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Regular Article

^{1}H-NMR-Based Metabolomics for the Classification of the Roots of *Paeonia lactiflora*, a Constituent of Kampo Medicines

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The root of *Paeonia lactiflora* (PAEONIAE RADIX) is a constituent of the traditional Japanese medicines (Kampo) and is known to have various effects. Peony roots cultivated in Japan and China are available in the Japanese market for medicinal use. In this study, the chemical diversity of ten available peony roots in the market that differed in their cultivation area was investigated using ^{1}H-NMR metabolomics techniques. Principal component analysis and hierarchical cluster analysis of the ^{1}H-NMR spectra of the peony roots methanolic extracts revealed a clear difference between the metabolic profiles of Japanese and Chinese peony roots. By preparative procedures using chromatography based on ^{1}H-NMR spectra measurements, oxypaeoniflorin and (+)-catechin were found to be specific compounds for Japanese peony root. All peony roots used in this study were listed in the Japanese Pharmacopoeia. Therefore, the differences in the constituents of these peony roots might be attributed to growing conditions than differences in species. Cultivation conditions also influence the quality of natural medicines.

Key words *Paeonia lactiflora*; ^{1}H-NMR metabolomics; multivariate analysis; oxypaeoniflorin; (+)-catechin

Introduction

The root of *Paeonia lactiflora* (PAEONIAE RADIX) is used as a natural medicine with analgesic and antispasmodic effects. This herbal medicine is frequently used as a constituent of the traditional Japanese medicines (Kampo) formula. According to the Japanese Pharmacopoeia, the paeoniflorin content of *P. lactiflora* is more than 2.0%.[1)] Notably, paeoniflorin is a chemical marker for controlling the quality of the root of *P. lactiflora*.[1)] The peony root roughly consists of two types: white peony root and red peony root. Unlike Kampo, white and red peony roots are used according to patient symptoms in Chinese medicine. Red peony root is often used to remove heat and blood stasis, while white peony root is used to calm the liver, tonify blood, and regulate menstruation based on Chinese medical theory. In the Chinese Pharmacopoeia, white peony root is prescribed as the boiled and peeled root of *P. lactiflora*. In contrast, red peony root is prescribed as the sun-dried root of *P. lactiflora* and *P. veitchii*.[2)] In the Japanese Pharmacopoeia, there is no distinction in the use of white and red peony roots.

Metabolite fingerprinting approaches using NMR spectroscopy can generate valuable metabolic signatures for complex natural medicine extracts. The advantage of ^{1}H-NMR spectroscopy over other metabolomics techniques such as MS is that the signal intensity in the NMR spectrum depends only on the molar concentration of each compound in the solution, thereby enabling direct comparisons of the concentrations of all compounds present in the sample. Our previous report demonstrated that an NMR-based metabolomics approach could differentiate species, production areas, and utility applications of commercially available cinnamon bark.[3)] An NMR metabolomics approach has also been employed to screen the antimicrobial activity of yucca extracts used as food additives.[4)]

In this study, we report the investigation of ten commercially available roots of *P. lactiflora* used as medicines through ^{1}H-NMR spectroscopy coupled with multivariate statistical analysis, such as principal component analysis (PCA) and hierarchical cluster analysis (HCA). Based on the experimental data, we evaluated a comprehensive profile of *P. lactiflora* roots based on their geographical origins (Japan or China) and explored marker compounds specific for their production area through chromatographic separation procedures.

