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Isolation, Quality Evaluation, and Seasonal Changes of Bakkenolide B in *Petasites japonicus* by HPLC

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The leaves of *Peatasites japonicus* are a traditional oriental medicine with diverse biological activities. A simple and specific analytical method for the quantitative determination of bakkenolide B constituents from methanolic extract of the leaves of *P. japonicus* was developed. Bakkenolide B was isolated from the leaves of *P. japonicus,* and its structure was elucidated based on 1D, 2D NMR, and GC-MS spectral data. A liquid chromatographic method was developed to evaluate the quality of *P. japonicus* through determination of major active compound, bakkenolide B. The wavelengths at 254 and 215 nm were chosen to determine bakkenolide B. The recovery of the method was in the range of 98.6 to 103.1%, and bakkenolide B showed good linearity (r^2=0.999) within test ranges. The developed method was applied to the determination of bakkenolide B in the plant part and seasonal changes. The results showed that the content of bakkenolide B in the leaf was higher than in the petiole and rhizome. In this study, a simple, rapid, and reliable high-performance liquid chromatography method was used to determine the percentage and composition of bakkenolide B in *P. japonicus* procured from different *Petasites* species plants in South Korea. The method can be employed in routine quantitative analysis and quality control of different products in the market.

Key words : Bakkenolide B, HPLC, *Petasites japonicus*, quality evaluation, seasonal change

Introduction

Petasites japonicus (Sieb. et Zucc.) Maxim, is a perennial herbaceous, belonging to the family Compositae, distributed in Korea, Japan and East China. It has been consumed as a wild leafy vegetable in recent years [14, 16]. *P. japonicus* has been utilized as a folk medicine for the treatment of furunculosis, contusion, wounds, and snakebites [10]. Previous phytochemical investigations on *P. japonicus* contained the main presence of sesquiterpenoids, especially eremophilane-type sesquiterpenoids [9, 15, 17, 22, 23, 24]. In our survey process on biologically active substances in medicinal plants, considerable attention has been given to the occurrence of compounds with anti-inflammatory, anticancer, and neuroprotective effects [7, 9, 12, 13], since these substances are expected to be potentially useful for the treatment and prevention of Parkinson's diseases [5]. As a continuation of our work on the search for bioactive substances from traditional folk medicines or medicinal food [7, 12, 13], we reported on the structural elucidation of sesquiterpene lactones from the leaf of *P. japonicus*, and confirmed that the bakkenolide B exhibited significant anti-allertic activity [9]. Bakkenolide-B is the major sesquiterpene lactones in *P. japonicus* and can be utilized to evaluate their quality. The distribution of the medicinal constituents plays an important role in raw material selection for medical extract production. However, there is no report about analytical methods for the seasonal changes and plant parts of bakkenolide B in *P. japonicus*. There is a great interest in establishing an analytical method for the quality control of *P. japonicus* for better applications in the medicinal herb industry. Therefore, we focused our investigations on the isolation and characterization of bakkenolide B, and its contents in different seasons

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and parts of *P. japonicus* were compared to establish the effectiveness of the method.

Materials and Methods

Materials

P. japonicus were planted at the Agricultural Experimental Field (Miryang, Gyeongnam) of Pusan National University in November, 2012 and leaves, petioles and rhizomes collected with one month interval from March to August, 2013. These samples were stored at -20℃ prior to testing. A voucher specimen (accession number MW-PRDR-11) was deposited at the Herbarium of Pusan National University. HPLC-grade water and acetonitrile were purchased from Fisher (Fair Lawn, NJ, USA). Distilled water was produced from Milli-pore water purified system. All the solutions were degassed by an ultrasonic bath before use. Bakkenolide B was extracted and purified from the leaves of *P. japonicus* with minimum purity of 98.0% by GC-MS (Gas chromatography - mass spectrometry). The chemical structure of bakkenolide B was identified by spectral method (^{1}H NMR, ^{13}C NMR, 2D NMR and MS) and confirmed by comparing with those in literature [1]. The ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR spectra were recorded on a Bruker DMX-500NMR spectrometer (Bruker, Billerica, Massachusetts, USA) using TMS as an internal standard. Silica gel (60-200 mesh) (Merk, Darmstadt, Germany) was used for column chromatography.

