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Simultaneous determination of seven major diterpenoids in *Pseudolarix kaempferi* by high-performance liquid chromatography DAD method

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Abstract

A reversed phase high-performance liquid chromatography method was established for the first time to simultaneously qualify the seven major diterpenoids in *Pseudolarix kaempferi*, namely pseudolaric acid B *O*-β-D-glucopyranoside (**1**), pseudolaric acid C_2 (**2**), pseudolaric acid C_1 (**3**), deacetylpseudolaric acid A (**4**), pseudolaric acid A *O*-β-D-glucopyranoside (**5**), pseudolaric acid B (**6**) and pseudolaric acid A (**7**). The optimal conditions of separation and detection were achieved on an Inertsil ODS-3 column with gradient elution of methanol and 0.5% aqueous acetic acid (v/v) at the flow rate of 0.6 ml min^{-1} within 40 min and detection wavelength set at 262 nm. All calibration curves showed good linear regression ($r^2 > 0.9999$) within test ranges. This method provided good accuracy with recoveries in the range of 94.3–106.1% and good precision with R.S.D.s of repeatability and intermediate precision less than 0.57% and 4.67%, respectively. The method was successfully applied to qualitative and quantitative determination of 20 *P. kaempferi* among the 54 samples collected from different areas. The results revealed that the commercial crude drugs were seriously confused and the developed HPLC assay could be used as a suitable qualitative and quantitative determination method for *P. kaempferi*.

Keywords: RP-HPLC; *Pseudolarix kaempferi*; Determination; Quality control; *Cleistocalyx operculatus*

1. Introduction

The root and trunk bark of *Pseudolarix kaempferi* Gord. (Pinaceae), known as "Tu-Jing-Pi" in traditional Chinese medicine, have been historically used for treatment of skin diseases caused by fungal infections in China. In the past few decades, systematic chemical studies have been performed on the bark, seeds and leaves of *P. kaempferi* and more than 60 compounds have been isolated including diterpenoids [1–3], triterpenoids [4,5], triterpene lactones [6–8] and phenolic compounds [9,10]. Among them, the characteristic diterpenoids obtained from the bark were reported to be responsible for the antifungal [3,11] and antifertility [12] activities. They were also found to have significant in vitro cytotoxic activities [2], as well as potent in vivo antitumor effects [13]. In recent years, extensive investigations on the mechanism of their cytotoxic activities were conducted and the cytotoxicities were found to be mediated by inhibition of angiogenesis [14], induction of cell apoptosis [15] and microtubule destabilization [13]. Therefore, the quality control of *P. kaempferi* should be focused on the determination of the diterpenoids, which are of great significance for the quality of this crude drug. However, previous research was insufficient to control the quality of *P. kaempferi* because only a single chemical marker was determined by TLC [16] or HPLC [17]. The aim of the present paper was to develop a simple and rapid HPLC method to simultaneously qualify the seven major diterpenoids in *P. kaempferi*. At the same time, it was also found that this method can be used to identify the authenticity of *P. kaempferi* in view of the confusion of the source of the commercial crude drug.

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2. Experimental

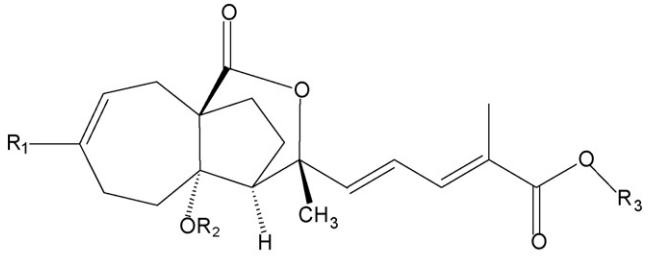
2.1. Materials and chemicals

Crude drugs were purchased from local drug stores in different provinces. Authentic crude drugs were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol, analytical grade acetic acid and other solvents used for compound isolation were all purchased from Beijing Chemical Engineering Factory (Beijing, China). The deionized water was prepared from Millipore water purification system (Millipore, Milford, MA, USA) and was filtered with 0.45 μm membranes.

