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Quantitative Determination of Bakkenolide D in *Petasites japonicus* and *Farfugium japonicum* by HPLC/UV

Norman G. Quilantang[1], Ki Ho Lee[1], Dong Gu Lee[1], Ju Sung Lee[1], EunJu Cho[2], Hyun Young Kim[3,*], and Sanghyun Lee[1,*]

[1]*Department of Integrative Plant Science, Chung-Ang University, Anseong 17546, Korea*
[2]*Department of Food Science and Nutrition & Kimchi Research Institute, Pusan National University, Busan 46241, Korea*
[3]*Department of Food Science, Gyeongnam National University of Science and Technology, Jinju 52725, Korea*

Abstract – A quantitative analysis of bakkenolide D in the different parts of *Petasites japonicus* and *Farfugium japonicum* was performed by HPLC. A gradient HPLC elution system with a mobile phase consisting of water: acetonitrile solution (20:80 to 0:100 for 45 min) was followed and an INNO C_{18} column was used for the chromatographic separation. The injection volume, flow rate, and UV detection were 10 μL, 1 mL/min, and 290 nm, respectively. Results show that both species showed the highest amount of bakkenolide D in the roots being 107.203 and 166.103 mg/g for *P. japonicas* and *F. japonicum,* respectively. Content analysis on the different parts of both plants displayed remarkably lower values which ranged from 0.403 - 4.419 and 7.252 - 32.614 mg/g for *P. japonicas* and *F. japonicum,* respectively. The results show that the roots of both plants are rich in bakkenolide D showing a promising use in the development of nutraceuticals and industrial application of the compound.
Keywords – Bakkenolide D, *Petasites japonicus, Farfugium japonicum,* HPLC/UV

Introduction

Petasites japonicus and *Farfugium japonicum* are perennial plants belonging to the Asteraceae family and are native to many East Asian countries such as Korea, Japan and China.[1] They are commonly utilized as garden ornaments due to their beautiful foliage and are primarily consumed as food in many countries.[2-3] Both plants are also widely used in traditional herbal medicine preparations as a remedy for various illnesses and discomforts. *P. japonicus* is reportedly used in treating asthma, hypertension, headaches, and gastrointestinal problems, whereas *F. japonicum* is used to heal sore throats, fever, eczema, and coughs.[4-6] Moreover, extracts from these genera have exhibited various biological activities such as anti-allergenic, neuroprotective, and anti-inflammatory effects.[7-9] Consequently, many studies have been performed to determine the phytochemicals responsible for their medicinal properties revealing that both species are abundant in many bioactive compounds such as terpenoids, sterols, fatty acids, phenolic compounds, and sesquiterpenes.[10-13] Among them, the eremophilane and bakkenane sesquiterpenes were frequently isolated and have been extensively studied in literature.[14] Recently, there has been great interest on the bakkenolides isolated from both plants due to their unique structure and varied pharmacological properties.[6,15]

Although there are several studies on the phytochemical isolation of bakkenolides and other sesquiterpene lactones from *P. japonicus* and *F. japonicum*, there are no reports regarding the quantitative determination of bakkenolide D in them. Hence, this study aims to develop a simple and reliable HPLC analytical method for the determination and content analysis of bakkenolide D in the different parts of *P. japonicus* and *F. japonicum.* The analytical method used in this study will also provide a basis for future studies regarding the quantitative analysis of the compound in these plants and other related species.

Experimental

Plant materials – Dried leaves, stems, and roots of *P. japonicus* were provided by the Korea National Arboretum and a voucher specimen (No. LEE 2009-07) was deposited

*Author for correspondence
Sanghyun Lee, Department of Integrative Plant Science, Chung-Ang University, Anseong 17546, Korea
Tel: +82-31-670-4688; E-mail: slee@cau.ac.kr

Hyun Young Kim, Department of Food Science, Gyeongnam National University of Science and Technology, Jinju 52725, Korea
Tel: +82-55-751-3277; E-mail: hykim@gntech.ac.kr

at the herbarium of the Department of Integrative Plant Science, Chung-Ang University, Korea. The methanol (MeOH) extracts of the aerial parts, roots, and flowers of *F. japonicum* were procured from the Korean Research Institute of Bioscience and Biotechnology (KRIBB), Korea.