Isolation of bakkenolide B

The fresh leaves of *P. japonicus* (1.0 kg) were chopped to fine particles with an electric mixer (HMF-3100 S, Hanil Electric, Seoul, Korea) and then extracted with 70% ethanol (3 l × 3) under sonicator for one hour at room temperature (Fig. 1). The solvent was evaporated under reduced pressure to obtain EtOH extracts. This residue was suspended in H_2O (500 ml) and successively partitioned with hexane (1 l × 3), ethyl acetate (1 l × 3), BuOH (500 ml × 3) and aqueous extract. The hexane extract (2.673 g) from total hexane extract (13.60 g) was evaporated in vacuo, and then chromatographed on a silica gel (40 μm, Baker, NJ) column (100×4.0 cm) using a step gradient 2.5%, 15%, 25% acetone in dichloromethylene and 15% and 25% MeOH in chloroform to obtain 62 fractions. Fraction 9 (MWLSH9, 304.9 mg) was separated on a Sephadex column (100×3.0 cm) using MeOH as eluant to obtain 7 fractions. The fraction 3 (MWLSH9IC, 209.7 mg) was further separated on a Sephadex column (100×3.0 cm) using MeOH to obtain five fractions. Fractions 2 and 3 (MWLSH9ICIB, 202.3 mg) were passed through a silica gel column (100×4.0 cm) using 1.5% acetone in CH_2Cl_2 as eluant to yield bakkenolide B (173.8 mg). Pure bakkenolide B was identified by HPLC on a Phenomenex Luna C_{18} column (Phenomenex, 150×4.6 mm ID; 5 μm particle size, Phenomenex, Torrance, CA, USA) using an acetonitrile-water gradient at a flow rate of 0.4 ml per minute. Bakkenolide B isolated from *P. japonicus* leaves was identified using ^{1}H, ^{13}C, and Dept (distortionless enhancement of polarization transfer) nuclear magnetic resonance spectroscopy in $CDCl_3$ by comparison with previously reported spectral data [6, 11].

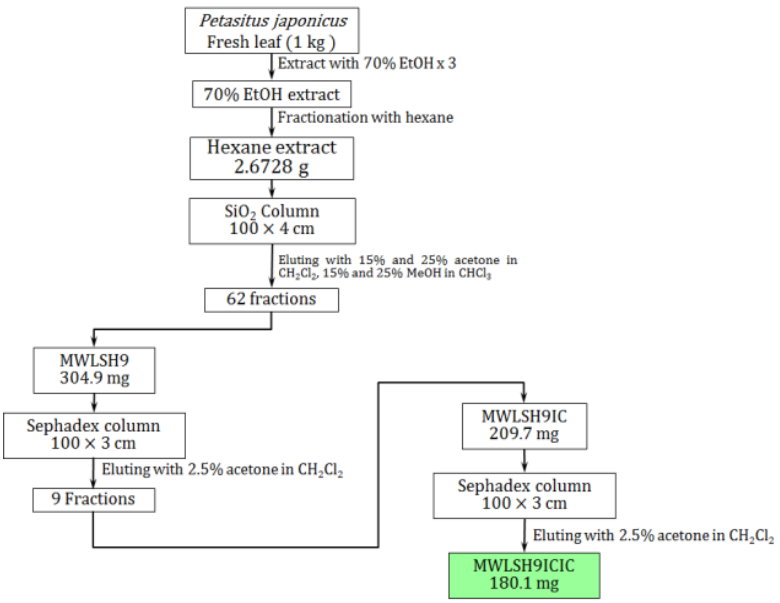

Fig. 1. Isolation scheme of bakkenolide B from leaves of *Petasitus japonicus*. The fresh leaves of *P. japonicus* (1.0 kg) were chopped to a fine particle with an electric mixer and then extracted with 70% ethanol (3 l × 3) under sonicator for one hour at room temperature. The hexane extract was chromatographed on a silica gel column using a step gradient 2.5%, 15%, 25% acetone in dichloromethylene and 15% and 25% MeOH in chloroform to obtain 62 fractions. Fraction 9 was successively separated on a Sephadex and normal silica column to yield bakkenolide B (173.8 mg).

