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HPLC Analysis of Bioactive Diterpenoids from the Root Bark of *Pseudolarix kaempferi*

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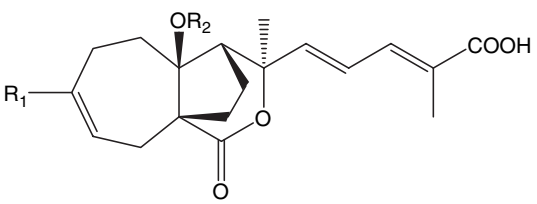
ABSTRACT

The root bark of *Pseudolarix kaempferi*, a famous traditional Chinese medicine, has been commonly used in China to treat skin diseases caused by fungal infections. To evaluate its pharmatherapeutic quality, a sensitive, simple and accurate high-performance liquid chromatography (HPLC) method was developed for the quantitative analysis of three major bioactive diterpenoids, pseudolaric acids A-C in *P. kaempferi*. A Zorbax XDB RP-C_{18} (4.6 mm × 250 mm, 5 μm) was used in this HPLC system, with UV detection at 260 nm. A gradient elution was effected by a mobile phase composed of acetonitrile and 1% aqueous acetic acid, where acetonitrile concentration was changed from 30% to 60% in 30 min. These three constituents were well resolved by this method with good linear relationships in the range of 0.982-98.2 μg/mL for pseudolaric acid A, 0.618-61.8 μg/mL for pseudolaric acid B, and 1.052-105.2 μg/mL for pseudolaric acid C, respectively. The correlation coefficients of the calibration curves were all over 0.999. In addition, these three compounds in six batches of the root barks of *P. kaempferi* were quantitatively determined to evaluate the effectiveness of this method.

Key words: *Pseudolarix kaempferi*, pseudolaric acid A, pseudolaric acid B, pseudolaric acid C, HPLC, determination

INTRODUCTION

Pseudolarix kaempferi Gord. (Pinaceae) is a plant indigenous to the eastern part of China. Its root bark is well-known as Tu-Jing-Pi in China, and has been used in traditional Chinese medicine for the treatment of skin diseases caused by microbial infection. A series of novel diterpenoids have been discovered from the barks in the past decades[1-8]. Some of them have been demonstrated to have a variety of biological activities. For example, pseudolaric acids A and B were reported to possess significant antifungal, antifertile and cytotoxic activities[9-12]. Pseudolaric acid B, the best antifungal agent isolated from higher plants, showed potent antifungal activities against *Candida* and *Torulopsis* species[10]. With the increasing application of the antifungal barks in the medicinal industry, it is essential to establish an analytical method for quality control. In previous studies, pseudolaric acid B in *P. kaempferi* has been used as a chemical marker and quantitatively analyzed by TLC Scanning and HPLC methods[13-14]. In our present study, pseudolaric acids A-C (Figure 1), the major antifungal diterpenoids existing in the root bark of *P. kaempferi*, were simultaneously determined by HPLC for the first time. As multiple constituents can be easily analyzed by this established method, the quality control of *P. kaempferi* can be addressed more efficiently.



	R_1	R_2
pseudolaric acid A	CH_3	$COCH_3$
pseudolaric acid B	$COOCH_3$	$COCH_3$
pseudolaric acid C	$COOCH_3$	H

Figure 1. Structures of pseudolaric acids A-C

MATERIALS AND METHODS

I. *Plant Materials and Chemicals*

Eight batches of the bark samples were purchased from herb stores in different cities. Six of them were identified as targeted samples, while two of them were revealed to be adulterants, the barks of *Cleistocalyx operculatus* (Roxb.) Merr. et Perry. The voucher specimens were identified and deposited at the Chinese Medicine Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong, P. R. China.

The Diterpenoid standard pseudolaric acid B was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Pseudolaric acids A and C were provided by Dr. Wei-Min

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Zhao, Institute of Materia Medica, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. Their purity is higher than 98% as assayed by HPLC with UV detection.

Distilled water was prepared and distilled twice using MILLI-Q SP reagent water system before use. HPLC grade acetonitrile, methanol, and acetic acid were products of International Lab, USA.

II. *Sample Preparation*

An accurately weighed portion (0.5 g) of each sample and 50 mL methanol was mixed in a 100 mL round bottom flask. The flask with samples was accurately weighed. After refluxing for an hour and cooling down to room temperature, the flask was weighed again. Methanol was used to make up the weight. Then the methanol extract was filtered through a 0.45 μm membrane directly into a HPLC sample vial for later use.

III. *Analytical Method*

The HPLC was operated on an Agilent 1100 serial system, equipped with a quaternary pump, online degasser, auto-sampler, column heater and variable wavelength detector. Separation was achieved on a reversed phase column (Zorbax XDB RP-C_{18}, 4.6 mm × 250 mm, pore size 5 μm, Agilent, USA) with a C_{18} guard column. The mobile phase is composed of acetonitrile and 1% aqueous acetic acid, in which acetonitrile was linearly changed from 30% to 60% in 30 min. The flow rate was 1.0 mL/min, and the elution profiles were monitored (and peaks were identified) by UV absorbance at 260 nm. The temperature was maintained at 25°C. The injection volume was 10 μL.