The bark of *P. kaempferi* (15 kg) was refluxed with 45 L 95% EtOH for twice. The resulting EtOH extract was concentrated (2 kg), suspended in H_2O and partitioned successively with petroleum ether (PE), EtOAc and *n*-BuOH. The EtOAc fraction (154.7 g) was then subjected to silica gel column chromatography (CC) eluted by $CHCl_3$-MeOH to furnish fractions A–G. Fraction B was applied to silica gel using a gradient solvent system of PE-EtOAc, a ODS open CC and then recrystallized with MeOH to afford **7** (0.6023 g) and **6** (5.0615 g), respectively. Fraction D was subjected to silica gel CC and the major fraction recrystallized with MeOH to afford **3** (1.7654 g), then the filtrate and other fractions of D were combined and subjected to ODS open CC using a gradient elution with MeOH-H_2O and further purified with HPLC to furnish **4** (108 mg) and **2** (120 mg), respectively. Fraction F was subjected to silica gel CC eluted by EtOAc-Me_2CO and ODS open CC then HPLC to obtain **5** (20 mg) and **1** (150 mg), respectively. Their structures (Fig. 1) were confirmed by comparison of UV, ESIMS and NMR spectroscopic data with published values [1,18,19], and their purities were not less than 97% by HPLC analysis.

2.2. HPLC apparatus and conditions

An Agilent 1100 liquid chromatograph system (Agilent Technologies, Palo Alto, CA, USA) consisting of a quaternary pump, an autosampler and a photodiode array detector coupled with Agilent Chemstation was used. Separations were carried out with an Inertsil ODS-3 reversed-phase column (250 mm × 4.6 mm, 5 μm) (GL Sciences Inc., Japan). The mobile phase was gradient of methanol–0.5% aqueous acetic acid (v/v) (0 min, 55:45; 40 min, 90:10). The flow rate was 0.6 ml min^{-1}. The UV detection wavelength was set at 262 nm, and absorption spectra of compounds were recorded between 200 and 400 nm. The column temperature was at 40 °C, and the sample injection volume was 10 μl. Those compounds were identified by comparing their retention times and UV spectra with those of the markers.



	R_1	R_2	R_3
1	COOMe	Ac	Glc
2	COOH	Ac	H
3	COOMe	H	H
4	Me	H	H
5	Me	Ac	Glc
6	COOMe	Ac	H
7	Me	Ac	H

Fig. 1. Structures of seven marker compounds.

2.3. Calibration curve

Each marker compound, **1**–**7**, was accurately weighed and dissolved in 60% methanol to give eight concentrations within the ranges of 184.80–9.24 μg ml^{-1}, 6.12–0.31 μg ml^{-1}, 47.60–2.38 μg ml^{-1}, 7.60–0.38 μg ml^{-1}, 37.36–1.87 μg ml^{-1}, 263.74–13.19 μg ml^{-1} and 50.80–2.54 μg ml^{-1}, respectively. All calibration curves were obtained from peak areas of the standard solutions over the concentrations. Concentrations of these compounds in samples were calculated from this regression analysis.

2.4. Sample preparations

The dried powders of *P. kaempferi* samples (0.2 g, 60 mesh) were accurately weighed and soaked in 10 ml 60% methanol solution at room temperature for 0.5 h then extracted at 80 °C for 0.5 h. The resultant mixture was adjusted to the original weight and filtered through filter paper and then 0.45 μm membrane.

3. Results and discussion

3.1. Extraction method

Varied extraction methods, solvents, temperatures and times were evaluated so as to obtain the best extraction efficiency. The results revealed that refluxing was better than ultrasonic bath extraction, so the further experiments were carried out with refluxing. Various solvents including water, 20%, 40%, 60%, 80% and 100% methanol were screened successively and 60% methanol exhibited complete extraction of all the major constituents. The samples were then extracted with 60% methanol at room temperature, 40, 60, 80 and 100 °C, respectively. It was found that the best extraction temperature was at 80 °C, which assured the maximum extraction of the target compounds. The samples were then refluxed with 60% methanol at 80 °C for 0.5, 1 and 2 h, respectively. There was no obvious difference among the three extraction times and therefore 0.5 h was selected to be the extraction time with a view to convenience.