Instruments and reagents – An Eyela rotary evaporator system (Japan) was used in the study. Quantitative analysis was performed with a Waters HPLC-UV/Vis system (MA, USA). A Jeol JMS-600W (Tokyo, Japan), Bruker Avance 300 and 500 NMR (Rheinstetten, Germany) were used for spectroscopic analysis. HPLC grade water, acetonitrile, and chloroform were obtained from J.T. Baker Chemicals (PA, USA).

Isolation of bakkenolide D from *P. japonicas* – Dried and powdered leaves of *P. japonicus* (2.95 kg) were extracted with MeOH under reflux at (8 L × 5; 65 - 75 ºC). The resulting extract was evaporated to dryness (541.90 g) and then partitioned with different solvents namely, *n*-hexane (Hx), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (BuOH). Twenty grams of the concentrated $CHCl_2$ fraction was used for open column chromatography with silica gel as packing material and was eluted with a solution of *n*-hexane: ethyl acetate (100:0 to 0:100) followed by ethyl acetate: methanol (100:0 to 0:100). Compound **1** (357 mg) was isolated and was identified by spectroscopic analysis (Table 1).

Content analysis of bakkenolide D by HPLC/UV – A quantitative analysis on bakkenolide D present in the different parts of *P. japonicus* and *F. japonicum* was performed in the study. A gradient HPLC elution system with a mobile phase consisting of a water: acetonitrile solution (20:80 to 0:100 for 45 min) was followed. An INNO C_{18} column (4.6 × 250 mm, 5 μm) was utilized for the chromatographic separation. The injection volume and the flow rate were 10 μL and 1 mL/min, respectively. The UV detection was set at 290 nm.

Limit of detection and limit of quantification – The limit of detection (LOD) and limit of quantification (LOQ) of bakkenolide D in the MeOH extracts of *P. japonicus* and *F. japonicum* were determined for the validation of the HPLC method. The LOD and LOQ were calculated using the linear regression equation. The LOD and LOQ were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Calibration curve – Stock solution of bakkenolide D was prepared by dissolving 1 mg in 1 mL chloroform. The working solutions used for the calibration curve was prepared by diluting the stock solution to desired concentration. The amount of the analyte present in the samples was determined from the calibration curve constructed where (Y) corresponds for the peak area and (X) for the concentration of the reference compound (μg/10 μL).

Table 1. ^{1}H- and ^{13}C-NMR spectral data for bakkenolide D in $CDCl_3$

No.	δ_H	δ_C
1	5.14 m	70.2
2	1.74-1.97 m	26.7
3	1.69 m	29.4
4	1.56, 1.38 m	35.2
5	-	43.1
6	1.96, 2.23 d (J = 14.5 Hz)	45.7
7	-	54.7
8	-	177.3
9	5.75 d (J = 11.5 Hz)	80.7
10	2.75 dd (J = 5.0, 11.0 Hz)	51.6
11	-	147.7
12	4.67 m	70.4
13	5.19 d (J = 16.0 Hz)	108.1
14	0.91 d (J = 6.5 Hz)	15.4
15	1.35 s	21.0
1'	-	169.7
2'	2.02 s	19.1
1"	-	165.4
2"	5.62 d (J = 10.0 Hz)	112.4
3"	7.54 d (J = 10.0 Hz)	152.6
4"	2.39 s	19.4

Chemical shifts were reported in parts per million (δ), and coupling constants (*J*) were expressed.

