve of the conocarpan

Compound	Linearity range (μg/ml)	Slope (a)	Intercept (b)	(r^2)
Conocarpan	31.25–500	55797	−595314	09991

r^2, correlation coefficient.

Table 2
Repeatability and intermediate precision data for the determination of conocarpan by HPLC

Compound	Concentration (μg/ml)	Repeatability (R.S.D%)	Intermediate precision (R.S.D%)
Conocarpan	31.25	1.18	4.14
	125	2.16	4.70
	500	1.42	2.83

R.S.D., relative standard deviation. For each sample $n = 3$.

3.2.3. Limit of detection and quantification

The limit of detection, defined as the lowest concentration of conocarpan in a sample, which can be detected but not necessary quantified under the stated experimental conditions, was 1.68 μg/ml. The limit of quantification, defined as the lowest concentration of conocarpan in a sample that can be determined with acceptable precision and accuracy, was 5.60 μg/ml.

3.2.4. Accuracy

The accuracy of the method was evaluated with the recovery test. Table 3 shows the recovery data, which were obtained by relationship between the amount of added standard and the amount detected. The method produced a medium recovery of 104.55% with R.S.D. below 4% for all analysed concentrations, confirming accuracy of the method.

3.2.5. Stability of the analyte during analysis

The analytes in solution did not show any appreciable change in chromatographic profile for at least 72 h. No degradation products were observed, confirming the stability of the samples under the studied conditions.

3.3. Analysis of leaves, stems and roots of P. regnellii

The retention times of the standards conocarpan, eupomatenoid-6 and eupomatenoid-5, were used to identify the corresponding peaks in the extracts of *P. regnellii*. For determination of conocarpan content in the extracts of *P. regnellii* was used the regression equation of $y = 55797x - 595314$. The concentrations of eupomatenoid-5 and eupomatenoid-6 were expressed in conocarpan. Fig. 3a–c shows the chromatograms of the extracts of *P. regnellii* obtained from leaves, stems and roots, respectively.

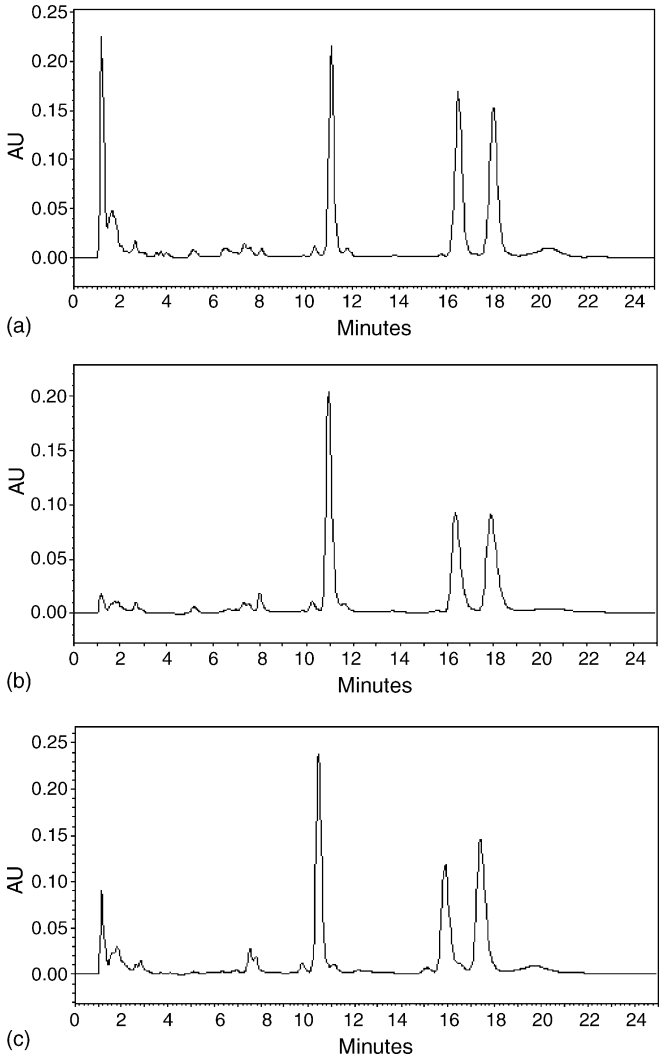

Fig. 3. Chromatograms of the *P. regnellii* extracts, (a) leaves; (b) stems and (c) roots. *Chromatographic conditions*: Metasil ODS column; mobile phase: acetonitrile/water (60:40, v/v) with 2% acetic acid; flow rate: 1.0 ml/min; temperature: 30 °C; detection: 280 nm.

The extracts demonstrate the same chromatographic profile, but there were differences in the concentrations of neolignans according to Table 4, that shows the content of neolignans in the different parts of the plant. As can be seen, roots presented a higher concentration of conocarpan than the leaves and stems, but the difference was significant ($p < 0.05$) in relation to leaves. Leaves and roots presented higher content of eupomatenoid-5

Table 3
Results of the recovery test for conocarpan from the extract of *P. regnellii*

Compound	Spiked concentration (μg/ml)	Recovery (%) (mean ± S.D.)	Mean ± S.D.	R.S.D. (%)
Conocarpan	31.25	100.69 ± 2.00	104.55 ± 3.48	3.33
	125	107.45 ± 0.09		
	500	105.49 ± 1.07		

S.D., standard deviation; R.S.D., relative standard deviation. For each sample $n = 3$.

Table 4
Quantification of neolignans in hydroethanolic extracts of the leaves, stem and root of *P. regnellii* by HPLC

Material	Conocarpan (μg/ml)	Eupomatenoid-6[a] (μg/ml)	Eupomatenoid-5[a] (μg/ml)
Leaves	71.88* ± 1.05	79.20* ± 1.10	81.55 ± 1.14
Stems	79.09 ± 1.46	60.28* ± 2.33	71.97* ± 5.41
Roots	84.32* ± 2.11	66.76 ± 3.16	85.97* ± 0.61

* $p < 0.05$, $n = 3$.
[a] Calculated as conocarpan.

and eupomatenoid-6, respectively, and both showed significant difference ($p < 0.05$) in relation to stems.

4. Conclusion

The HPLC method developed allowed the detection and quantification of the neolignans conocarpan, eupomatenoid-5 and eupomatenoid-6 in the extracts of *P. regnellii*. The validation procedure demonstrated the method presented linearity, precision and accuracy in the range studied. This procedure confirms that developed technique affords reliable analysis of the neolignans and is appropriate for the quality control of extracts and phytopharmaceutical preparations produced with *P. regnellii*. Moreover, the validated method complies with regulatory requirements if the plant is to be used by the pharmaceutical industry.

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