Results and Discussion

In the present study, ten different peony root samples were employed, as listed in Table 1. All 345 variables in the bucketed regions were equally accounted for in the datasets. PCA models are depicted as score plots and consist of two synthetic variables: principal component (PC) 1 (the greatest data variance) and PC2 (the second greatest data variance, orthogonal to PC1). These samples were primarily divided into two groups (first group: #1, #3, #4, #5, #8, #9; second group: #2, #6, and #7), and one sample (#10) was excluded because it was located far from these two groups on the PCA score plot, as shown in Fig. 1. The two groups were related to their cultivation area: the Japanese group (#1, 3, 4, 5, 8, and 9) and the Chinese group (#2, 6, and 7). In addition, an HCA constellation dendrogram of peony root was divided into two groups, namely #10 and others, as seen in Fig. 2. The processing of

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Table 1. Market-Available Peony Roots Used in This Study

Sample No.	Production area	Supplier	Lot No.
1	Japan (Wakayama, Nara, Mie Prefecture)	UW	G6S0259
2	China (Anhui)	UW	F8T0221
3	Japan (Nagano, Niigata)	UW	G950257
4	Japan (Wakayama, Nara, Mie)	UW	G7K0258
5	Japan (Nagano, Niigata)	UW	G960256
6	China (Anhui)	TT	5318002
7	China (Sichuan)	TT	5318003
8	Japan (Hokkaido)	TT	5317007
9	Japan (unknown)	TT	5317008
10	China (Anhui)	TT	5318004

UW, UCHIDAWAKANYAKU Ltd.; TT, Tochimoto Tenkaido Co., Ltd.

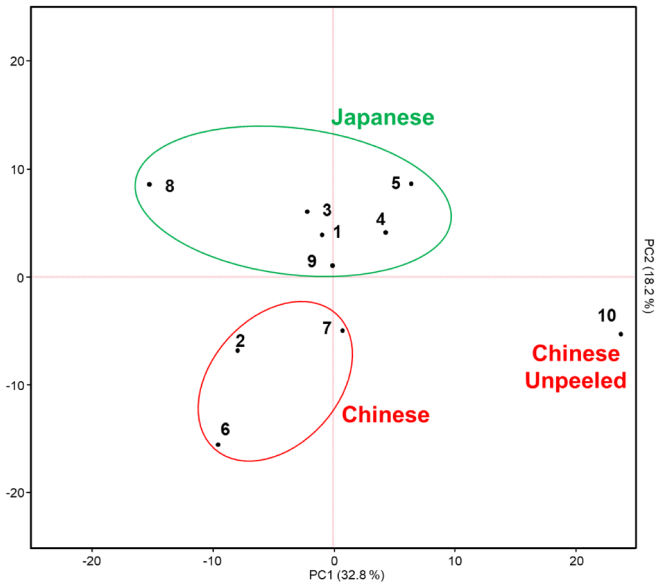

Fig. 1. The Principal Component Analysis (PCA) Score Plot of the Peony Roots, in Which 345 Variables Were Equally Accounted for in the Datasets

The PCA score plot clearly showed two groups related to their cultivation area, *i.e.*, Japanese group (#1, #3, #4, #5, #8, and #9) and Chinese group (#2, #6, and #7). Notably, the only unpeeled sample (#10) was located far from the other samples on PCA.

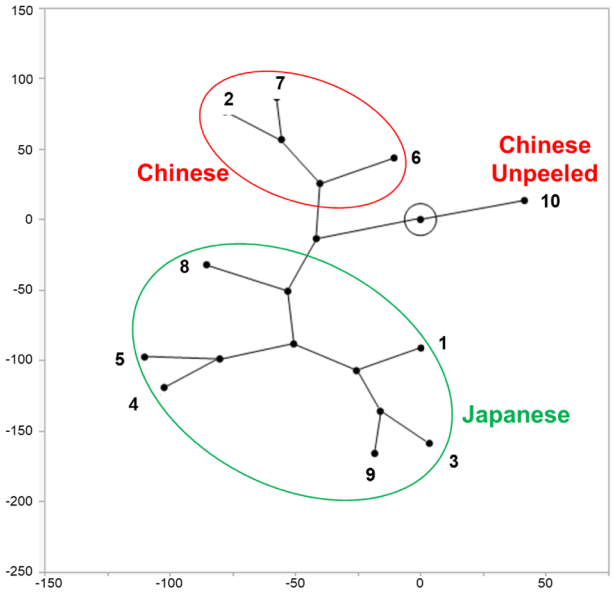

Fig. 2. Constellation Dendrogram Obtained Using the ^{1}H-NMR Spectral Data of Peony Roots as Illustrated by Hierarchical Cluster Analysis (HCA)

Peony roots used in this study were divided in two groups, unpeeled (#10) and peeled (others). Furthermore, peeled peony roots were categorized according to their cultivation area (Japan and China).