Result and Discussion

The chromatographic separation of the CH_2Cl_2 fraction of *P. japonicus* led to the isolation of a white powder with a molecular ion peak at *m/z* 408 $[M]^+$ in the EI-MS spectrum showing a compatibility with the molecular formula of $C_{21}H_{28}O_6S$. ^{1}H-NMR data displayed two fundamental methyl groups at δ 0.91 (2H, d, J = 6.5 Hz, H-14) and 1.35 (3H, s, H-15). Moreover, two special functional groups where one is a methyl group of acetate δ 2.02 (3H, s, H-2') and the other is a functional group peak of methyl thiopropenoyloxy group were observed at δ 2.39 (3H, s, H-4"), 5.62 (1H, d, J = 10.0 Hz, H-2") and 7.54 (1H, d, J = 10.0 Hz, H-3"). A total of 21 carbon resonances including a single C=C double bond (δ 112.4, C-2" and 152.6, C-3"), three carbonyl groups, and four methyl groups were observed in the ^{13}C-NMR spectrum. Carbonyl signals were observed at δ 177.3 (C-8), 169.7 (C-1'), and 165.4 (C-1"). Based on the data obtained in the study (Table 1) and a comparison of spectral data from previous literatures, the identity of the isolated compound was determined as bakkenolide D and its structure shown in Fig. 1.[16-17]

The genus *Petasites* and *Farfugium* have been extensively researched due to their reported use in traditional medicine as treatment for many diseases which led to the isolation of sesquiterpene lactones considered as the major bioactive components found in these genera. Bakkenolides belong to this category of compounds which has also been reported to exhibit various biological activities.[8] Particularly, bakkenolide D is focused in this study. The MeOH extracts of the various parts of *P. japonicus* and *F. japonicum* were subjected to HPLC/UV analysis to determine the amount of bakkenolide D present in them utilizing a reverse phase system and a water-acetonitrile solution as mobile phase. The standard calibration curve for the analysis of bakkenolide D showed good linear regression ($r^2 = 0.9999$) within test ranges as shown in Table 2. The LOD and LOQ values were determined as 0.0046 and 0.0136 mg/mL, respectively (Table 2). The HPLC conditions followed in the experiment

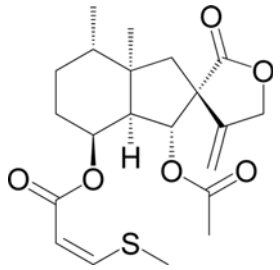

Fig. 1. Structure of bakkenolide D.