3.2. Optimization of separation conditions

An appropriate chromatographic condition was particularly required in this method for the considerable structure similari-

ties of the marker compounds. Firstly, different chromatographic columns were tested including ODS Hypersil, Zorbax Extend C18, Zorbax Eclipsed XDB C8, Waters Xterra Rp_{18} and Inertsil ODS-3 columns. There were two pairs of peaks that had close retention times in the chromatogram of crude drug, viz. marker **2** and **3**, as well as marker **7** and an interfered peak. Only the utilization of Inertsil ODS-3 column could simultaneously obtain the baseline separation. Secondly, the composition of mobile phase was investigated. Methanol possessed better resolution of the peaks than acetonitrile and acetic acid was better than phosphoric acid and trifluoroacetic acid in separation. It was also found that the concentration of acetic acid was crucial for the simultaneous baseline separation of the aforementioned two pairs of peaks and the ratio of 0.5% (v/v) was screened as the best concentration. Finally, the monitoring wavelength was set at 262 nm, which is the characteristic and maximum absorption of this type of diterpenoid according to their 3D ultraviolet absorption spectra. The relative higher column temperature (40 °C) and lower flow rate (0.6 ml min^{-1}) were employed to obtain better resolution based on the analyses of different conditions.

3.3. Linearity and Range

In this method, linearity was determined by constructing seven calibration curves with external standard method at eight concentration levels. All seven calibration curves exhibited good linearity ($r^2 > 0.9999$) under the established chromatographic conditions (Table 1). The ranges of the calibration curves were specified as approximately 80–120% of the test concentration of the samples. These ranges covered the amounts of the analytes of all samples and provided suitable level of linearity, accuracy and precision.

3.4. Accuracy

The ratio of observed concentration and nominal concentration of the mixed standard solutions at three concentration levels (low, medium and high) were in the range of 98.97–104.43% (Table 2), indicating the good accuracy of the method.

Recovery tests were carried out to further investigate the accuracy of the method by adding three concentration levels of the mixed standard solutions to known amounts of *P. kaempferi* samples. The resultant samples were then extracted and analyzed with the described method. The results were calculated with the value detected versus added amounts. The recoveries of the method were in the range of 94.3–106.1%, with R.S.D. less than 2.94% as shown in Table 3, suggesting the good accuracy of the method.

3.5. Precision

Repeatability, viz. intra-assay precision for each marker compound at three concentration levels was listed in Table 2. The R.S.D.s of the intra-assay precision data were in the range of 0.09–0.57%.

Intermediate precision was performed by utilizing different instruments to determine the same sample with the developed method by different analysts and the R.S.D. values were less than 4.67%. Inter-day precision for each marker compound at three concentration levels was also investigated with R.S.D.s in the range of 0.26–1.18% as listed in Table 2. All these data revealed that the described method has an accepted degree of precision.

3.6. LOD and LOQ

LOD and LOQ were the concentrations of a compound at which its signal-to-noise ratios were detected as 3:1 and 10:1, respectively. They were determined by serial dilution of standard solution using the described HPLC conditions. The results were showed in Table 1.

3.7. Sample analysis

The established method was applied to determine 54 commercial samples collected from all over the country. It was found that only 20 of them are *P. kaempferi*, as well as 9 of them are *Cleistocalyx operculatus* (Myrtaceae), which were confirmed by comparison of the chromatograms of the samples with those of the authentic crude drugs of *P. kaempferi* and *C. operculatus*, respectively. The bark of *C. operculatus* is also used for treatment of dermal infections in certain area of China with name of "Tu-jin-pi" [20], which can easily be confused with *P. kaempferi*. The other 25 samples could not be identified solely based on the chromatograms. The result suggested that the commercial crude drugs of *P. kaempferi* were seriously confused in the market. The described procedure therefore provides a feasible chromatographic method for the qualitative identification of the confusable crude drugs (Fig. 2).