#10 was different since this peony root was the only unpeeled sample used in the experiment. The others were further divided into two groups based on their cultivation area on the HCA constellation dendrogram. These results were consistent with those of PCA. The constituents of peony root stipulated by the Japanese Pharmacopoeia depend on their cultivation area. Furthermore, ^{1}H-NMR spectra were compared to identify signals specific to their cultivation area. As shown in Fig. 3, a doublet signal at δ 7.83 appeared in the ^{1}H-NMR spectra of Japanese peony roots. The signals appearing at δ 5.67 and δ 5.87 were also specific to Japanese peony roots. To identify the compounds specific to Japanese peony roots, the methanolic extract of #1 was fractionated based on the identified ^{1}H-NMR spectral signals. The methanolic extract was loaded onto an octadecyl silyl (ODS) open column and eluted with water, aqueous methanol, and methanol to arrive at the corresponding elution. Following this, 50% aqueous methanol elution was further purified by reversed-phase HPLC to yield compounds **1** (2.95 mg) and **2** (1.64 mg). These compounds were identified as oxypaeoniflorin and (+)-catechin by spectroscopic analysis.[5,6] The specific proton signal for Japanese peony roots observed at δ 7.83 (d, $J = 8.8$ Hz) was assigned to H-2 (H-6) of oxypaeoniflorin. On the other hand, the signals that appeared at δ 5.67 (d, $J = 2.2$ Hz) and δ 5.87 (d, $J = 2.2$ Hz) were attributed to H-8 and H-6 of (+)-catechin, respectively. The results showed that oxypaeoniflorin and (+)-catechin are characteristic compounds of Japanese *P. lactiflora*. Previous research has reported that Japanese peony root has a higher content of oxypaeoniflorin than Chinese peony root,[7] supporting our experimental results. Although Komatsu and colleagues reported a high level of (+)-catechin content in red peony root,[8] the fact that (+)-catechin is a distinguishing compound for Japanese peony has never been reported to our knowledge. Furthermore, there are no recent studies showing that peony root cultivated in Japan have a higher content of (+)-catechin than those cultivated in China. It has been reported that paeoniflorin and oxypaeoniflorin were able to attenuate advanced glycation end products, inducing oxidative damage and inflammation in mesangial cells.[9] Furthermore, the positive effect of oxypaeoniflorin in myocardial ischemia/

