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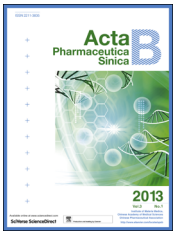

SHORT COMMUNICATION

Simultaneous determination of eight bioactive bakkenolides of *Petasites tatewakianus* Kitam by HPLC–UV

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KEY WORDS

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Abstract A high-performance liquid chromatography coupled with variable wavelength detection (HPLC–UV) was developed to evaluate the quality of *Petasites tatewakianus* Kitam through a simultaneous determination of eight bakkenolides: bakkenolide-L, bakkenolide-D, bakkenolide-B, bakkenolide-Ia, bakkenolide-VIIa, bakkenolide-IVa, bakkenolide-IIIa and homofukinolide. With a C18 analytical column, the eight analytes were efficiently separated using tetrahydrofuran–acetonitrile–water as the mobile phase in a constant program. The limits of detection and limits of quantitation of the method ranged 0.42–2.56 μg/mL and 1.22–8.40 μg/mL, respectively. The intra- and inter-day precisions of the method were all less than 1.83%. All the recoveries for the spiked analytes ranged 97.8%–102.4%. There were statistically significant differences among the contents of the eight bakkenolides in different parts and origins of *P. tatewakianus* ($P<0.01$). The content of total bakkenolides in rhizome was higher than that in other parts of the plant. The content of total bakkenolides in rhizome from Baishan was higher than those in other localities. The result suggested that rhizome may be the most valuable part of *P. tatewakianus*, and *P. tatewakianus* from Baishan may be the best plant resource. Our results might serve as a sound foundation for further study and application of this plant.

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1. Introduction

The genus *Petasites* (Asteraceae) comprises 18 species, 5 of which are scattered in the northwest, east and southeast of mainland China, including *Petasites rubellus* (J. F. Geme.) Toman, *Petasites japonicas* (Seib. et Zucc) Maxim, *Petasites tricholobus* Franch., *Petasites versipilus* Hand.-Mazz and *Petasites tatewakianus* Kitam[1]. They were used as folk medicines to treat hypertension, coughs, palsy and snakebite in China. The reported characteristic chemical components of *Petasites* species included sesquiterpenes (bakkenolides) and triterpenoids[2–6]. The isolation of bakkenolide type compounds from *P. tricholobus* and their neuroprotective effects[7], as well as the determination of four major active bakkenolides in different parts of *P. tricholobus*[8], have been described in our previous paper. However, there are limited reports of phytochemical and pharmacological research on *Petasites tatewakianus* Kitam up to now[9–11]. In our continued research of the genus *Petasites*, several bakkenolides were extracted, purified and identified from *Petasites tatewakianus* Kitam including bakkenolide-VIIa, homofukinolide, bakkenolide-Ia, bakkenolide-IIIa, bakkenolide-IVa, bakkenolide-L, bakkenolide-B and bakkenolide-D (Fig. 1), and were proved to be the main active constituents of *P. tatewakianus*[12]. Accordingly, these bakkenosides could be of great importance for identification and quality control of *P. tatewakianus*.

As a simple, accurate, effective and routine method, high-performance liquid chromatography with a variable wavelength detector (HPLC–UV) was widely used for quality control of traditional Chinese medicine. On the basis of satisfactory accuracy and effectiveness, a simple method is preferred to be developed for quality evaluation of *P. tatewakianus*. In the present study, a HPLC–UV method was developed to simultaneously determine eight active bakkenolides in *P. tatewakianus*. It is the first HPLC–UV method for the simultaneous determination of the eight active bakkenolides in this plant. This method can be applied to monitor these active compounds in order to obtain quality control of this plant. It is of benefit to evaluate correct planting, collection, storage and preparation of this medicinal resource. Our results might serve as a sound foundation for further study and better application of this plant in the medicinal herb industry.

2. Materials and method

2.1. Samples, chemicals and reagents

Samples of rhizome, blade and petiole of *P. tatewakianus* were herborized from six different localities: Raohe County (SY090901), Shuangyashan (SY090902), Luobei County (SY090903), Yichun (SY090904), and Fangzheng County (SY090905) of Heilongjiang Province, and Baishan (SY090906) of Jilin Province. All samples were identified by Professor Meili Guo (Department of Pharmacognosy, Second Military Medical University, Shanghai, China). Voucher specimens were deposited at the Department of Pharmacognosy, Second Military Medical University, Shanghai, China.

