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Structure Determination of Heishuixiecaoline A from *Valeriana fauriei* and Its Content from Different Cultivated Regions by HPLC/PDA Analysis

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Abstract – A germacrane-type sesquiterpenoid was isolated and purified from a methanol extract of the roots of *Valeriana fauriei* (RVF) through open column chromatography using silica gel. This compound was verified to be heishuixiecaoline A by spectroscopic analysis. This compound was isolated for the first time from RVF. Quantitative analysis of heishuixiecaoline A from RVF cultivated from three different regions (Eumseong, Jinbu, and Jinan regions) was performed by combining high-performance liquid chromatography with a photodiode array detector. The extract of RVF cultivated in the Jinbu region showed the highest content (9.23 mg/g). In addition, a significant amount of the compound was detected in all RVF samples, which could be expected since it is a characteristic compound of RVF. The sesquiterpenoid group heishuixiecaoline A was isolated from RVF, a resource for various pharmacological substances, and quantitative analysis of RVF cultivated from three different regions was performed. As a result of these experiments, basic data on RVF that can be used in the development and application of pharmaceuticals and health functional foods in the future were obtained.
Keywords – *Valeriana fauriei*, heishuixiecaoline A, cultivation, HPLC

Introduction

Natural fragrances used in food and medicine can be divided into two categories: plant and animal origins. The main source is aromatic plants. Aromatic plants are high-value plants because of their characteristic fragrances in their flowers, fruits, leaves, and stems, and they are highly useful in various fields. Aromatic plants belong to several species including *Valeriana fauriei*, *Lavandula angustifolia*, *Juglans regia*, *Melilotus officinalis*, etc.[1] Among them, *V. fauriei* has been traditionally used as a medicine and has value as food and for flavor.[2] *V. fauriei* has lower usability and accessibility than other aromatic plants due to its characteristic scent; thus, research on its constituents is needed. Although synthetic fragrances can be substituted for natural fragrances, various studies are needed as the demand for natural fragrances is gradually increasing due to the quality and side effects of synthetic fragrances.[3]

V. fauriei, belonging to the family Valerianaceae, is a perennial plant endemic to Korea and distributed in temperate regions of Europe, Asia, and North America.[4] The height of *V. fauriei* reaches 40–80 cm, and the root has a strong scent.[5] Since ancient times, the roots of *V. fauriei* have been used to treat neuropsychiatric disorders such as anxiety, insomnia, sleep disorders, and neuralgia.[6-11] In addition, *V. fauriei* shows significant anti-oxidant and anti-obesity activities.[12-14] Its pharmacological activity is attributed to valerenic acid and valepotriates, which are the main components of *V. fauriei*.[15] Among its essential oil components, valerenic acid is known to act by directly binding to GABA receptors in the brain that respond to GABA as a neurotransmitter in the central nervous system of vertebrates.[16-18] In addition, the essential oils obtained from the roots of *V. fauriei* have been used as a flavoring

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for food, medicine, tobacco, etc.[19] Furthermore, the roots of *V. fauriei* (RVF) are called "valerian root" and has been used to treat blood circulation diseases such as hyperlipidemia in Oriental medicine.[20–22]

Various germacrane-type sesquiterpenoids have been isolated from *V. fauriei*, including valerianins A-B, volvalerenic acids A-D, and volvalerenals A-G.[23] *V. officinalis*, a species of the same family (Valerianaceae) as *V. fauriei*, has been reported to be used for anti-inflammatory treatment in traditional European medicine. The extract had inhibitory activity against NF-κB and a protective function against excitotoxicity.[24] Plants tend to have higher contents of secondary metabolites when treated with elicitors. *V. fauriei* contains various valepotriate compounds with pharmacological effects.[25] The content of valerenic acid and valepotriates, which were detected in *V. fauriei* and reported as major pharmacologically active components, were found to be increased in the atypical roots generated after elicitor treatment.[26]

In this study, to identify and analyze phytochemical constituents from aromatic plants with various degrees of development potential for food and medicine, RVF from three different regions in Korea were evaluated for the contents phytochemical substances by repeated column chromatography and quantitative analysis by high-performance liquid chromatography (HPLC) with a photodiode array detector (PDA).