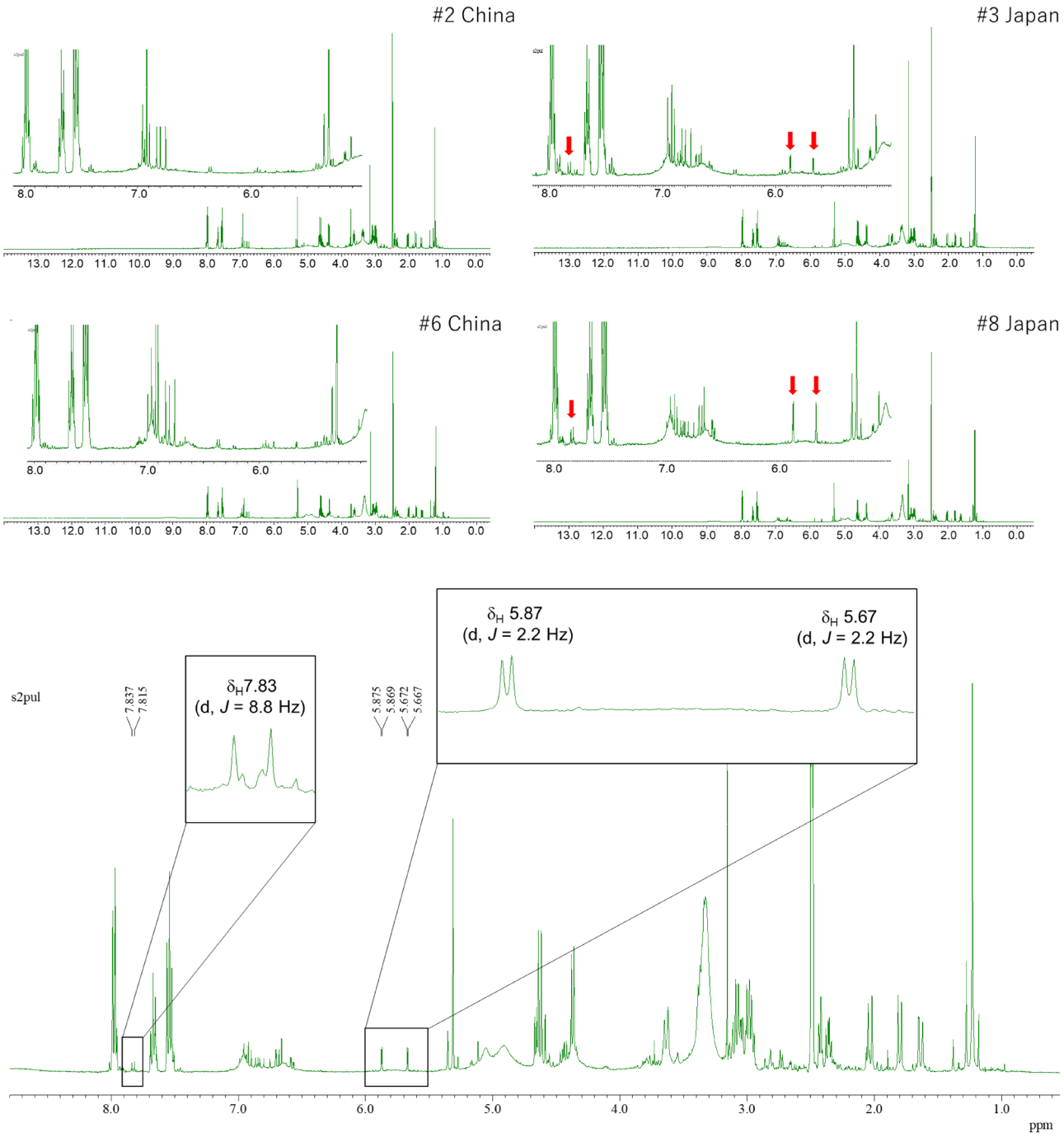

Fig. 3. [1]H-NMR Spectra of Japanese (#3 and #8) and Chinese Peony Root Extracts (#2 and #6); Expanded [1]H-NMR Spectrum of Japanese Peony Root (#8)

Arrows show signals specific to Japanese peony roots.

reperfusion injury has been reported.[10] Because the Japanese Pharmacopoeia has specified the use of peony root as medicine, the high content rate of (+)-catechin in Japanese peony might be attributed to similar growth conditions rather than differences in species. Determining the environmental factors influencing oxypaeoniflorin and (+)-catechin content of peony root is important for maintaining the quality of natural medicines. For subsequent studies, we plan to determine the factors that exist in the growing soil.

Conclusion

The constituents of Japanese and Chinese peony roots differed based on their cultivation area on PCA score plots derived from their [1]H-NMR spectral data. Oxypaeoniflorin and (+)-catechin were identified as marker compounds for Japanese peony root. As peony roots used in this experiment are specified by the Japanese Pharmacopoeia, the differences in their components might be attributed to the similarity in cultivation conditions. Clarifying the cultivation factors influencing the components of peony root will be crucial for the quality control of natural medicines.