Eight bakkenolides (bakkenolide-L, bakkenolide-D, bakkenolide-B, bakkenolide-Ia, bakkenolide-VIIa, bakkenolide-IVa, bakkenolide-IIIa and homofukinolide) were previously isolated and purified from the rhizome of *P. tatewakianus* in our laboratory and their structures were identified on the basis of chemical characters and spectral methods (^{1}H NMR, ^{13}C NMR, 2D NMR, MS, UV and IR). The spectral data of the eight bakkenolides agree with those in literatures[7,12–15]. The purity of each compound was determined to be more than 98.5% by normalization of the peak areas detected by HPLC.

Acetonitrile and tetrahydrofuran (LC-grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Double distilled water was self-prepared. Other chemicals used were at least of analytical reagent grade and purchased from China National Medicines Corporation Ltd. All the solutions were filtered through 0.45 μm membrane filters (Schleicher & Schuell, Germany) and degassed by an ultrasonic bath before injecting into HPLC.

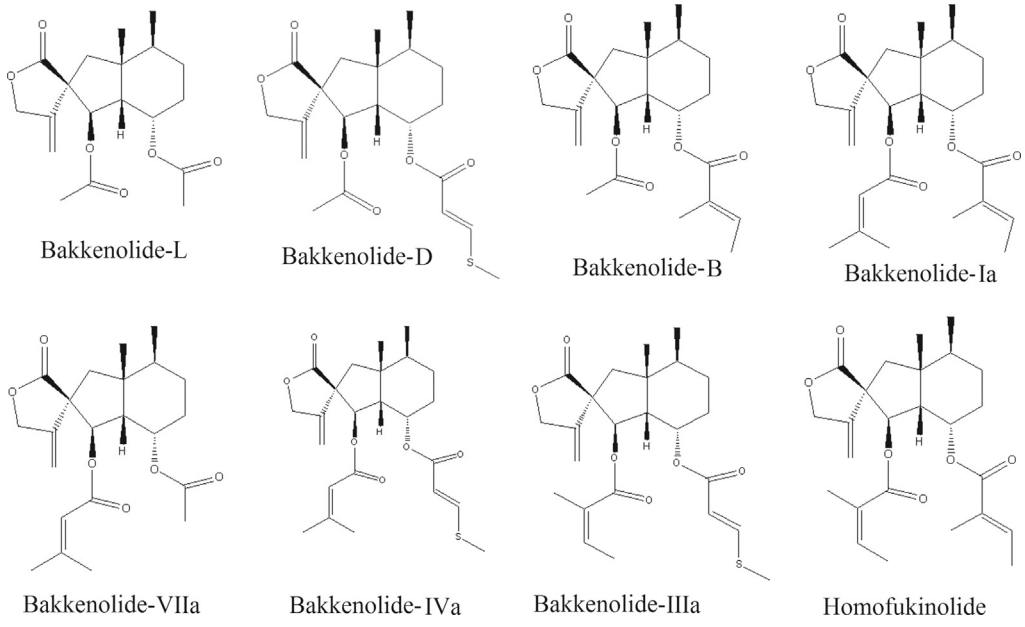

Figure 1 Structures of the eight bakkenolides.

2.2. Instrumentation and chromatographic conditions

HPLC analysis was carried out on an Agilent Technologies (Agilent Technologies, USA) 1100 high-performance liquid chromatography system equipped with a G1322A vacuum degasser, a G1311A quaternary pump, a G1316A thermostatic oven device, a G1314A variable wavelength detector and a G1313A autosampler. The chromatographic data and chromatograms were acquired and analyzed using a Chem Station software (Revision B.04.03, Agilent Technologies, USA). Compounds were separated on an Agilent Zorbax-C18 column (250 mm × 4.6 mm, i.d., 5 μm) with a mobile phase consisting of tetrahydrofuran–acetonitrile–water (28:2:70, *v*/*v*/*v*) at the flow rate of 1.0 mL/min. An aliquot of 20 μL was injected into the HPLC for analysis with the variable wavelength detector operated at 235 nm and column temperature at 30 °C.