Table 1
Linear relation between peak area and concentration ($n = 8$)

Marker compounds	Regression equation	r^2	Range (μg ml^{-1})	LOD (μg ml^{-1})	LOQ (μg ml^{-1})
1	$y = 47.567x + 3.078$	0.9999	184.80–9.24	0.028	0.083
2	$y = 54.200x - 2.8784$	0.9999	6.12–0.31	0.046	0.153
3	$y = 63.978x - 9.5067$	1.0000	47.60–2.38	0.036	0.119
4	$y = 79.262x - 1.0467$	0.9999	7.60–0.38	0.033	0.095
5	$y = 34.604x - 1.6053$	0.9999	37.36–1.87	0.066	0.249
6	$y = 67.898x + 69.878$	0.9999	263.74–13.19	0.035	0.176
7	$y = 70.995x - 1.1428$	0.9999	50.80–2.54	0.038	0.111

y = peak area, x = concentration (μg ml^{-1}). Triplicate assay about the different concentration ($n = 8$).

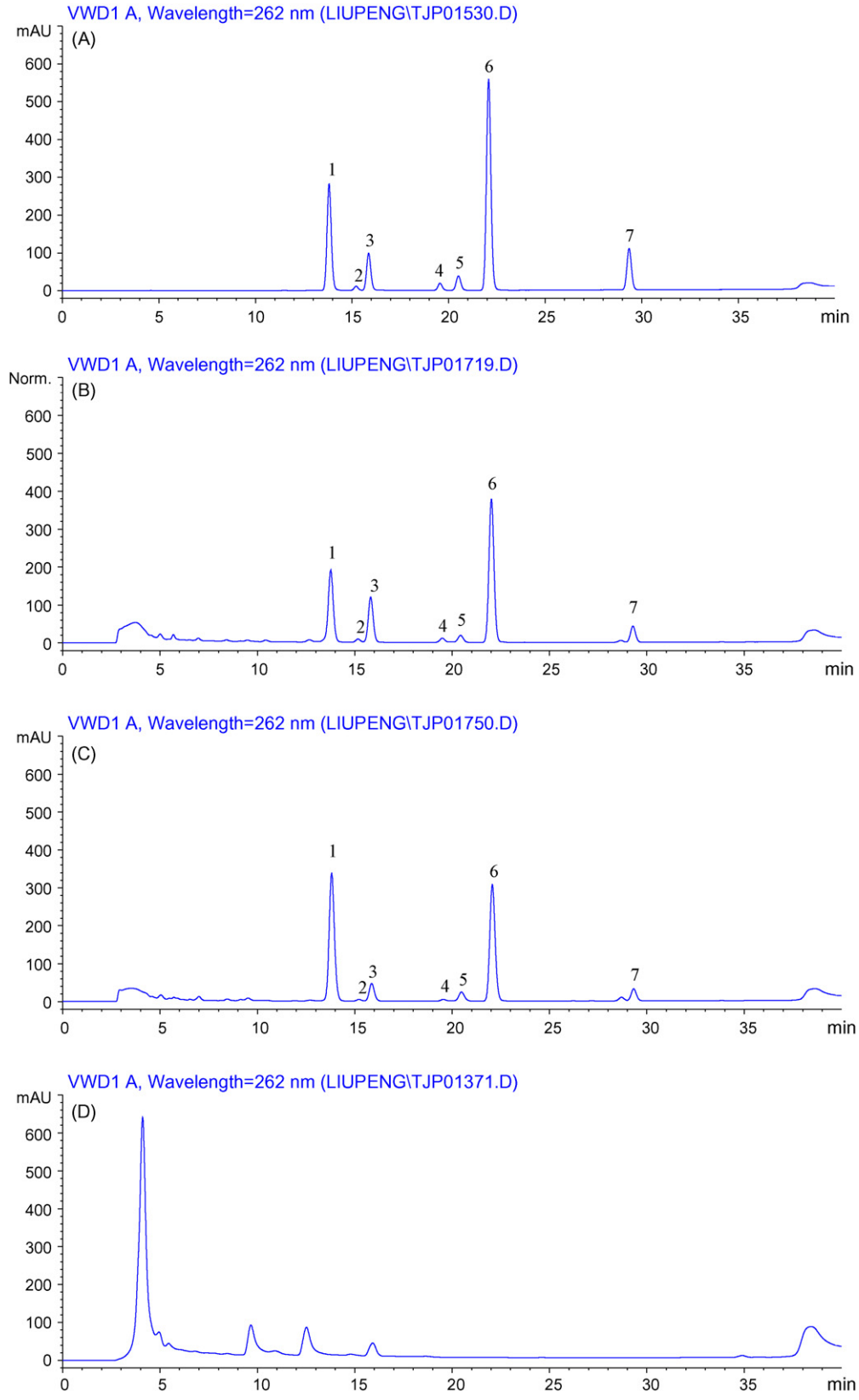


Fig. 2. Representative HPLC chromatograms of (A) standard solution at medium concentration, (B) *P. kaempferi* (Hengshui, Hebei province), (C) *P. kaempferi* (Jilin province), (D) *C. operculatus* (authentic crude drug). **1**, Pseudolaric acid B *O*-β-D-glucopyranoside; **2**, pseudolaric acid C_2; **3**, pseudolaric acid C_1; **4**, deacetylpseudolaric acid A; **5**, pseudolaric acid A *O*-β-D-glucopyranoside; **6**, pseudolaric acid B; **7**, pseudolaric acid A.

Table 2
Accuracy and precision data for the HPLC method

Marker compounds	Nominal concentration ($\mu g\,ml^{-1}$)	Observed concentration ($\mu g\,ml^{-1}$) ± S.D.		Accuracy (%)		Precision R.S.D. (%)	