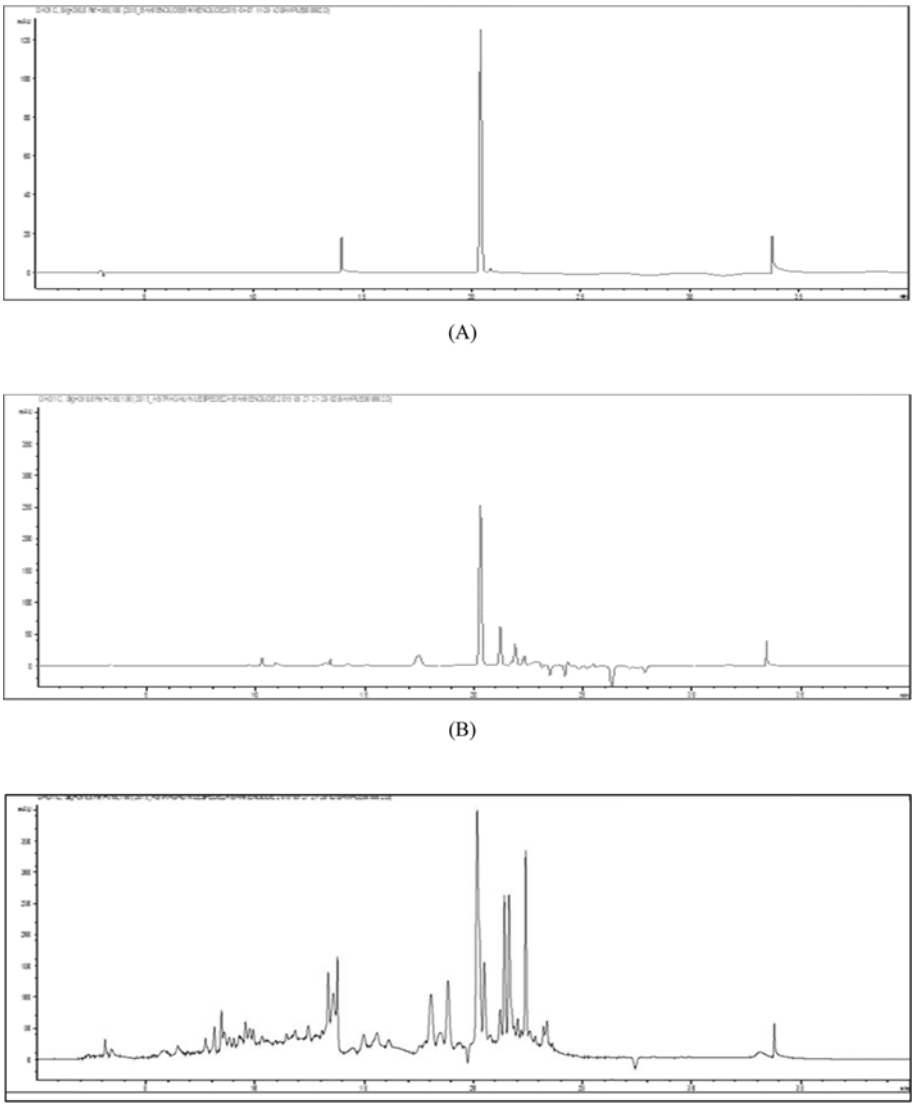

Fig. 2. HPLC chromatograms of bakkenolide D (A) and the MeOH extracts of *P. japonicus* (B) and *F. japonicum* (C).

Table 2. LOD and LOQ values of bakkenolide D

Compound	Regression equation	r^2	Linear range (mg/mL)	LOD (mg/mL)	LOQ (mg/mL)
Bakkenolide D	Y = 124.2X + 0.5969	0.9999	0.006 – 0.1	0.0046	0.0136

Y = Peak area, X = Concentration of standard (mg/mL)
r^2 = Correlation coefficient for three data points from calibration curve

Table 3. Contents of bakkenolide D in the MeOH extracts of *P. japonicus*

Part	Content (mg/g)
Leaves	0.403 ± 0.017
Roots	107.203 ± 6.603
Stems	4.419 ± 0.275

Table 4. Contents of bakkenolide D in the MeOH extracts of *F. japonicum*

Part	Content (mg/g)
Leaves	32.614 ± 1.738
Roots	166.103 ± 7.711
Flowers	7.252 ± 0.243

generated similar chromatograms in both plants exhibiting that such chromatographic conditions may be applicable to other related species for the analysis of bakkenolide D. To our knowledge, this is also the first study which reports on the quantitative analysis of the compound in these plant species. The results of the analysis show that in both plants, the roots contain the highest amount of the bakkenolide D being 107.203 and 166.103 mg/g for *P. japonicas* and *F. japonicum,* respectively (Tables 3 and 4). This is consistent with other studies conducted with other species belonging in the same genera.[18-19] On the other hand, the other parts examined showed relatively lower amount of the compound ranging from 0.403 - 4.419 and 7.252 - 32.614 mg/g for *P. japonicas* and *F. japonicum,* respectively. Moreover, the amount of bakkenolide D in the two plants analyzed were remarkably higher than those reported in other species where the values are 1.67 and 1.00 mg/g for *P. tatewakianus* and *P. tricholobus,* respectively indicating that *P. japonicas* and *F. japonicum* are rich natural sources of the compound.[18-19]

This study showed a fast and reliable method for the quantitative analysis of bakkenolide D in *P. japonicus* and *F. japonicum* which may be used for the analysis of other related species. The results show that the roots of both plants contain high amounts of bakkenolide D showing a promising use in the development of nutraceuticals and other pharmacological products involving the use of the compound.

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