Sample preparation

All samples (including leaves, petioles and rhizomes) were stored at -20℃ for use. A sample of 1 g fresh weight was mixed by using mortar and pestle and sonicated in 5 ml MeOH for 60 min three times. The extract was centrifuged at 3,000 rpm for 10 min and collected supernatant was then adjust to 15 ml with MeOH. The sample solution was filtered through a 0.45 μm membrane before HPLC (performance liquid chromatography) analysis.

Analytical method

Chromatographic analyses were performed on Agilent Technologies (Waldbronn, Germany) G1100 systems equipped with a vacuum degasser, quaternary pump, thermostated oven device and a variable wavelength UV detector. The chromatographic data were acquired and analyzed using Agilent chromatographic Work Station software. Analyses were carried out on a Luna C_{18} column (5 μm, 150×3.0 mm i.d. Phenomenex, Torrance, CA, USA) with a mobile phase gradient of acetonitrile - water (0 to 100) for 35 min. The injection volume was 10 μl sample. The column temperature was kept constant at 30℃, and the mobile phase flow rate was 0.4 ml min^{-1} with UV detection at 210, 215, 235, 254 nm and 360 nm.

Calibration curves, limits of detection and quantification

Stock solution of bakkenolide B was prepared by solving 2.0 mg bakkenolide B to 2 ml methanol. The concentration of bakkenolide B was 1.0 mg ml^{-1}. The stock solution was further diluted to make different concentration ranges. The calibration curve was performed with at least five appropriate concentrations in triplicate. These concentrations were appropriate for the calibration curves: 500, 250, 125, 62.5 and 31.25 mg/ml of the original concentration. The solutions were brought to room temperature and filtered through a 0.45 μm membrane filter and an aliquot of 10 μl was injected into the HPLC for analysis. Their regression equations were calculated in the form of y=ax+b, where x and y were peak area and contents of compound, respectively. The diluted solution of bakkenolide B was further diluted to a series of concentrations with methanol for gaining the limits of detection (LOD) and quantification (LOQ). The LOD and LOQ under the present chromatographic conditions were determined at a signal-to-noise ratio of 3 and 10, respectively.

Precision, repeatability and accuracy

The intra- and inter-day precision was determined by analyzing calibration samples during a single day on three different days. To confirm the repeatability, three different working solutions were prepared from the leaves sample. The RSD (relative standard deviation) was taken as a measure of precision and repeatability. Recovery test was used to evaluate the accuracy of this method by adding 50 μg bakkenolide B to approximately 1 g of fresh leave extract of *P. japonicus*. The average recoveries were counted by the formula: recovery (%)=(amount found-original amount)/amount spiked×100%, and RSD (%)=(standard deviation/mean)×100%.

Results

Extraction and isolation of bakkenolide B

The hexane extract (2.673 g) from total hexane extract (13.60 g) was subjected to column chromatography packed with silica gel and gradiently eluted with mixed 15, 25 acetone in CH_2Cl_2, 15% and 25% in $CHCl_3$ to give 62 fractions. Fraction 9 (MWLSH 304.9 mg) was chromatographed on a sephadex (100×3.0 cm) further fractionated using a 2.5% acetone in CH_2Cl_2 to yield pure compound 180.1 mg (MWLSH9ICIC). GC-MS data suggested that the purity of the compound was more than 97%.