Experimental

Plant materials – The seeds of *V. fauriei* were collected from Pyeongchang, Gangwon Province, Korea and authenticated by Dr. S. H. Han, Rural Development Administration, Eumseong, Korea and planted in the test fields in the Eumseong (ES), Jinbu (JB), and Jinan (JA) regions. After planting (2014), the cultivated RVF in these three regions were used for experiments (2015). Voucher specimens from ES (LEE-CAU-2015-01), JB (LEE-CAU-2015-02), and JA (LEE-CAU-2015-03) were deposited at Department of Plant Science and Technology, Chung-Ang University, Anseong, Korea.

Instrumentation, chemicals, and reagents – The solvents ethanol (EtOH), methanol (MeOH), *n*-hexane (*n*-Hx), chloroform ($CHCl_3$), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) used for extraction and separation were purchased from Samchun Pure Chemicals Co., (Pyeongtaek, Korea). HPLC-grade solvents such as water, trifluoroacetic acid (TFA), and acetonitrile (ACN) were purchased from J. T. Baker (Phillipsburg, PA, USA). The column filler used for open column chromatography was silica gel (40–60 μm, 60–200 mesh, intertec® Silicagel, Bosnia and Herzegovina). Thin-layer chromatography analysis was performed on silica gel 60-coated glass using Kiesel gel 60 F_{254} (Merck Co., Darmstadt, Germany). Nuclear magnetic resonance (NMR) spectra were recorded on an AVANCE III HD 500 NMR spectrometer (Bruker, Germany). The electron ionization mass spectrum (EI-MS) was recorded using a JEOL-MS spectrometer (Tokyo, Japan). Analyses were performed by using an HPLC system (Waters Alliance e2695 Separations Module, MA, USA) that consisted of an auto-sampler, pump, and photodiode array detector (Waters 2998 PDA detector, MA, USA).

Extraction and isolation – The underground part of dried RVF (5 kg) was pulverized to make a powder. The RVF powder was repeatedly extracted three times for 3 h at 65ºC in 100% MeOH under reflux. The resulting extract solution was filtered through filter paper (5–8 μm pore size) and concentrated using a rotary vacuum concentrator to obtain crude MeOH extract (670 g). The MeOH extract of RVF was suspended in distilled water, and equal volumes of *n*-Hx (117 g), $CHCl_3$ (16 g), EtOAc (27 g), and *n*-BuOH (90 g) were sequentially added according to polarity, followed by fractionation and concentration to obtain fractions. The *n*-Hx fraction of the MeOH extract of RVF was eluted with a gradient solvent system of *n*-Hx:EtOAc (100:0-0:100; v/v) using open-column chromatography filled with silica gel. Compound **1** (12 mg) was obtained from a vial in sub-fraction 67 by recrystallization with MeOH.

Sample preparation and HPLC analysis – The underground part of RVF grown in the test fields in three regions in Korea was extracted with MeOH (65ºC, repeated three times for 3 h) and filtered to obtain an extract. Each MeOH extract of RVF and compound **1** was dissolved in MeOH to prepare 50 and 1 mg/mL solutions. After that, all samples were sonicated with an ultrasonicator for 20 min and filtered using a syringe filter (0.45 μm pore size). Chromatographic separation using HPLC with a PDA was performed using an INNO C18 column (4.6 × 250 mm, 5 μm pore size). The mobile phase used was run under gradient elution conditions and consisted of 0.1% TFA in water (solvent A) and ACN (solvent B) for a total of 60 min. Gradient elution of the mobile phase was started with 83% of solvent A and held for 10 min. Solvent A was gradually reduced to 30% over 30 min and was further reduced to 0% after 5 min. Then, solvent A was increased to 83% again for 5 min and maintained for 10 min. The injection volume was set to 10 μL, and the flow rate was 1.0 mL/min. The UV detection wavelength

was set to 275 nm for analysis.