2.3. Sample preparation

All samples (including rhizome, blade and petiole) were powdered to a homogeneous size, sieved through a No. 40 mesh, and further dried at 60 °C until a constant weight was obtained. Powdered samples (1.50 g) were ultrasonicated twice for 30 min with 30 mL methanol at room temperature. Appropriate volume of methanol was used to compensate for the loss of the solvent, and mixed well. After centrifugation at 3000 rpm for 10 min, the supernatant was collected. The extracts were combined, filtered, evaporated under reduced pressure, and then the residue was reconstituted to exactly 25 mL with 50% aqueous methanol using a volumetric flask. After filtration through a 0.45 μm membrane filter, 20 μL of the successive filtrate was analyzed by the HPLC–UV system. Each sample was independently prepared and analyzed in triplicate.

2.4. Preparation of standard solution

Stock standard solutions containing the eight reference compounds at concentration of 0.60 mg/mL for bakkenolide-L, bakkenolide-D and homofukinolide, as well as 1.0 mg/mL for bakkenolide-B, bakkenolide-Ia, bakkenolide-VIIa, bakkenolide-IVa and bakkenolide-IIIa, were prepared by dissolving accurately weighed portions of the standards in 50% aqueous methanol. 6.0 g bakkenolide-L, 6.0 g bakkenolide-D, 10.0 g bakkenolide-B, 10.0 g bakkenolide-Ia, 10.0 g bakkenolide-VIIa, 10.0 g bakkenolide-IVa, 10.0 g bakkenolide-IIIa and 6.0 g homofukinolide were accurately weighed and transferred to a 10 mL volumetric flask. The volume was made up of 50% aqueous methanol, and then the solution was mixed well. The stock standard solution was further diluted to a series of concentrations with 50% aqueous methanol. The solutions at a series of concentrations were prepared in triplicate in batches and stored in the dark at 4 °C before use.

2.5. Calibration curves, limits of detection and quantitation

Linear regression analysis for each of the eight bakkenolides was performed by the external standard method. Standard solutions with different concentrations (1/2, 1/4, 1/10, 1/20, 1/40 and 1/50 of the concentration of stock standard solution) were injected into the LC system in triplicate. The integrated peak (Y) of each compound at different concentrations and concentrations (X) were analyzed by linear regression to calculate the calibration curve and correlation coefficient (r).

The stock solution containing the eight reference compounds was further diluted to a series of lower concentrations, and an aliquot of the diluted solutions was injected into HPLC for analysis. The limit of detection (LOD) and limit of quantitation (LOQ) for each analyte were determined with the corresponding standard solution at the signal-to-noise ratio (S/N) of 3 and 10, respectively.

2.6. Precision and repeatability

The standard solutions at a certain concentration (0.30 mg/mL for bakkenolide-L, bakkenolide-D and homofukinolide, as well as 0.50 mg/mL for bakkenolide-B, bakkenolide-Ia, bakkenolide-VIIa, bakkenolide-IVa and bakkenolide-IIIa) were used to determine the precision of the analytic method. The precision was determined by calculating the relative standard deviation (RSD) for repeated measurements in intra- and inter-day analysis. The intra-day precision was tested on six repeated injections on the same day and the inter-day precision was tested on three consecutive days. To confirm the repeatability, six solutions with known content of analytes (1.85 mg/g bakkenolide-L, 1.12 mg/g bakkenolide-D, 4.67 mg/g bakkenolide-B, 6.06 mg/g bakkenolide-Ia, 0.61 mg/g bakkenolide-VIIa, 0.33 mg/g bakkenolide-Iva, 0.36 mg/g bakkenolide-IIIa and 2.74 mg/g homofukinolide) were prepared from the same sample collected from Shuangyashan, Heilongjiang Province, under the method described in Section 2.3 and analyzed using HPLC–UV. RSD was calculated to evaluate the method repeatability.

2.7. Recovery

The recovery under the chromatographic conditions described above was calculated to evaluate the method. The content of the eight analytes in a sample was calculated according to their respective calibration curves. Accurate amounts of the eight analytes (1.51 mg bakkenolide-L, 1.23 mg bakkenolide-D, 4.54 mg bakkenolide-B, 6.13 mg bakkenolide-Ia, 0.60 mg bakkenolide-VIIa, 0.35 mg bakkenolide-IVa, 0.42 mg bakkenolide-IIIa and 2.98 mg homofukinolide) were added to 1.0 g of the sample from Shuangyashan, Heilongjiang Province. Then, the fortified samples were extracted and analyzed under the procedures as described above. The average recoveries and RSD were calculated.