IV. *Calibration*

A mixed stock solution consisting of standard pseudolaric acids A-C (0.491 mg/mL, 0.309 mg/mL, 0.526 mg/mL, respectively) was prepared. 0.02, 0.1, 0.2, 0.4, 1.0, and 2.0 mL of the stock solution was each diluted to 10 mL with methanol and used for the standard curves calibration.

RESULTS AND DISCUSSION

I. *Linearity*

Typical HPLC chromatograms of the bark sample are shown in Figure 2. The retention time of pseudolaric acids A-C were 25.9, 19.9 and 11.0 min, respectively. A good linearity was achieved in the ranges of 0.982-98.2 μg/mL for pseudolaric acid A, 0.618-61.8 μg/mL for pseudolaric acid B, and 1.052-105.2 μg/mL for pseudolaric acid C. The regression equations and correlation coefficients for the references were [y = 2128.9x + 0.5204] (R^2 = 0.9999) for pseudolaric acid A, [y = 6002.7x + 0.8639] (R^2 = 0.9999) for pseudolaric acid B, and [y = 1936.6x - 2.4948] (R^2 = 0.9999) for pseudolaric acid C.

II. *Reproducibility*

Commercial sample from Nanjing (Batch 4) of Cortex Pseudolaricis was extracted and analyzed in duplicate. The procedure was repeated five times to evaluate the reproducibility of extraction protocol. The ranges of relative standard deviations were from 1.97% to 2.33% as shown in Table 1, indicating that the satisfactory reproducibility of pseudolaric acids A-C extraction from individual source.

III. *Precision*

Standards of pseudolaric acid A (4.91, 19.64, 49.10 μg/mL), pseudolaric acid B (3.09, 12.36, 30.90 μg/mL)

Table 1. Reproducibility of pseudolaric acids A-C extracted from Cortex Pseudolaricis (mean ± S.D., n = 5)

Compound	Content (%)	RSD%
pseudolaric acid A	0.145 ± 0.007	2.04
pseudolaric acid B	0.364 ± 0.021	1.97
pseudolaric acid C	0.093 ± 0.022	2.33

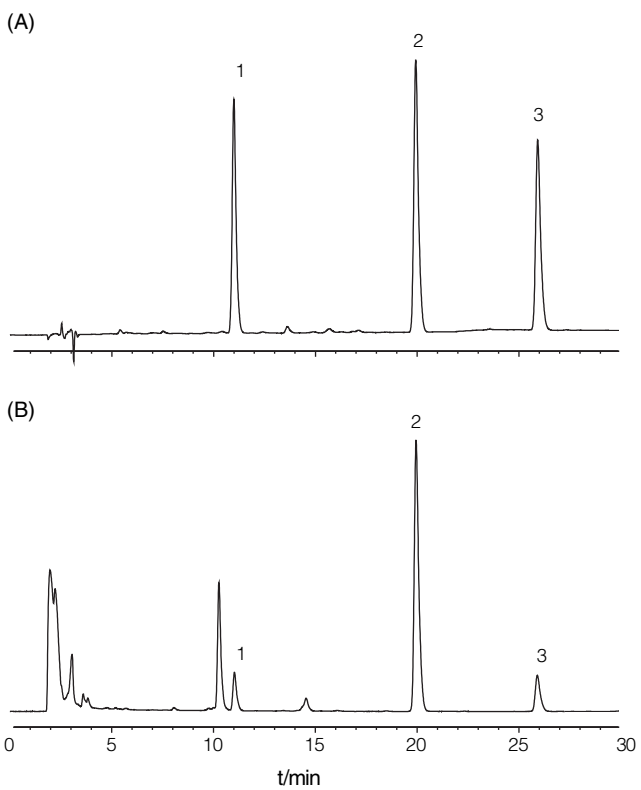

Figure 2. Typical HPLC chromatograms of chemical references (A) and sample (B): 1 pseudolaric acid C, 2 pseudolaric acid B, 3 pseudolaric acid A

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Table 2. Reproducibility of intra-day and inter-day analysis of pseudolaric acids A-C