		Intra-assay[a]	Inter-day[b]	Intra-assay	Inter-day	Intra-assay	Inter-day
1	36.96	36.75 ± 0.10	36.86 ± 0.14	99.44	99.72	0.27	0.38
	92.40	92.65 ± 0.37	93.16 ± 0.73	100.27	100.82	0.40	0.78
	147.84	148.84 ± 0.22	149.56 ± 0.85	100.68	101.16	0.15	0.57
2	1.22	1.28 ± 0.01	1.28 ± 0.01	104.43	104.25	0.55	0.89
	3.06	3.18 ± 0.02	3.20 ± 0.02	103.76	104.44	0.57	0.75
	4.90	5.10 ± 0.01	5.12 ± 0.03	104.10	104.62	0.09	0.59
3	9.52	9.57 ± 0.03	9.60 ± 0.04	100.50	100.82	0.30	0.39
	23.80	23.93 ± 0.10	24.07 ± 0.20	100.55	101.13	0.42	0.83
	38.08	38.40 ± 0.07	38.62 ± 0.25	100.83	101.41	0.18	0.64
4	1.52	1.51 ± 0.01	1.51 ± 0.01	99.38	99.45	0.42	0.36
	3.80	3.80 ± 0.02	3.82 ± 0.03	100.04	100.54	0.53	0.75
	6.08	6.13 ± 0.02	6.16 ± 0.04	100.88	101.27	0.27	0.68
5	7.47	7.43 ± 0.04	7.44 ± 0.02	99.38	99.52	0.55	0.26
	18.68	18.69 ± 0.08	18.79 ± 0.14	100.05	100.57	0.42	0.74
	29.89	30.06 ± 0.04	30.21 ± 0.19	100.57	101.08	0.15	0.61
6	52.75	52.20 ± 0.15	52.45 ± 0.20	98.97	99.43	0.30	0.39
	131.87	132.73 ± 0.57	133.49 ± 1.07	100.65	101.23	0.43	0.80
	210.99	212.15 ± 0.36	213.37 ± 1.37	100.55	101.13	0.17	0.64
7	10.16	10.12 ± 0.04	10.20 ± 0.07	99.58	100.41	0.35	0.71
	25.40	25.49 ± 0.09	25.78 ± 0.28	100.36	101.50	0.36	1.07
	40.64	40.92 ± 0.07	41.34 ± 0.49	100.68	101.73	0.17	1.18