2.8. Statistical analysis

Differences between average contents of total bakkenolides in different parts and origins of *P. tatewakianus* were analyzed by one-way ANOVA followed by Dunnett's test using SPSS version 13.0 (SPSS Inc, Chicago, Ill). Differences were considered statistically significant when $P < 0.05$.

3. Results and discussion

3.1. HPLC separation optimization

The chromatographic separation conditions were optimized to obtain chromatograms with good resolution of adjacent peaks within a short analysis time. To confirm the test wavelength, wavelengths from 190 nm to 600 nm were scanned for the eight reference compounds. On the basis of ultraviolet absorption of the eight bakkenolides, all of them showed better ultraviolet absorption at 235 nm and therefore, the detection wavelength was set at 235 nm.

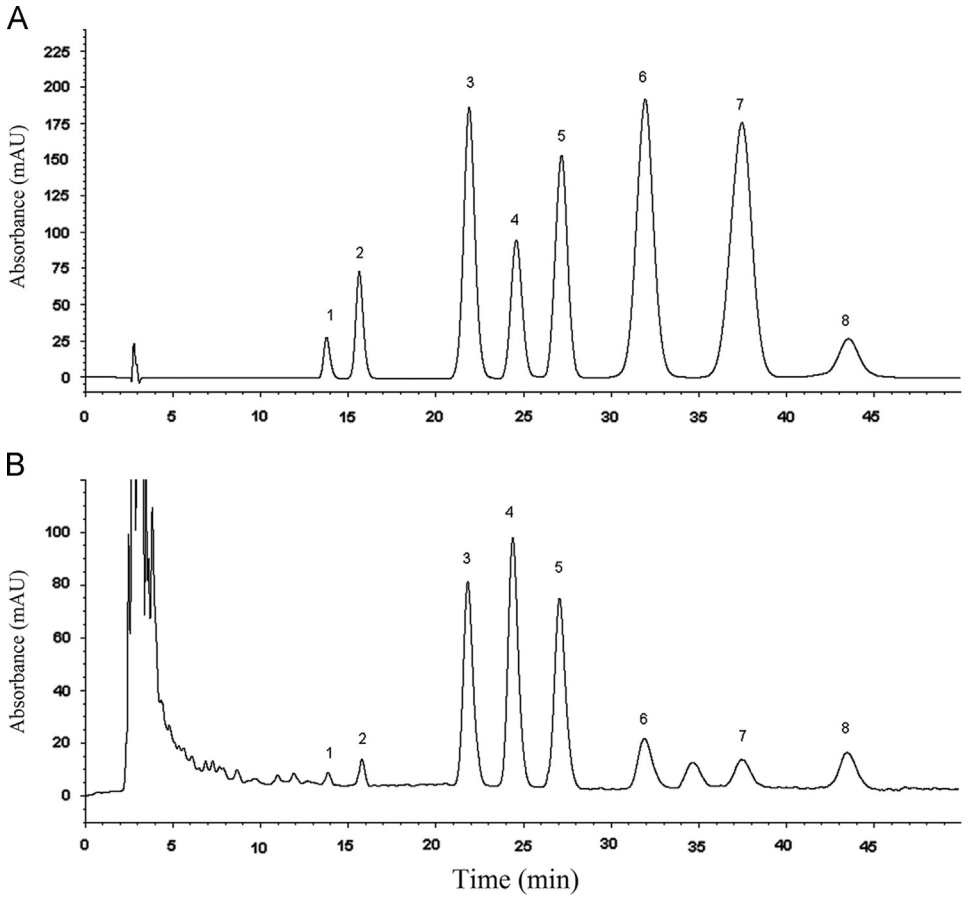

Figure 2 Representative chromatograms of the eight reference standards (A) and the extract of a sample from Shuangyashan, Heilongjiang Province (B). Peaks: 1, bakkenolide-L; 2, bakkenolide-D; 3, bakkenolide-B; 4, bakkenolide-Ia; 5, bakkenolide-VIIa; 6, bakkenolide-IVa; 7, bakkenolide-IIIa; 8, homofukinolide.