Calibration curve – To prepare a calibration curve, compound **1** was diluted to different concentrations (0.0625–1.0 mg/mL). A calibration curve was obtained by plotting each peak area according to the concentration, and the linearity was determined using the correlation coefficient (r^2). The concentration of compound **1** in the sample was calculated using the obtained calibration curve. The calibration function was determined using the mean standard deviation (n = 3) with the peak area set as the Y-axis and the concentration (mg/mL) of the sample as the X-axis.

Statistical analysis – All data were analyzed by an f-test or ANOVA using statistical analysis system software 9.4 (SAS Institute, NC, USA). ANOVA was performed on the real sample at 95% confidence level on statistical analysis system program. The results of the quantitative analysis of the HPLC for comparing different cultivated regions of RVF were performed by ANOVA test.

Results and Discussion

According to previous studies, RVF were investigated for pain and protein expression in fibromyalgia. Regulatory responses to brain-derived neurotrophic factor signaling pathways were confirmed in the prefrontal cortex and hippocampus.[27,28] As a result, the likelihood of developing mental disorders such as schizophrenia and depression related to pre-pregnancy stress in women was reduced.[29]

Compound **1** was isolated from the *n*-Hx fraction of the MeOH extract of RVF by open-column chromatography using silica gel. Compound **1** had a germacrane-type sesquiterpenoid structure (Fig. 1).

Compound **1** was a white powder, and its molecular structure was defined as $C_{17}H_{24}O_3$ through EI-MS (m/z 276 $[M]^+$). In the ^{1}H-NMR spectrum of compound **1**, a singlet signal with 4 methyl groups [δ_H 1.19 (H-12), 1.20 (H-13) 1.36 (H-15), 2.06 (H-17)] was detected, and one oxygenated methine proton multiplet [δ_H 4.48 (H-8)] was found. In addition, one aldehyde proton [δ_H 9.30 (H-14)] appeared as a singlet, and two olefinic protons [δ_H 5.25 (H-1), 6.37 (H-5)] were detected. Three methylene groups [δ_H 2.13, 2.17 (H-2), 2.08, 2.76 (H-3), 2.24, 2.28 (H-9)] were confirmed. In the ^{13}C-NMR spectrum, 17 carbon

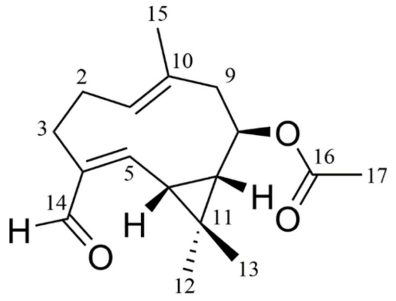


Fig. 1. Chemical structure of heishuixiecaoline A (**1**).

Table 1. NMR assignments of compound **1**

No.	δ_H	δ_C
1	5.25 (1H, dd, 5.0, 11.0)	127.6
2	2.13 (1H, m), 2.17 (1H, m)	28.4
3	2.08 (1H, m), 2.76 (1H, m)	24.1
4	-	143.9
5	6.37 (1H, d, 9.7)	153.3
6	1.73 (1H, t, 9.7)	30.8
7	1.31 (1H, dd, 9.7, 11.0)	39.6
8	4.48 (1H, dt, 3.0, 11.0)	72.2
9	2.28 (t, 11.0), 2.24 (dd, 3.0, 11.0)	46.5
10	-	133.0
11	-	22.5
12	1.19 (3H, s)	27.5
13	1.20 (3H, s)	15.9
14	9.30 (1H, s)	194.5
15	1.36 (3H, s)	18.2
16	-	170.5
17	2.06 (3H, s)	21.5

signals were identified. An aldehyde carbon group [δ_C 194.5 (C-14)], a carbonyl carbon [δ_C 170.5 (C-16)], two trisubstituted double bonds [δ_C 127.6 (C-1), 133.0 (C-10), 143.9 (C-4), 153.3 (C-5)], three methylenes, three methines, and four methyls were present (Table 1). Compound **1** was identified as heishuixiecaoline A by comparing the results with data from previous studies of *V. amurensis*.[30] Heishuixiecaoline A (**1**) isolated in this study is a substance belonging to the germacrane-type sesquiterpenoid family and is contained in the roots of the family Valerianaceae.[31] This compound was isolated for the first time from *V. fauriei*.

