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Conduritol F, the discriminant marker between *C. wilfordii* and *C. auriculatum* by ^{1}H NMR spectroscopy

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ABSTRACT

Quantitative ^{1}H nuclear magnetic resonance (qNMR) spectroscopy is a powerful and versatile technique to enable the absolute quantification of specific components in a mixture with excellent reproducibility and robustness. In the present study, qNMR analysis was applied to conduritol F, a chemical marker with the potential to distinguish between *C. wilfordii* and *C. auriculatum*. We found that the signals of H-5 and H-6 of conduritol F were well-separated from others with purities sufficient to be used to distinguish between *C. wilfordii* and *C. auriculatum*. This simple methodology can be applied to identify one or two species in products or powdered herbs widely distributed in the markets.

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1. Introduction

Medicinal plants have long been used for the treatment of a wide range of symptoms or diseases all over the world. In Asian countries, medicinal plants have been histologically utilized as medicines or dietary functional supplements. In particular, some traditional herbs in three Asian countries, Korea, China and Japan, where similar geographical and cultural backgrounds have influenced each other, have been used as substitutes for similar herbal resources from the other countries. Some herbs were replaced by another species from the same genus or a species with similar morphological features. Although different species originating from the same genus often contain similar components, the chemical or biological differences by adulteration or misidentification of similar species might often result in unexpected adverse effects. Thus, the right uses of the exact identified species are necessary for the health of patients and the safety of consumers.

For several years, dietary functional supplements containing *C. wilfordii* (CW) have been popular to prevent or treat climacteric symptoms in Korea. The Ministry of Food and Drug Safety (MFDS) in Korea approved its use with annual sales eclipsing $100 million in 2014. This popularity has caused price increases as well as a scarcity of CW in herbal markets. In 2015, the misuses of *Cynanchum auriculatum* (CA) instead of CW have become a big social issue in Korea [1]. CA was not listed in 11th Korean Pharmacopoeia or 4th Korean Herbal Pharmacopoeia, and the MFDS strictly restricted the use of CA as the dietary supplement source. However, since CA looks similar to CW, grows faster and gets bigger than CW, CA farms rapidly replaced CW farms. Recently, the chemical marker which helps distinguish between the two species has been reported [2,3]. MFDS reported these identification and discrimination methods using DNA and TLC analyses, and has also funded a set of genome sequencing of CW and CA to provide the basic genetic information for identifying both substances.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful and versatile technique for structure elucidation of both small and macro molecules [4,5]. Recently, NMR has been used for both qualitative (metabolic profiling) and quantitative (qNMR) analyses of mixtures in plant extracts [6,7]. Although NMR spectra often suffer from spectral overlap and low sensitivity compared to mass spectrometry (MS), its high selectivity and reproducibility are advantageous for metabolic profiling in the mixture of metabolites. The assignment of known metabolites in plant extracts can be easily determined by comparing with standard NMR spectra deposited in commercial or public databases [8,9]. In addition to qualitative metabolic profiling, NMR analysis has been utilized for absolute quantification of metabolites in the field of natural products research. Quantitative NMR can also be used to determine the purity of a sample by comparison with an internal standard [10].

In the present study, we developed new and alternative analytical methods for the identification and discrimination of CW and CA by NMR spectroscopy. We verified that conduritol F, the chemical marker of CW, could be quantified in CW samples without interference from other signals and was used for the discriminant marker between CW and CA samples.

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Table 1
Chemical shifts and purity of the signals for compound **1** for qNMR study.

Proton	Chemical shift (δ_H, J in Hz)[a]	Purity[b]
H-1	3.41, dd (4.3, 10.3)	99.4%
H-2	3.61, dd (7.7, 10.3)	93.5%
H-3	3.92, dt-like (7.4)	95. 9%
H-4	4.15, t (4.5)	96.5%
H-5	5.79, ddd (2.0, 4.9, 10.0)	97.3%
H-6	5.71, dd (2.1, 10.1)	96.4%

[a] Spectra were referenced to the signal of CD_3OD at δ_H 3.30.
[b] Each signal was calculated using the equation described in the Methods and materials section.

2. Methods and materials

2.1. Plant materials

CW (10 kg) and CA (10 kg), collected in 2015, were purchased from a local herbal market in Geumsan-county, South Chungcheong province based on a suggestion from Dr. Rak Sun Sung, Herbal Medicinal Products Division of Ministry of Food, Drug and Safety (Osong, Korea). The weight for each piece of CW and CA root was, on average, in the range of approximately 10–40 g and 30–70 g, respectively. We separated the pieces of CW and CA into 50 groups to verify that there was no-cross-contamination between the CA and CW samples. Each piece of CA was weighed, separated into 50 samples and labeled as CA-1–50. Since the average mass of CW pieces was smaller than those of CA, the pieces of CW close enough to the average mass of a piece of CA were used and separated into 50 samples (CW-1–50). For the discriminative study between CW and CA distributed in herbal markets, nine samples (7 labeled as CW and 2 labeled as CA) were purchased from different Korean herb markets. For HPLC and NMR analyses, each sample was ground into powder form using an analytical grinding mill (A-11, IKA® Werke GmbH & Co. KG, Germany). For DNA analysis, it was cut to a length of 1–2 cm, frozen in liquid nitrogen and ground into a fine powder. Total genomic DNA was extracted using a plant DNA isolation mini kit (DNeasy Plant Mini Kit, Qiagen, Hilden, Germany).