Considering similar polarity of the eight compounds, the isocratic elution was used to achieve better separation. Several mobile phases were tested, including methanol–water, acetonitrile–water and tetrahydrofuran–acetonitrile–water. Finally, the optimum separation was achieved using an isocratic system with tetrahydrofuran–acetonitrile–water.

Different types of chromatographic columns were investigated for separation, such as Agilent Zorbax SB-C18 (250 mm × 4.6 mm, 5 μm), Hypersil ODS (250 mm × 4.6 mm, 5 μm) and Diamonsal C18 (250 mm × 4.6 mm, 5 μm). As a result, the Agilent Zorbax SB-C18 column (250 mm × 4.6 mm, 5 μm) was selected because it provided the best peak shape and resolution in the extracts of herbs as well as in the standard mixture solution.

Identification of the marker compounds in the samples was carried out by comparing their retention time with that of each reference compound. Representative chromatograms for the eight standard analytes and a sample are shown in Fig. 2. Fig. 2A indicates that the eight standard analytes are well separated within 50 min and their solution between any two compounds is greater than 1.5. Fig. 2B exhibits that other compounds in the sample do not interfere with the analysis of the eight anaytes.

3.2. *Method validation*

3.2.1. *Linearity, limits of detection and limits of quantitation*

Table 1 lists the linear calibration curve with r^2, linear range, LOD and LOQ of each analyte. The results show that an excellent linear correlation existed between the peak area and concentration of each analyte. All calibration curves have good linear regression ($r^2 > 0.99$) within test ranges. LOD ranged 0.42–2.56 μg/mL and LOQ ranged 1.22–8.40 μg/mL.

3.2.2. *Precision and repeatability*

Table 2 shows the precisions and repeatabilities of the eight analytes. It indicates that the RSDs of the overall intra- and inter-day variations are less than 1.83% for all the eight analytes. Besides, validation studies of this method showed that this assay has good repeatability with RSDs less than 2.30% for the eight analytes. Thus, the precision and repeatability of the method were considered to be acceptable.

3.2.3. *Recovery*

A sample was assayed in triplicate by the LC system and the contents of the eight analytes were calculated to be 1.75, 1.12, 4.67, 6.06, 0.61, 0.33, 0.36 and 2.74 mg/g, correspondingly, according to their calibration curves. Appropriate amount of each standard was spiked into 1.0 g of the sample to obtain fortified sample at different concentrations. These fortified samples were processed and measured as described above and the recoveries for the eight analytes were calculated. The recoveries of these fortified samples are shown in Table 3. The recoveries ranged 97.8%–102.4% and were acceptable.

Table 1 Linear equations, limits of detection (LOD) and limits of quantitation (LOQ) for analytes.

Analyte	Calibration curve[a]	r^2[b]	Linear range (mg/mL)	LOD[c] (μg/mL)	LOQ[d] (μg/mL)
Bakkenolide-L	$Y=2511.37X-4.23$	0.9957	0.012–0.30	2.40	8.24
Bakkenolide-D	$Y=7574.50X-12.35$	0.9990	0.012–0.30	1.66	4.94
Bakkenolide-B	$Y=17087.88X-23.41$	0.9992	0.02–0.50	1.16	3.62
Bakkenolide-Ia	$Y=8678.3X-14.87$	0.9982	0.02–0.50	1.56	4.60
Bakkenolide-VIIa	$Y=15275.4X-18.26$	0.9998	0.02–0.50	0.80	2.31
Bakkenolide-IVa	$Y=26810.8X-35.94$	0.9999	0.02–0.50	0.51	1.57
Bakkenolide-IIIa	$Y=30521.2X-63.38$	0.9993	0.02–0.50	0.42	1.22
Homofukinolide	$Y=8296.23X-9.87$	0.9976	0.012–0.30	2.56	8.40

[a] y is the peak area and x is the concentration (mg/mL).
[b] Correlation coefficient.
[c] Limit of detection.
[d] Limit of quantitation.

Table 2 Precision and repeatability of the eight analytes.