[a] Intra-assay precision test at six times in 1 day.
[b] Inter-day precision on 5 different days.

Table 3
Recoveries of seven marker compounds[a]

Marker compounds	Initial amount (mg)	Added amount (mg)	Detected amount (mg)	Recovery (%)	R.S.D. (%)
1	0.7171	0.2004	0.9296	106.1	0.57
		0.4008	1.0951	94.3	0.35
		0.6012	1.3198	100.2	1.28
2	0.0273	0.0069	0.0339	96.4	2.94
		0.0138	0.0414	102.4	1.96
		0.0207	0.0480	100.0	2.74
3	0.3280	0.0414	0.3696	100.5	2.74
		0.0828	0.4105	99.6	0.73
		0.1242	0.4584	105.0	2.28
4	0.0304	0.0095	0.0398	98.8	2.57
		0.0190	0.0500	103.0	2.40
		0.0285	0.0600	103.7	2.41
5	0.1064	0.0208	0.1279	103.6	2.80
		0.0415	0.1499	104.9	0.78
		0.0519	0.1609	105.1	2.76
6	0.9839	0.2355	1.2274	103.4	1.43
		0.4710	1.4345	95.7	1.26
		0.7065	1.6969	100.9	1.77
7	0.1101	0.0468	0.1575	101.2	0.12
		0.0936	0.2076	104.2	2.85
		0.1404	0.2545	102.8	2.15

[a] Triplicate assay at each concentration level.

Table 4
Contents of seven analytes in different *P. kaempferi* samples[a]