Structure elucidation of bakkenolide B

In the course of our survey on pharmacologically active substances in medicinal plants [7, 12, 13], much attention has been given to the occurrence of compounds with anti-allergic effects, since these compounds are expected to be potentially useful for the treatment and prevention of Parkinson's disease. We investigated the chemical constituents of the leaves of *P. japonicus*, of which the hexane extract showed moderate anti-allergic effects [9]. As a result of further chemical investigation for bioactive products, bakkenolide B were isolated from the leaves of *P. japonicus*. Herein, we describe the isolation and structural elucidation of bakkenolide B (Table 1. and Fig. 2).

Molecular formula of Bakkenolide B (BB), $C_{22}H_{30}O_6$, was determined by GC-MS (m/z 390 for $C_{22}H_{30}O_6$). The ^{1}H NMR data (Table 1) for BB exhibited five methyl groups at δ 0.82 (3H, d, J=6.5 Hz, H-14), 1.03 (3H, s, H-15), 1.84 (3H, s, -COCH_3, H-2''), 1.70 (3H, s, -COC(CH_3)=CHCH_3, H-5), and 1.79 (1.79, d, J=6.5 Hz, -COC(CH_3)=CHCH_3, H-4), two olefinic protons at δ 5.10 (2H, s, H-13), two oxygenated methines at δ 5.09 (1H, br, H-1) and 5.66 (1H, d, J=11.0 Hz,

Table 1. ^{1}H and ^{13}C NMR spectroscopic data of bakkenolide B (^{1}H NMR 500 MHz, ^{13}C NMR 125 MHz in $CDCl_3$)*

Position	δ_C		δ_H	HMBC
1	70.40	CH	5.09, s	
2	26.77	CH_2	1.73, dd, J=9.5/6.5	
3	29.50	CH_2	1.63, br	
4	35.22	CH	1.56, br	
5	43.28	C		
6	45.81	CH_2	2.14, d, J=14.0	177.38, 147.75, 54.91, 43.28, 35.22, 19.53
			1.87, d, J=14.0	
7	54.91	C		
8	177.38	C		
9	80.75	CH	5.66, d, J=11.0	177.38, 169.81, 147.75, 70.40
10	51.41	CH	2.72, br	80.75, 70.40, 43.28, 35.22, 26.77, 19.53
11	147.75	C		
12	70.52	CH_2	4.57, t	
13	108.22	CH_2	5.10, d, J=14.0	70.52, 54.91
14	15.48	CH_3	0.82, d, J=6.5	43.28, 35.22, 29.50
15	19.53	CH_3	1.03, s	51.41, 45.81, 43.28, 35.22
1′	167.17	C		
2′	128.20	C		
3′	136.58	CH	5.86, d,d	
4′	15.48	CH_3	1.79, d, J=6.5	136.58, 128.20
5′	20.29	CH_3	1.70, s	167.17, 136.58, 128.20, 20.90
1"	169.81	C		
2"	20.90	CH_3	1.84, s	169.81

*Assignments of chemical shifts of bakkenolide B was confirmed by DEPT, gHMQC, gHMBC