Compound	Concentration (μg/mL)	Mean ± S.D. (RSD%)	
		Intra-day (n = 5)	Inter-day (n = 4)
pseudolaric acid A	4.91	4.88 ± 0.05 (0.99)	4.90 ± 0.07 (1.21)
	19.64	19.60 ± 0.25 (1.56)	19.59 ± 1.21 (1.77)
	49.10	49.46 ± 0.45 (1.14)	48.17 ± 2.22 (2.13)
pseudolaric acid B	3.09	3.05 ± 0.04 (1.03)	3.10 ± 0.09 (1.34)
	12.36	12.35 ± 0.27 (1.88)	12.29 ± 1.37 (2.04)
	30.90	30.62 ± 0.58 (2.22)	30.88 ± 1.98 (2.42)
pseudolaric acid C	5.26	5.18 ± 0.07 (0.74)	5.23 ± 0.09 (1.83)
	21.04	20.93 ± 0.14 (1.64)	20.86 ± 1.27 (1.53)
	52.60	52.47 ± 0.74 (0.83)	52.26 ± 0.66 (1.97)

and pseudolaric acids C (5.26, 21.04, 52.60 μg/mL) were injected both at intra-day (injecting each concentration five times within 24 hr) and inter-day (injecting each concentration four times during 5 days with each injection separated by at least 24 hr) intervals to check the precision. The results were shown in Table 2 and indicated high precision, with coefficients of variation of intra-day and inter-day test ranging 0.74-2.22% and 1.21-2.42%, respectively.

IV. *Recovery*

The recovery was used to evaluate the accuracy of the method. Five of six parallel solutions were accurately spiked with various concentrations of reference solutions just prior to the extraction. The results were shown in Table 3. Good recoveries from the analysis were obtained as follows: 96.62-101.22% for pseudolaric acid A, 97.21-99.28% for pseudolaric acid B and 97.46-101.02% for pseudolaric acid C, respectively.

V. *Limits of Detection (LOD) and Limits of Quantification (LOQ)*

Serial dilutions of pseudolaric acids A-C were analyzed by HPLC method. The limit of detection and limit of quantification were obtained with the signal to noise ratio of 3 and 10. LOD represents the lowest concentrations of pseudolaric acids A-C that can be detected, whereas the LOQ represents the lowest concentrations of pseudolaric acids A-C that can be determined with acceptable precision and accuracy. The LOD and LOQ were found to be 0.0982 and 0.3273 μg/mL for pseudolaric acid A, 0.0309 and 0.1030 μg/mL for pseudolaric acid B, 0.1052 and 0.3507 μg/mL for pseudolaric acid C, respectively. This indicated that the new method exhibited a good sensitivity for the quantification of pseudolaric acids A-C in Cortex Pseudolaricis. In order to obtain more accurate regression, the lower limit of linearity was adjusted to be higher than LOQ. The concentrations of pseudolaric acids A-C in sample solutions were within the range of linearity.

Table 3. Recovery of pseudolaric acids A-C in Cortex Pseudolaricis (n = 5)

Compound	Concentration (μg/mL)	Recovery (%) Mean ± S.D. (RSD%)
pseudolaric acid A	4.91	96.62 ± 0.73 (1.21)
	19.64	98.43 ± 0.88 (1.33)
	49.10	101.22 ± 1.31 (1.37)
pseudolaric acid B	3.09	97.21 ± 0.92 (1.85)
	12.36	99.28 ± 1.16 (1.32)
	30.90	98.19 ± 0.85 (2.22)
pseudolaric acid C	5.26	97.46 ± 0.79 (0.94)
	21.04	101.02 ± 1.14 (1.49)
	52.60	98.83 ± 0.94 (2.11)

VI. *Quantitative Determination*

Eight samples were extracted, as described above and analyzed by HPLC. The content of each compound was determined by the corresponding regression equation and is summarized in Table 4.

The results indicated that all three diterpenoids were detected in six true samples, in which pseudolaric acid B was obviously dominant. While in two adulterants, none of these three markers was detected. In all true barks analyzed, pseudolaric acids A-C contents varied in a relatively narrow windows of 0.097-0.153%, 0.266-0.406% and 0.037-0.098%, respectively.

For the first time, a simple, accurate and rapid HPLC method was developed for the simultaneous determination of three major antifungal diterpenoids, pseudolaric acids A-C in the barks of *P. kaempferi*. The assay is proved to be accurate, reproducible, and sensitive. Furthermore, it has been successfully employed for the determination of these three compounds in different commercial bark

Table 4. Contents of pseudolaric acids A-C in eight batch of commercial samples of Cortex Pseudolaricis (mean ± S.D., n = 3)

Batch	Purchased from	Contents (%)		
		pseudolaric acid A	pseudolaric acid B	pseudolaric acid C
1	Beijing	0.139 ± 0.017	0.406 ± 0.024	0.082 ± 0.018
2	Shanxi	0.102 ± 0.021	0.308 ± 0.027	0.037 ± 0.020
3	Nanjing	0.097 ± 0.028	0.266 ± 0.016	0.093 ± 0.006
4	Nanjing	0.146 ± 0.009	0.367 ± 0.022	0.092 ± 0.025
5	Nanjing	0.153 ± 0.022	0.400 ± 0.018	0.052 ± 0.014
6	Nanjing	0.110 ± 0.006	0.269 ± 0.026	0.098 ± 0.019
7[1)]	Hong Kong	–	–	–
8[1)]	Guangzhou	–	–	–

[1)] Adulterants, the barks of *Cleistocalyx operculatus* (Roxb.) Merr. et Perry
- Not detected.

samples. It was found that samples from different sources show a similar but unique HPLC chromatogram, thus making this method useful for the quality control of the crude drugs.

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