No.	Collected place	Content[b] (mg g^{-1} crude drug)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
1	Zhejiang	2.47 ± 0.01	0.10 ± 0.00	0.81 ± 0.01	0.11 ± 0.00	0.43 ± 0.01	4.67 ± 0.01	0.85 ± 0.01
2	Shanghai	2.47 ± 0.01	0.24 ± 0.01	0.30 ± 0.00	0.02 ± 0.00	0.39 ± 0.01	8.88 ± 0.02	1.09 ± 0.01
3	Huangshi, Hubei	2.60 ± 0.01	0.14 ± 0.00	0.66 ± 0.01	0.08 ± 0.00	0.38 ± 0.00	6.67 ± 0.02	0.92 ± 0.00
4	Shijiazhuang, Hebei	3.35 ± 0.01	0.14 ± 0.00	1.26 ± 0.01	0.09 ± 0.00	0.38 ± 0.01	5.71 ± 0.01	0.63 ± 0.00
5	Liaoning	1.89 ± 0.01	0.07 ± 0.01	0.92 ± 0.01	0.06 ± 0.00	0.28 ± 0.01	3.18 ± 0.04	0.42 ± 0.00
6	Hengshui, Hebei	3.55 ± 0.03	0.13 ± 0.00	1.59 ± 0.02	0.13 ± 0.01	0.52 ± 0.01	4.97 ± 0.03	0.54 ± 0.01
7	Wuchang, Hubei	2.97 ± 0.01	0.12 ± 0.01	0.21 ± 0.01	0.03 ± 0.00	0.41 ± 0.00	4.57 ± 0.01	0.54 ± 0.01
8	Zhangzhou, Fujian	2.94 ± 0.02	0.10 ± 0.01	0.42 ± 0.01	0.05 ± 0.00	0.50 ± 0.01	4.49 ± 0.01	0.69 ± 0.01
9	Hubei	1.79 ± 0.01	0.10 ± 0.00	0.82 ± 0.01	0.07 ± 0.00	0.17 ± 0.00	6.57 ± 0.01	0.61 ± 0.00
10	Xuzhou, Jiangsu	1.51 ± 0.01	0.04 ± 0.00	0.32 ± 0.01	0.03 ± 0.00	0.20 ± 0.00	1.47 ± 0.01	0.17 ± 0.01
11	Fuzhou, Fujian	3.43 ± 0.01	0.11 ± 0.00	0.68 ± 0.01	0.06 ± 0.00	0.53 ± 0.01	4.97 ± 0.01	0.64 ± 0.00
12	Anhui	1.04 ± 0.01	0.15 ± 0.00	0.76 ± 0.01	0.09 ± 0.00	0.11 ± 0.00	8.52 ± 0.02	1.33 ± 0.01
13	Jilin	6.80 ± 0.03	0.08 ± 0.01	0.69 ± 0.01	0.06 ± 0.00	0.74 ± 0.01	4.34 ± 0.01	0.43 ± 0.00
14	Hefei, Anhui	1.06 ± 0.01	0.12 ± 0.00	1.80 ± 0.01	0.28 ± 0.01	0.25 ± 0.01	4.34 ± 0.02	0.51 ± 0.01
15	Sichuan	2.71 ± 0.01	0.11 ± 0.00	0.61 ± 0.01	0.04 ± 0.01	0.34 ± 0.01	5.81 ± 0.02	0.68 ± 0.01
16	Beijing	7.02 ± 0.02	0.18 ± 0.00	0.21 ± 0.00	0.03 ± 0.00	1.31 ± 0.01	7.05 ± 0.03	1.15 ± 0.01
17	Beijing	4.96 ± 0.01	0.24 ± 0.00	0.15 ± 0.00	0.02 ± 0.00	1.40 ± 0.00	8.37 ± 0.01	1.68 ± 0.01
18	Mudanjiang, Heilongjiang	5.84 ± 0.04	0.07 ± 0.01	0.45 ± 0.01	0.03 ± 0.00	0.55 ± 0.01	5.00 ± 0.02	0.45 ± 0.01
19	Liuan, Anhui	2.24 ± 0.01	0.12 ± 0.00	1.00 ± 0.01	0.09 ± 0.00	0.29 ± 0.00	6.19 ± 0.01	0.71 ± 0.01
20	Authentic drug	5.63 ± 0.01	0.15 ± 0.00	0.30 ± 0.00	0.03 ± 0.00	0.84 ± 0.01	5.88 ± 0.01	0.69 ± 0.01

[a] Samples 1–19 were purchased from drug stores. Sample 20 was purchased from the National Institute for the Control of Pharmaceutical and Biological Products. (Beijing, PR China).
[b] Content = mean ± S.D., $n = 3$.

The seven marker compounds were simultaneously detected in the 20 samples of the title plant and their contents were determined by the external standard method (Table 4). The total amounts of diterpenoids in 20 samples varied from 3.73 to 16.96 mg g^{-1}, with 4.5-fold variation in conjunction with the maximum variation of the content of single constituent with the value of 12.4-fold, indicating the contents of diterpenoids differed greatly among samples, which may consequently result in the difference of therapeutic effect. Therefore, the factors influenced the quality, such as plant resource, harvesting time, processing and storage conditions, should be standardized to obtain steady quality so as to ensure the therapeutic effect.

In addition, only pseudolaric acid B (**6**) was considered as a quantitative constituent to control the quality of *P. kaempferi* in the Chinese Pharmacopoeia (Version 2005) [21] for its significant antifungal and cytotoxic activities. According to the content of each compound in 20 samples, the average proportion of pseudolaric acid B (**6**) in the detected total diterpenoids was calculated with the value of 50.58%, and those of other active compounds including pseudolaric acid A (**7**), pseudolaric acid B *O*-β-D-glucopyranoside (**1**) and pseudolaric acid A *O*-β-D-glucopyranoside (**5**) [2,3] were expressed with the value of 40.48% in total. Therefore, all these active components should be simultaneously qualified for the purpose of evaluating the quality of *P. kaempferi* properly.