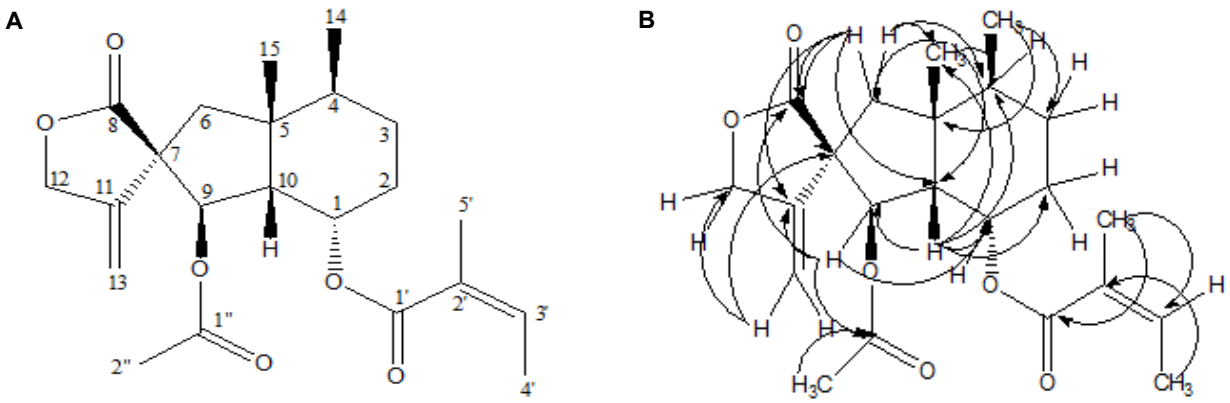

Fig. 2. Structure (A) and key HMBC (B) of bakkenolide B.

H-9), and an oxygenated methylene at δ 4.57 (2H, t, H-12). Bakkenolide B was determined to be optically active and to contain a bakkenolide-type skeleton with two substituents on C-1 and C-9 based on their ^{1}H-NMR and ^{13}C-NMR spectra (Table 1) and by comparison with previously reported data [1, 21] and completely assigned based on the two-dimensional (2D) NMR spectra. The ^{13}C NMR spectrum of bakkenolide B, in combination with the DEPT and HMQC spectra, showed twenty two carbon signals that were classified into five methyls [δ 15.48 (C-14), 19.53 (C-15), 20.90 (-CO*CH*$_3$, C-2"), 15.48 (-COC(CH$_3$)=CH*CH*$_3$, C-4′), and 20.29 (-COC(*CH*$_3$)=CHCH$_3$, C-5′)], five methylenes [δ 26.77 (C-2), 29.50 (C-3), 45.81 (C-6), 70.52 (C-12), and 108.22 (C-13)], five methines [δ 70.40 (C-1), 35.22 (C-4), 80.75 (C-9), 51.41 (C-10), and 136.58 (-COC(CH3)=*CH*CH$_3$, C-3′)], and seven quaternary carbons [δ 43.28 (C-5), 54.91 (C-7), 178.38 (C-8), 147.74 (C-11), 169.81 (-*C*OCH3, C-1"), 128.20 (-CO*C*(CH3)=CHCH$_3$, C), and 167.17 (-*C*OC(CH$_3$)=CHCH$_3$)]. Close similarities of the chemical shifts from C-1 to C-15 for BB with reported values for similar compounds implied that 1 was a bakkeno-

lide-type sesquiterpene with an acetoxy and an angeloyloxy [1, 18, 21]. The further analysis of HSQC and HMBC spectra confirmed the presence of the bakkenolide-type skeleton, the acetoxy, and the angeloyloxy groups. The HMBC correlations of H-9 to the carbonyl carbon of the acetoxy, and H-1 to the carbonyl carbon of the angeloyloxy, revealed that the acetoxy and the angeloyloxy were attached at C-1 and C-9, respectively. By analysing the HSQC, HMBC, and ^{1}H-^{1}H COSY spectra (Fig. 2), all the proton and carbon signals were assigned unambiguously. Thus, the planar structure of BB was disclosed.

Analytical conditions

HPLC conditions, especially the mobile phase and its elution program, are also of importance for the determination of the compound in the biological matrix. In this study, we found that a mobile phase consisting of acetonitrile and H_2O can separate bakkenolide B (Fig. 3). The selection of the LC conditions was guided by the requirement for obtaining chromatograms with better resolution. The LC conditions developed in this study produced full peak-to-baseline resolution of the major active bakkenolide B present in *P. japonicus* sample. On the basis of UV maximal absorption of the bakkenolide B shown in Fig. 3, the choice of detection at 215 nm for quantitative analysis was appropriate.