The quantitative analysis of heishuixiecaoline A (**1**) isolated from RVF was conducted using MeOH extracts from three different regions including ES, JB, and JA. As a result of comparing RVF grown in different test fields

Table 2. Calibration curve of heishuixiecaoline A (**1**)

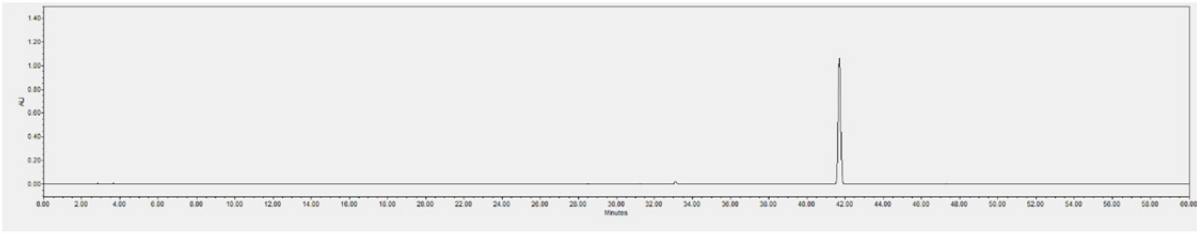
Compound	t_R	Range (mg/mL)	Calibration equation [a]	r^2 [b]
1	41.7	0.0625 – 1.0	Y = 18595X – 164544	0.9998

[a] Y = peak area, X = concentration of the standard (μg/mL)
[b] r^2 = correlation coefficient for five data points in the calibration curve

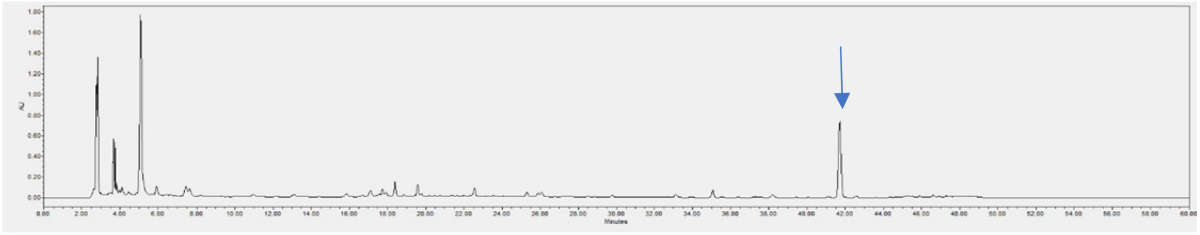
Table 3. Content of heishuixiecaoline A (**1**) in RVF from different cultivation regions including ES, JB, and JA

Cultivation region	Content (mg/g ext.)	SS	df[a]	MS	F value
ES	6.80 ± 0.00				
JB	9.23 ± 0.01	46.552164	2	23.276082	12240.5
JA	3.67 ± 0.01				

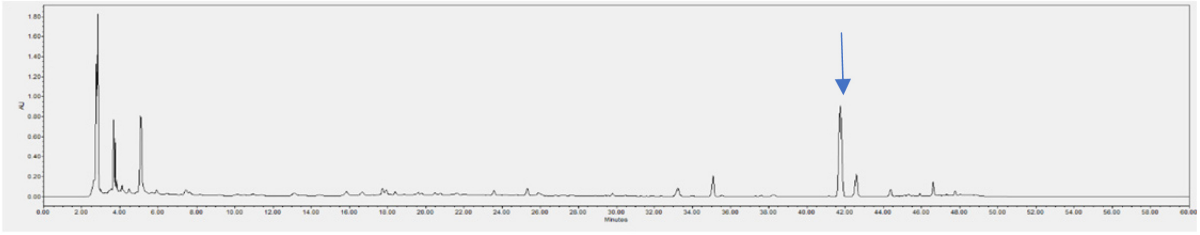
SS, sum of square; df, degree of freedom; Ms, mean squares.
[a] Degreesof freedom for between groups: h-1; h, number of cultivation regions.