4. Conclusion

A HPLC method was developed for simultaneous determination of seven major diterpenoids in *P. kaempferi* for the first time. It was proved to be simple, rapid and precise. This HPLC assay can be readily utilized as a suitable quality control method for the determination of the major biologically active constituents in *P. kaempferi*. Moreover, it can also be used to the qualitative identification of *P. kaempferi* and *C. operculatus* in consideration of their serious confusion in the market.

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References

[1] Z.L. Li, D.J. Pan, C.Q. Hu, Q.L. Wu, S.S. Yang, G.Y. Xu, Huaxue Xuebao 40 (1982) 447–457.
[2] Z.L. Li, D.J. Pan, C.Q. Hu, K. Chen, J.J. Chang, K.H. Lee, Planta Med. 56 (1990) 383–385.
[3] S.P. Yang, L. Dong, Y. Wang, Y. Wu, J.M. Yue, Bioorg. Med. Chem. 11 (2003) 4577–4584.
[4] K. Chen, Z.L. Li, D.J. Pan, Huaxue Xuebao 48 (1990) 591–595.
[5] S.P. Yang, J.M. Yue, Bioorg. Med. Chem. Lett. 11 (2001) 3119–3122.
[6] G.F. Chen, Z.L. Li, K. Chen, C.M. Tang, X. He, D.J. Pan, D.R. McPhail, A.T. McPhail, K.H. Lee, Tetrahedron Lett. 31 (1990) 3413–3416.
[7] G.F. Chen, Z.L. Li, D.J. Pan, J. Nat. Prod. 56 (1993) 1114–1122.
[8] T.X. Zhou, H.P. Zhang, N.Y. Zhu, P. Chiu, Tetrahedron 60 (2004) 4931–4936.
[9] S.P. Yang, Y. Wang, Y. Wu, J.M. Yue, Nat. Prod. Res. 18 (2004) 439–446.
[10] P. Liu, H.Z. Guo, Y. Tian, Q. Wang, D.A. Guo, Phytochemistry 67 (2006) 1395–1398.
[11] E. Li, A.M. Clark, C.D. Hufford, J. Nat. Prod. 58 (1995) 57–67.
[12] Y.L. Zhang, R.Z. Lu, A.L. Yan, Acta Pharmacol. Sin. 11 (1990) 60–62.

[13] V.K.W. Wong, P. Chiu, S.S.M. Chung, L.M.C. Chow, Y.Z. Zhao, B.B. Yang, B.C.B. Ko, Clin. Cancer Res. 11 (2005) 6002–6011.
[14] W.F. Tan, X.W. Zhang, M.H. Li, J.M. Yue, Y. Chen, L.P. Lin, J. Ding, Eur. J. Pharmacol. 499 (2004) 219–228.
[15] X.F. Gong, M.W. Wang, Z. Wu, S. Tashiro, S. Onodera, T. Ikejima, Exp. Mol. Med. 36 (2004) 551–556.
[16] X.F. Zhai, Z.R. Zhang, G.H. Li, W.J. Sun, Zhong Cao Yao 35 (2004) 93–94.
[17] H. Chen, B.K. Feng, Y.Y. Li, S.H. Wu, L. Ru, Zhong Yao Cai 29 (2006) 451–452.
[18] Z.L. Li, D.J. Pan, Q.L. Wu, Huaxue Xuebao 40 (1982) 757–761.
[19] Z.L. Li, K. Chen, D.J. Pan, G.Y. Xu, Huaxue Xuebao 43 (1985) 786–788.
[20] D.P. Zhang, Zhong Yao Cai 25 (2002) 615–616.
[21] The State Pharmacopoeia Commission, Chinese Pharmacopoeia, Chemical Industry Press, Beijing, 2005, 14 pp.