Analyte	Precision				Repeatability($n=6$)	
	Intra-day ($n=5$)		Inter-day ($n=5$)		Mean ± SD (mg/g)	RSD (%)
	Mean[a] ± SD	RSD (%)	Mean[a] ± SD	RSD (%)		
Bakkenolide-L	730 ± 11.14	0.85	719 ± 20.84	1.60	1.85 ± 0.020	1.08
Bakkenolide-D	2260 ± 24.90	1.10	2273 ± 32.45	1.43	1.12 ± 0.008	0.71
Bakkenolide-B	8398 ± 87.83	1.05	8305 ± 100.10	1.21	4.67 ± 0.051	1.10
Bakkenolide-Ia	4078 ± 49.82	1.22	4100 ± 70.23	1.71	6.06 ± 0.059	0.97
Bakkenolide-VIIa	7613 ± 78.57	1.03	7681 ± 85.89	1.12	0.61 ± 0.014	2.30
Bakkenolide-IVa	13451 ± 101.34	0.75	13455 ± 113.25	0.84	0.33 ± 0.005	1.50
Bakkenolide-IIIa	15425 ± 118.58	0.77	15442 ± 121.58	0.79	0.36 ± 0.004	1.11
Homofukinolide	2325 ± 29.91	1.29	2298 ± 42.12	1.83	2.74 ± 0.042	1.53

[a] Peak area.

Table 3 Recoveries of the eight analytes.

Analyte	Original mean (mg)	Spiked mean (mg)	Found mean (mg)	Recovery (%) ($n=5$)
Bakkenolide-L	1.75 ± 0.028	1.51 ± 0.028	3.26 ± 0.028	99.7 ± 1.36
Bakkenolide-D	1.12 ± 0.017	1.23 ± 0.008	2.37 ± 0.034	101.7 ± 1.28
Bakkenolide-B	4.67 ± 0.077	4.54 ± 0.061	9.32 ± 0.106	102.4 ± 0.88
Bakkenolide-Ia	6.06 ± 0.058	6.13 ± 0.070	12.24 ± 0.135	100.8 ± 2.37
Bakkenolide-VIIa	0.61 ± 0.012	0.60 ± 0.013	1.20 ± 0.034	99.2 ± 1.64
Bakkenolide-IVa	0.33 ± 0.005	0.35 ± 0.071	0.67 ± 0.013	97.8 ± 1.82
Bakkenolide-IIIa	0.36 ± 0.064	0.42 ± 0.0068	0.78 ± 0.018	100.2 ± 1.76
Homofukinolide	2.74 ± 0.019	2.98 ± 0.021	5.69 ± 0.048	99.0 ± 2.53

Data are expressed as mean ± SD, $n=3$.

Therefore, the HPLC–UV method is precise, accurate and sensitive enough for simultaneous quantification of the eight compounds in *P. tatewakianus*.

3.3. *Extraction method development*

In order to obtain effective extraction, preparation conditions including extraction solvent, extraction times, ratio of samples to solvent and extraction time were optimized. Pure and aqueous ethanol or methanol was tried as the extraction solvent. The best solvent was found to be 100% pure methanol that allowed complete extraction of eight bakkenolides. After comparisons, ultrasonic extraction was selected as the preferred method for the present experiments, which is a simple and effective extraction method. The influence of the extraction time (15, 30, 45 and 60 min), ratio of samples to solvent (1:5, 1:10, 1:15 and 1:20) and extraction times (1, 2 and 3) affecting the extraction efficiency was also examined. As a result, the optimized extraction method was confirmed as follows: 1.50 g of the powdered sample was

Table 4 Contents of the eight analytes in samples of *P. tatewakianus*.