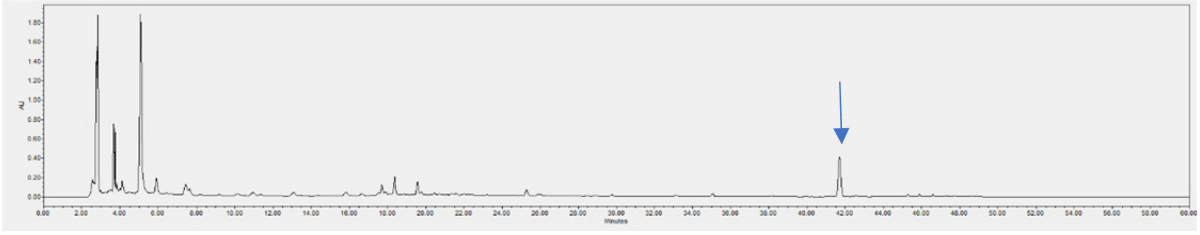
(A)



(B)



(C)



(D)

Fig. 2. HPLC chromatograms of heishuixiecaoline A (A) in RVF from different cultivation regions [ES (B), JB (C), and JA (D)].

during the same year, heishuixiecaoline A (**1**) content was detected in all three regions, and the content was calculated. The correlation coefficient (r^2) of the calibration curve calculated by detecting heishuixiecaoline A (**1**), the standard material, at continuous concentrations was 0.9998, indicating excellent linearity, and the retention time was 41.7 min (Table 2). Among the samples, the area with the highest content was JB (9.23 mg/g ext.), and the area with the lowest content was JA (3.67 mg/g ext.). Although there were differences between regions, all samples had significant content, and the results of ANOVA are summarized (Table 3). In addition, heishuixiecaoline A is likely a representative component of RVF as it was well separated into a characteristic peak on the chromatograms (Fig. 2).

The average content of valerenic acid contained in RVF was 0.2925 mg/g ext.,[32,33] which was significantly lower than the content of heishuixiecaoline A (**1**) studied in this experiment. Since the RVF from all three regions used in this experiment contained a large amount of heishuixiecaoline A (**1**), it is expected that this is a characteristic component of RVF.

Previous studies on the presence of heishuixiecaoline A (**1**) contained in RVF have shown various biological activities. Heishuixiecaoline A (**1**) showed a protective effect against neurotoxicity in PC12 cells caused by $A\beta_{25-35}$, an amino acid peptide found in the brains of patients with Alzheimer's.[30,34] In previous studies, samples from *V. amurensis*, which is from the same family as *V. fauriei* were extracted using 95% EtOH, and the 50% EtOH fraction was subjected to open column chromatography filled with silica gel to isolate heishuixiecaoline A (**1**) to identify the structure.[29, 35] Through the results of this study, heishuixiecaoline A (**1**) was able to be isolated from RVF, and an isolation method for isolating heishuixiecaoline A (**1**) using an *n*-Hx fractionation layer was developed.

Heishuixiecaoline A (**1**) appeared as a characteristic peak of the MeOH extract of RVF on all HPLC chromatograms; therefore, it is expected to play a role as a representative component of *V. fauriei*. Although there was a difference in the heishuixiecaoline A (**1**) content from each region, a significant level of content was detected in all samples. Therefore, the results of this experiment provide basic data that can be used for various medical applications and uses of RVF. In addition, the content of heishuixiecaoline A (**1**) contained in RVF was calculated, providing basic data that can help develop medicines and health functional foods so that heishuixiecaoline A can be applied to the treatment of various diseases such as nervous system diseases, anxiety, and depression.

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