2.2. Determination of adulteration in CW and CA samples using HPLC-UV and DNA analyses

HPLC-UV analysis was performed to verify the presence of conduritol F in CW-1–50 and CA-1–50 samples [11]. Briefly, HPLC analyses were performed using an Agilent 1260 infinity HPLC-UV system connected to a UV detector (Agilent Technologies Mfg GmbH & Co. KG, Waldbronn, Germany) set at 205 nm and a Hector-M SIL column (250 mm × 4.6 mm; i.d. 5 μm, RStech, Daejeon, Korea) with a compatible phenomenex guard column (4 mm × 3 mm; i.d. 5 μm). The isocratic solvent condition was H_2O:acetonitrile (98:2) for 20 min and the flow rate was 1.0 ml/min.

For molecular authentication of these medicinal plants, the *trnH–psbA* region was amplified using specific primers trnHf_05 (5′-GTTATGCATGAACGTAATGCTC-3′) and psbA3 F (5′-CGCGCATGGTGGATTCACAATCC-3′). Each PCR (polymerase chain reaction) was performed in a 20-μl volume containing 20 ng template DNA, 2 μl 10 × PCR buffer, 0.20 mM dNTPs, 0.5 μM forward and reverse primer, and 0.5 U i-star max DNA polymerase (Intron Biotechnology, Seongnam, Korea). The PCR cycling conditions were as follows: pre-denaturation at 95 °C for 3 min, followed by 34 cycles of denaturation at 94 °C for 30 s, primer annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. The final cycle included a 5-min extension at 72 °C to ensure full extension. The PCR products were separated by 1.0% agarose gel electrophoresis, stained with ethidium bromide, and viewed under ultraviolet light.

2.3. Isolation of conduritol F from *C. wilfordii*

Conduritol F was isolated from CW by a slightly modified method from Jiang et al. [11]. Briefly, the dried roots of CW (3 kg) were ground and extracted with 100% MeOH in an ultrasonic apparatus (3 h × 2). The methanolic extract (147 g) was sequentially fractionated with n-

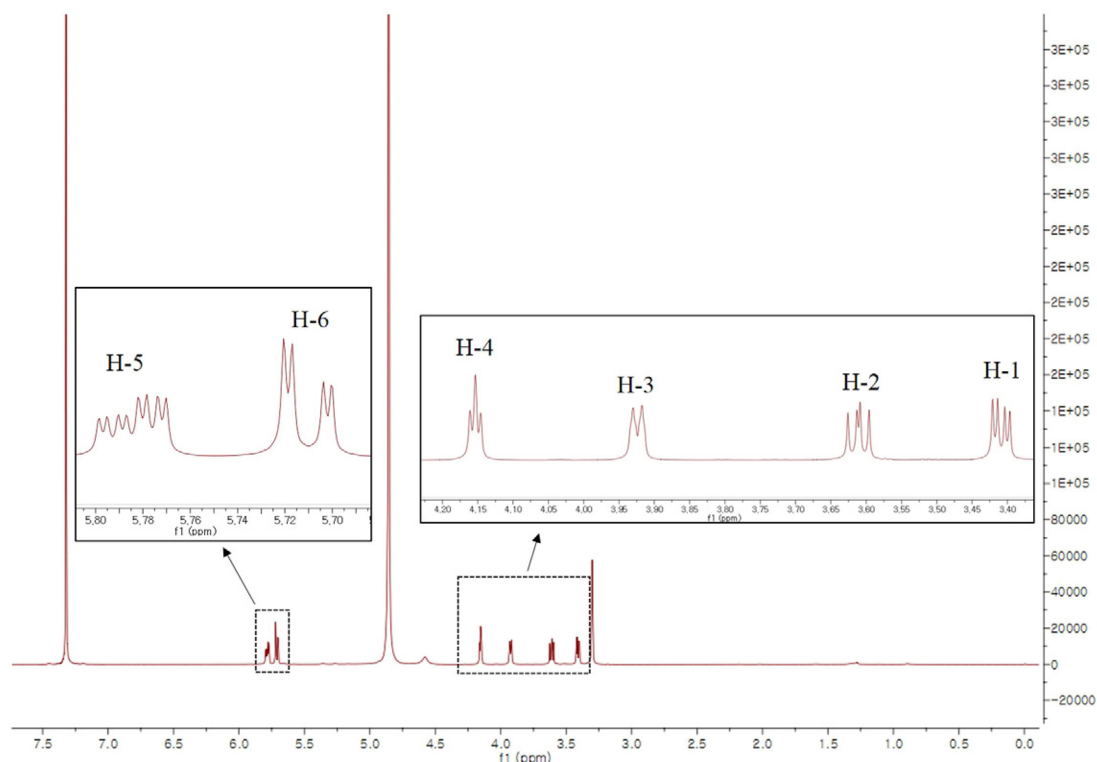

Fig. 1. ^{1}H NMR spectrum of compound 1 isolated from CW sample. Benzene-d_6 (δ_H 7.32) was used as the internal standard. The signals were recorded using CD_3OD (δ_H 3.30).

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