Experimental

The Reagents and Chemicals Dimethylsulfoxide-d_6 (DMSO-d_6) was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, U.S.A.) while (+)-catechin was obtained from Tokyo Chemical Industry Co., Ltd. (Chuo-ku, Tokyo, Japan).

Materials The roots of *P. lactiflora* used in this study are shown in Table 1. These samples were obtained from UCHIDAWAKANYAKU Ltd. (Arakawa-ku, Tokyo, Japan) and Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). A voucher specimen was deposited at the Laboratory of Natural products & Phytochemistry, Josai University.

Sample Preparation and Extraction Each sample was extracted using methanol in reflex three times (first time for 1 h, second and third times for 30 min). The methanolic extracts were filtered through a filter paper and evaporated to yield dried residues. These residues were applied to a Sep-Pak column C18 (Waters, Milford, MA, U.S.A.) and eluted subsequently with water, 50% aqueous methanol, and then methanol to yield corresponding fractions. The 50% aqueous methanol fraction was dissolved in DMSO-d_6 at a 10 mg/mL concentration for ^{1}H-NMR spectrum measurement.

Instrumentation One- and two-dimensional NMR spectra were recorded at 400 MHz on an Agilent 400MR-vnmrs 400 spectrometer (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.).

^{1}H-NMR Analysis for Metabolomics The ^{1}H-NMR spectra were recorded at 20 °C on a 400 MHz Agilent 400MR-vnmrs 400 spectrometer. Each spectrum consisted of 65536 complex data points and had a spectral width of 6410.3 Hz. The spectra were obtained over 16 scans with a repetition time of 5.0 s and a relaxation delay of 1.5 s per scan. The detection pulse flip angle was set at 45°.

Data Analysis: PCA and HCA Each NMR spectrum from 0.00 and 14.00 ppm was divided into 350 regions (0.04 ppm wide), with each segment of the spectral region being integrated. Any integrated regions from 2.40 to 2.52 ppm and 3.08 to 3.16 ppm that contained solvent and water signals were eliminated from the data table, reducing the total data to 345 regions. The remaining integral values for each spectrum were normalized over 100 total summed integrals to compensate for any differences in concentration among the roots of *P. lactiflora* extracts. Spectral processing was performed using ALICE2 for Metabolome version 5.0 (JEOL, Tachikawa, Tokyo, Japan). Multivariate analyses such as PCA and HCA were conducted using JMP Pro 13 (SAS Institute Inc., Cary, NC, U.S.A.). Variables were standardized with a mean of 0 and standard deviation of 1.

Isolation and Identification of Oxypaeoniflorin and (+)-Catechin The root of *P. lactiflora* (#1) (400 g) was extracted using methanol under reflux for 1 h, and the same procedure was repeated twice for 30 min. The methanolic extracts were combined and evaporated to produce dried residues (80 g). The residue (1 g) was subjected to ODS open column chromatography (ϕ 2.2 × 30 cm) and eluted subsequently with water, 50% aqueous methanol, and then methanol to yield corresponding fractions (water fraction, 600 mg; 50% aqueous methanol fraction, 170 mg; methanol fraction, 44 mg). The 50% aqueous methanol fraction (100 mg) was purified using HPLC with Senshu Pak ODS-4151-N (ϕ 10 × 150 mm, Senshu Scientific Co., Ltd., Suginami, Tokyo) and eluted using an isocratic system (methanol-water 1 : 2, 3 mL/min) to yield residue (43 mg). The residue (43 mg) was further purified using HPLC with Senshu Pak ODS-4151-N (ϕ 10 × 150 mm, Senshu Scientific Co., Ltd.) and eluted using an isocratic system (acetonitrile-water 9 : 91, 3 mL/min) to yield compounds **1** (2.95 mg) and **2** (1.64 mg). Compounds **1** and **2** were identified as oxypaeoniflorin and (+)-catechin, respectively. Their structures were confirmed by comparing their spectroscopic data, such as NMR, MS, and optical rotation data, with those found in literature and authenticated compounds.

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Conflict of Interest The authors declare no conflict of interest.

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