Locality	Analyte	Content (mg/g)			Total
		Rhizome	Petiole	Blade	
Shuangyashan	Bakkenolide-L	1.85 ± 0.024	1.65 ± 0.023	1.75 ± 0.015	
	Bakkenolide-D	1.12 ± 0.015	0.94 ± 0.001	0.85 ± 0.011	
	Bakkenolide-B	4.67 ± 0.054	1.29 ± 0.018	1.10 ± 0.014	
	Bakkenolide-Ia	6.06 ± 0.081	1.51 ± 0.057	1.04 ± 0.019	
	Bakkenolide-VIIa	0.61 ± 0.009	1.85 ± 0.025	0.48 ± 0.008	
	Bakkenolide-IVa	0.33 ± 0.005	0.25 ± 0.003	0.18 ± 0.004	
	Bakkenolide-IIIa	0.36 ± 0.005	0.24 ± 0.003	0.21 ± 0.003	
	Homofukinolide	2.74 ± 0.038	1.86 ± 0.020	1.02 ± 0.012	
	Total	17.73 ± 0.19	9.58 ± 0.12	6.62 ± 0.08	33.93 ± 0.42
Raohe County	Bakkenolide-L	0.87 ± 0.001	0.74 ± 0.010	0.68 ± 0.009	
	Bakkenolide-D	0.33 ± 0.004	0.23 ± 0.003	0.21 ± 0.003	
	Bakkenolide-B	3.52 ± 0.046	0.81 ± 0.011	0.64 ± 0.008	
	Bakkenolide-Ia	9.22 ± 0.103	9.60 ± 0.108	0.88 ± 0.011	
	Bakkenolide-VIIa	0.27 ± 0.004	0.91 ± 0.012	0.18 ± 0.003	
	Bakkenolide-IVa	0.06 ± 0.005	–	–	
	Bakkenolide-IIIa	0.15 ± 0.003	0.08 ± 0.001	0.10 ± 0.002	
	Homofukinolide	0.35 ± 0.005	–	–	
	Total	14.77 ± 0.17	12.37 ± 0.16	2.69 ± 0.03	31.63 ± 0.39
Luobei County	Bakkenolide-L	1.06 ± 0.013	0.66 ± 0.009	0.98 ± 0.012	
	Bakkenolide-D	1.67 ± 0.020	1.29 ± 0.015	0.91 ± 0.013	
	Bakkenolide-B	1.02 ± 0.015	0.16 ± 0.019	0.15 ± 0.003	
	Bakkenolide-Ia	5.20 ± 0.061	1.23 ± 0.018	0.11 ± 0.002	
	Bakkenolide-VIIa	0.64 ± 0.009	2.71 ± 0.033	2.96 ± 0.035	
	Bakkenolide-IVa	0.35 ± 0.005	0.27 ± 0.004	0.21 ± 0.003	
	Bakkenolide-IIIa	0.41 ± 0.006	0.20 ± 0.003	0.19 ± 0.003	
	Homofukinolide	0.32 ± 0.005	0.18 ± 0.003	–	
	Total	10.61 ± 0.13	6.69 ± 0.08	5.51 ± 0.06	22.81 ± 0.31
Yichun	Bakkenolide-L	1.13 ± 0.015	1.09 ± 0.014	0.74 ± 0.010	
	Bakkenolide-D	0.24 ± 0.004	0.19 ± 0.003	0.16 ± 0.003	
	Bakkenolide-B	0.18 ± 0.003	0.18 ± 0.003	0.13 ± 0.002	
	Bakkenolide-Ia	5.21 ± 0.062	1.53 ± 0.020	1.04 ± 0.013	
	Bakkenolide-VIIa	0.69 ± 0.009	2.53 ± 0.028	0.51 ± 0.006	
	Bakkenolide-IVa	0.54 ± 0.008	0.35 ± 0.004	0.23 ± 0.003	
	Bakkenolide-IIIa	0.17 ± 0.003	0.09 ± 0.001	–	
	Homofukinolide	0.42 ± 0.031	0.23 ± 0.003	–	
	Total	8.58 ± 0.11	6.19 ± 0.07	2.80 ± 0.04	17.57 ± 0.24
Fangzheng County	Bakkenolide-L	0.16 ± 0.003	0.13 ± 0.002	0.13 ± 0.002	
	Bakkenolide-D	–	–	–	
	Bakkenolide-B	0.11 ± 0.002	0.10 ± 0.002	0.12 ± 0.002	
	Bakkenolide-Ia	8.92 ± 0.098	13.4 ± 0.189	8.92 ± 0.101	
	Bakkenolide-VIIa	12.4 ± 0.141	4.8 ± 0.058	8.41 ± 0.094	
	Bakkenolide-IVa	0.38 ± 0.005	0.26 ± 0.004	0.19 ± 0.003	
	Bakkenolide-IIIa	0.23 ± 0.003	0.14 ± 0.002	0.12 ± 0.002	
	Homofukinolide	1.44 ± 0.018	1.13 ± 0.134	0.68 ± 0.009	
	Total	23.64 ± 0.33	19.96 ± 0.24	18.51 ± 0.23	62.11 ± 0.82
Baishan	Bakkenolide-L	0.66 ± 0.009	0.73 ± 0.011	0.57 ± 0.008	
	Bakkenolide-D	0.54 ± 0.008	0.52 ± 0.008	0.37 ± 0.005	
	Bakkenolide-B	3.07 ± 0.041	0.72 ± 0.009	0.56 ± 0.008	
	Bakkenolide-Ia	7.80 ± 0.082	0.51 ± 0.007	1.02 ± 0.012	
	Bakkenolide-VIIa	19.63 ± 0.210	3.50 ± 0.042	18.21 ± 0.203	
	Bakkenolide-IVa	0.69 ± 0.009	0.55 ± 0.008	0.48 ± 0.006	
	Bakkenolide-IIIa	0.32 ± 0.005	0.19 ± 0.003	0.17 ± 0.003	
	Homofukinolide	5.36 ± 0.063	3.56 ± 0.048	1.72 ± 0.021	
	Total	37.98 ± 0.48	10.26 ± 0.12	23.08 ± 0.31	71.32 ± 1.04