Validation of the method

The analysis method for bakkenolide B was validated based on linear calibration curve with r^2, linear range, -LOD and LOQ of bakkenolide B. As a result, calibration curve showed good linear regression (r^2=0.999) within test ranges; the LOD (S/N=3) and the LOQ (S/N=10) for the bakkenolide B were less than 1.05 and 3.38 μg in 210 nm and 2.26 and 6.84 μg in 254 nm, respectively (Table 2).

Table 3 shows the results of precision of the bakkenolide B. It indicates that the RSD of the overall 215 and 254 nm

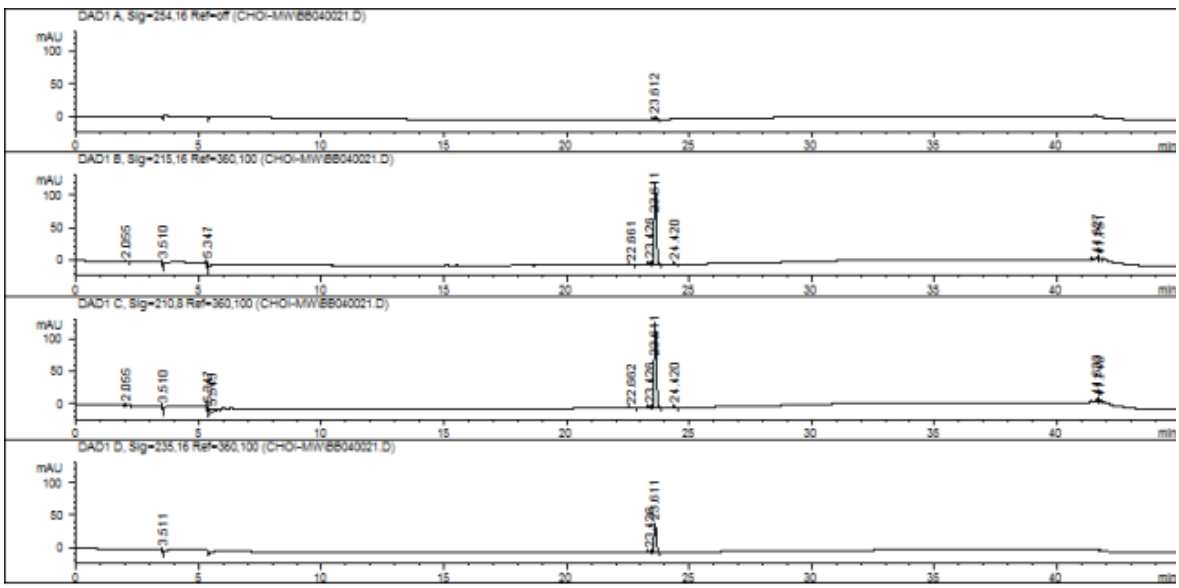

Fig. 3. Typical HPLC chromatograms of the reference bakkenolide B (100 μg/ml). Analyses were carried out on a Luna C_{18} column with a mobile phase gradient of acetonitrile - water (0 to 100) for 35 min. The injection volume was 10 μl sample. The column temperature was kept constant at 30, and the mobile phase flow rate was 0.4 ml min^{-1} with UV detection at 210, 215, 235, 254 nm and 360 nm.

Table 2. Regression data, LODs and LOQs for the bakkenolide B by HPLC

Wavelength (nm)	Calibration curve[z]	r^2	Linear range (μg)	LOD[y] (μg)	LOQ[x] (μg)
215 nm	y = 7.841x - 6.262	0.9999	25 - 500	1.049	3.379
254 nm	y = 0.226x - 0.475	0.9999	25 - 500	2.259	6.844

[z]x is the peak area in UV chromatograms, Y is the compound amount injected
[y]LOD refers to the limits of detection
[x]LOQ refers to the limits of quantification

Table 3. Precision of the bakkelolide B (n=6)

Replication	Retention time (min)	Peak area		Concentration (μg/ml)
		215 nm	254 nm	
1	23.611	745.2	22.5	100
2	23.612	742.1	22.2	100
3	23.611	739.7	22.1	100
4	23.610	739.2	22.1	100
5	23.610	738.9	22.1	100
6	23.591	740.7	22.2	100
Mean	23.608	740.967	22.200	
SD	0.0081	2.3779	0.1549	
%RSD	**0.0344**	**0.3209**	**0.6978**	

RSD (%)=(SD/mean)×100

Table 4. Recovery of the bakkenolide B of *P. japonicus* in leaves (n=3).