Data are expressed as mean ± SD, $n = 3$.
"–" Means undetected.

extracted using ultrasonication for 30 min twice with 30 mL of methanol.

3.4. Assay of samples from different localities

The optimized HPLC–UV method was applied to simultaneously determine the eight major active bakkenolides in different parts of *P. tatewakianus* from six localities. Three independent experiments of each sample were carried out following the developed method. The content of each analyte was calculated by the corresponding regression equation. The average contents of the three independent experiments are revealed in Table 4.

The content of each bakkenolide varied in different parts and localities. There were statistically significant differences between the total bakkenolide content of *P. tatewakianus* from every two localities ($P<0.01$), as well as between the total bakkenolide content of every two parts of *P. tatewakianus* from all the localities ($P<0.01$). By analyzing the result, it is found that the content of total bakkenolides in rhizome was higher than that in other parts of the plant from all the localities. The amounts of total bakkenolides in rhizome of plant ranged 8.58 ± 0.11 mg/g (from Yichun)–37.98 ± 0.48 mg/g (from Baishan). Also, total bakkenolides of the whole plant from Baishan was the highest (71.32 ± 1.04 mg/g) while the amount of whole plant from Yichun was the lowest (17.57 ± 0.24 mg/g). The result suggested that rhizome may be the most valueable part of *P. tatewakianus*, and *P. tatewakianus* from Baishan may be the best plant resource. The highest content of each bakkenolide was in different parts of the plant from different localities. For example, the content of bakkenolide-L (1.85 ± 0.024 mg/g) in rhizome of the plant from Shuangyashan had the highest level, and the amount of bakkenolide-Ia (13.4 ± 0.189 mg/g) in petiole of plant from Fangzheng County had the highest level. Among all these bakkenolides, bakkenolide-VIIa in rhizome of *P. tatewakianus* from Baishan had the most valuable quantity (19.63 ± 0.210 mg/g). However, some bakkenolides were not detected in certain part of the plant, such as bakkenolide-IVa in petiole and blade of the plant from Raohe County. From the results of this study, the plant that contained remarkably high level of the target bakkenolides could be considered as the best resource if any one of the eight bakkenolides was chosen for further study and application. The quality of *Petasites tatewakianus* Kitam can be evaluated through a simultaneous determination of eight bakkenolides, including bakkenolide-L, bakkenolide-D, bakkenolide-B, bakkenolide-Ia, bakkenolide-VIIa, bakkenolide-IVa, bakkenolide-IIIa and homofukinolide, using the developed method.

4. Conclusions

In this study, a simple quantitative method based on HPLC–UV was developed to simultaneously determine eight active bakkenolides in *P. tatewakianus*. Good linear calibration curves enabled quantitative determination of the eight marker compounds over a relatively wide concentration range. Method validation revealed acceptable accuracy, precision, sensitivity and repeatability. Samples (including rhizome, blade and petiole) from six different localities were assayed by this method. There were statistically significant differences ($P<0.01$) between the total bakkenolide contents of *P. tatewakianus* from any two localities, as well as the total bakkenolide contents of any two parts of *P. tatewakianus* from all the localities ($P<0.01$). The content of total bakkenolides in rhizome was higher than that in other parts of the plant. Thus the total content of bakkenolides could be used as the quantitiative marker to assess the quality of *P. tatewakianus*. The rhizome of *P. tatewakianus* and *P. tatewakianus* from Baishan may be the best plant resource to obtain bakkenolides. Our results might serve as a sound foundation for further study and application of this plant.

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