Spike (μg/ml)	Intra-day (n=6)		Inter-day (n=3)	
	Found mean ± SD	RSD (%) (n=3)	Found mean ± SD	RSD (%) (n=3)
50	50.11±0.44	0.99	44.44±0.26	1.00
100	104.30±1.28	1.01	100.70±1.42	0.99
200	211.81±0.88	1.00	211.74±4.61	0.98

Recovery = Peak area of sample/peak area of control.

wavelength variations were less than 1% for bakkenolide B. Besides, validation studies of this method proved that this assay has a good reproducibility with RSD also less than 1% for the bakkenolide B.

The developed analytical method has good accuracy with the overall recovery from 97.34 to 107.45% at intra-day and 100.22 to 105.91% at inter-day for 3 different spike levels (Table 4). The recovery efficiencies of RSD (%) for bakkenolide B at three different concentrations (50, 100 and 200 μg/ml) were 0.99 - 1.01%. Therefore, the method is precise, accurate and sensitive enough for bakkenolide B quantitative evaluation in *P. japonicus*.

Quantitative determination of bakkenolide B in *P. japonicus*

This newly developed HPLC assay method was subsequently applied to bakkenolide B determination for different season and parts of *P. japonicus*. The content of bakkenolide B was determined by the corresponding regression equation. The content of bakkenolide B in leaf was higher than in other parts and samples collected earlier in seasonal changes.

Discussion

Bakkenolide derivatives is one of the most important medical constituent of *Petasites* and its distribution is as significant as the differences observed with petasin derivatives. HPLC method for effective determination of bakkenolide B contents of *P. japonicus* was developed. The chromatogram of optimized separation on the column Luna C_{18} 100A column (150×3.0 mm) as gradient phase gave the most sufficient results with mobile phases: acetonitrile (A) and water at zero to 100% for 35 min (Fig. 3, Table 2). Avula et al. [2] developed two UPLC-UV and HPLC - ToF - MS methods for the analysis of major sesquiterpene constituents including petasin, isopetasin, S-petasin and 8β-H-eremophilanolide from roots of *P. hybridus* and dietary supplements containing *P. hybridus* extracts. Wang et al. [19] carried out the analyses on Agilent-ODS C_{18} column with mobile phase consisting of tetrahydrofuran - acetonitrile - water (28:12:60, v/v/v) to estimate simultaneous determination of four major active bakkenolides in *P. tricholobus*. The column temperature was at 55℃ and UV detections were at 265 nm between 0 and 15 min and at 235 nm between 15 and 32 min.

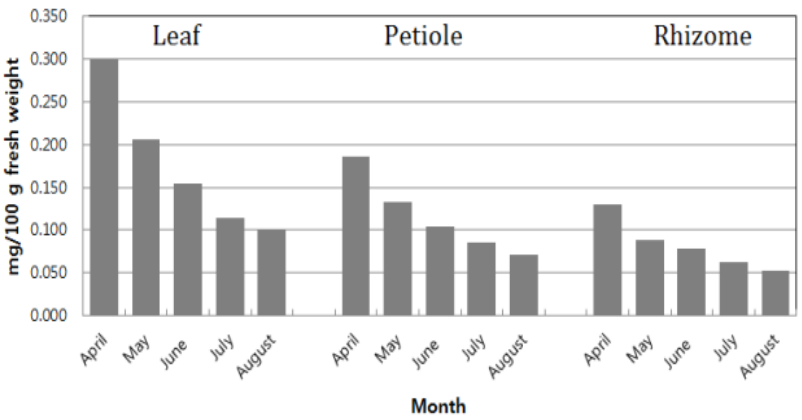

Fig. 4. Contents of bakkenolide B from different plant parts. Leaves, petioles and rhizomes were collected from April to August in 2013. Analyses were carried out on a Luna C_{18} column with a mobile phase gradient of acetonitrile - water (0 to 100) for 35 min. The injection volume was 10 μl sample. The column temperature was kept constant at 30℃, and the mobile phase flow rate was 0.4 ml min^{-1} with UV detection at 254 nm.

These literatures [19] and our results provide useful analysis method for determination of bekkenolide B. Wildi et al. [20] conducted the analyses of petasin distribution in rhizomes and leaves of the plant. The mean petasin content of populations ranged from 7.4 to 15.3 mg/g dry weight in rhizomes and from 3.3 to 11.4 mg/g dry weight in leaves of *P. hybridus*. The concentration of bakkenolides was found to be significantly higher in rhizomes than in flowers and leaves of *P. tricholobus* [19]. Chizzola et al. [4] analyzed the essential oil content of leaves, roots and flower stems of *P. japonicus* with GC and GC–MS. According to these results, contents of bakkenolode B and other bioactive compounds were different with plant parts, but bakkenolide B was generally found in younger metabolically active parts of leaves and it was also lower content in petioles and rhizomes (Fig. 4). The seasonal variation of bakkenolide B content in leaves, petioles and rhizomes decreased with later collection date. In this sense, the leaves are more favorable for harvesting with much higher bakkenolide B concentrations (Fig. 4) and petasin rich extracts [8, 20] since the changes in petasin concentrations between leaves and rhizomes of the plant are not tremendous [3]. In conclusion, a simple, accurate and rapid HPLC method was developed for bakkenolide B in *P. japonicus*. The HPLC assay is sensitive, reproducible and has been fully validated. The results indicate that samples from different season and parts of the plant show a specific HPLC chromatogram. This method can be used to determine the content of bakkenolide B from other *Petasites* species plants.

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초록 : 머위로부터 Bakkenolide B의 순수분리, HPLC분석 방법 및 채취 시기별 함량 분석

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머위(*Peatasites japonicus*)의 잎으로부터 생리활성물질을 탐색하여 항천식 효과가 있는 물질로 알려진 bakenolide B를 순수분리하였으며, 1D-NMR, 2D-NMR 및 GC-MS spectrum 데이터를 이용하여 구조를 동정하였다. 머위의 잎, 엽병 및 근경 등의 채취시기별 bakkenolide B의 함량을 평가하기 위하여 HPLC 분석 방법을 확립하였다. Bakkenolide B의 민감도는 210 nm와 215 nm의 파장에서 254 nm, 235 nm 및 265 nm보다 높았으며, 분석을 위한 회귀 직선식은 y=7.841-6.262(파장 215 nm)로서 상관값(r^2)이 0.999 이상으로서 유의성이 매우 높았다. 검출한계(LOD)는 1.05, 정량한계(LOQ)는 3.38이었다. 회수율은 3종류의 농도값에 대하여 98.6에서 103.1%로서 매우 높았다. 이러한 결과들로 미루어 볼 때 머위에서 bakkenolide B의 함량은 HPLC 분석방법으로서 가능한 것으로 증명되었다. 따라서 식물체의 부위 즉 잎, 엽병 및 근경에서의 bakkenolide B의 함량을 측정한 결과, 잎에서 가장 높았고, 다음은 엽병이었으며, 근경에서 함량이 가장 낮았다. 3월 초부터 8월 초까지 1개월 간격으로 식물체의 부위별 bakkenolide B의 함량을 조사한 결과, 3월부터 8월까지 채취시기가 지연될수록 감소하는 경향이었다. 따라서 HPLC를 이용한 *Petasites* species의 bakkenolides 분석은 단순하고, 급속하게 분석할 수 있는 방법으로 확인되었다.