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Quantitative analysis of phenolic metabolites from different parts of *Angelica keiskei* by HPLC–ESI MS/MS and their xanthine oxidase inhibition

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ABSTRACT

Angelica keiskei is used as popular functional food stuff. However, quantitative analysis of this plant's metabolites has not yet been disclosed. The principal phenolic compounds (**1**–**16**) within *A. keiskei* were isolated, enabling us to quantify the metabolites within different parts of the plant. The specific quantification of metabolites (**1**–**16**) was accomplished by multiple reaction monitoring (MRM) using a quadruple tandem mass spectrometer. The limit of detection and limit of quantitation were calculated as 0.4–44 μg/kg and 1.5–148 μg/kg, respectively. Abundance and composition of these metabolites varied significantly across different parts of plant. For example, the abundance of chalcones (**12**–**16**) decreased as follows: root bark (10.51 mg/g) > stems (8.52 mg/g) > leaves (2.63 mg/g) > root cores (1.44 mg/g). The chalcones were found to be responsible for the xanthine oxidase (XO) inhibition shown by this plant. The most potent inhibitor, xanthoangelol inhibited XO with an IC_{50} of 8.5 μM. Chalcones (**12**–**16**) exhibited mixed-type inhibition characteristics.

1. Introduction

Recently, quantitative metabolite profiling has been increasingly used in food chemistry to standardize botanical products or herbal medicines to ensure activity and safety (Jiang, David, Tu, & Barbin, 2010; Wang et al., 2011). In particular, quantitative analysis of metabolites is useful to optimise crops for specific applications by optimising cultivar, harvest time, or specific parts of the whole plant. *Angelica keiskei*, known under the Japanese name of Ashitaba, belongs to the apiaceae family of umbelliferae and is a perennial plant from the angelica genus. In folk medicine, it is claimed to be a diuretic and a tonic used to improve digestion and to promote healing of wounds. There is also pharmacological evidence that Angelica plants possess hepatoprotective activity, platelet anti-aggregatory effects, anti-tumor effects, anti-bacterial effects, and neuroprotective activities (Kang, Lee, Sung, & Kim, 2005; Lee, Lee, Jin, & Yun-Choi, 2003; Oh et al., 2002).

Coumarins and chalcones are the main bioactive components of *A. keiskei*. Coumarins have been shown to possess antioxidant, anti-cancer, antidepressant and acetylcolinesterase inhibitory effects (Aoki & Ohta, 2010; Akihisa et al., 2003; Capra et al., 2010; Wszelaki, Paradowska, Jamróz, Granica, & Kiss, 2011). Chalcones have been also extensively studied and are shown to be antioxidants, anticancer agents and inhibitors of α-glucosidases (Li et al., 2009; Luo, Wang, Wang, Ma, & Li, 2012).

A. keiskei has been cultivated in many Asian countries because of its above mentioned beneficial effects to human health. Its stem and leaves have been consumed commercially as a health food. Its roots have also been used as a medicine and in food additives considered to alleviate pain and the symptoms of diabetes mellitus (Bensky, Gamble, & Kaptchuk, 1993). Given the manifold properties ascribed to *A. keiskei*, it is necessary to investigate individual components extensively for further application of this valuable plant.

In this study, we quantified the phenolic compounds from different parts of *A. keiskei*. Sixteen phenolic compounds were isolated from the methanol extracts of *A. keiskei* root bark. These compounds were used as standards by which to assess the composition of the different plant components. In the meantime, the methanol extract was found to inhibit xanthine oxidase (XO) significantly. Further investigation showed that chalcones were responsible for this inhibitory effect. XO is an important source of free radicals and has been reported in various physiological and pathological models (Parcher, Nivorozhkin, & Szabó, 2006).

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XO is a cause of gout and can also cause oxidative damage to living tissues. This enzyme reduces molecular oxygen, leading to the formation of O_2^- and hydrogen peroxide. Regulation of XO activity is important during inflammation (Nakai, Kadiiska, Jiang, Stadler, & Mason, 2006). For instance, treatment with an XO inhibitor largely prevented the development of endothelial dysfunction and atherosclerosis in mice (Schröder et al., 2006).

2. Materials and methods

2.1. Plant material and chemicals

A. keiskei was collected from a farm at Kimhae, Korea in June, 2012. Each whole plant was separated into leaves, stems, root barks and root cores and then dried in the shade. Analytical grade water and methanol were purchased from Merck (Darmstadt, Germany). Xanthine oxidase from bovine milk (E.C. 1.1.3.22), xanthine, allopurinol, sodium pyrophosphate, chloroform-d and acetone-d_6 were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Silica gel (70–230 and 230–400 mesh), NP F254 and RP−18 F254s TLC plates were obtained from Merck (Darmstadt, Germany). Spherical C18 100 Å reversed phase silica gel (particle size: 20–40 μm) was obtained from SILICYCLE (Quebec, Canada). Methanol, acetone, ethyl acetate, chloroform and *n*-hexane were purchased from Duksan Co. (Gyoenggi Korea).

2.2. Instruments

1D and 2D NMR spectra (^{1}H, ^{13}C, DEPT-90, DEPT-135, COSY, HMQC and HMBC) were recorded on a Bruker (AM 500 MHz) spectrometer, using $CDCl_3$, and acetone-d_6, as solvent and tetramethylsilane (TMS) as an internal standard. Electron ionisation (EI) and EI-high resolution (HR) mass spectra were obtained on a JEOL JMS-700 instrument (Japan). The HPLC−DAD−MS analysis was performed using a Agilent (USA) 1100 series system equipped with a dual low-pressure gradient pump, an auto sampler, a column compartment, a diode array detector and an Applied Biosystems 3200 ion trap mass spectrometer with an ESI interface (Applied Biosystems, Forster, CA, USA). Enzymatic assays were carried out on a SpectraMaxM3Multi-Mode Microplate Reader (Molecular device, USA).

2.3. Extraction, fractionation and isolation of A. keiskei

The dried root bark (1.5 kg) of *A. keiskei* was extracted using methanol (3 × 5 l) at room temperature to give crude extract (43.5 g). Crude extract (8 g) was purified by MPLC (PuriFlash 450, Interchim, France) over reversed phase silica gel (20–40 μm, 250 g) and eluted by using a stepwise H_2O/methanol gradient (10–100%, 25 ml/min) to give 120 fractions. This MPLC procedure was repeated five times using the same conditions before further isolation. Fractions 80–95 (6.2 g) were purified over silica gel (50 μm, 120 g) eluting with a hexane–EtOAc gradient (0–25% EtOAc, 10 ml/min) to give 60 subfractions. Subfractions 13–16 (430 mg) were further purified by repeated silica gel chromatography (CC) to give **12** (81 mg) and **14** (30 mg). Repeated silica gel CC of subfractions 17–21 (760 mg) yielded **13** (16 mg), **15** (114 mg) and **16** (22 mg). Fractions 55–67 (3.8 g) were fractionated over silica gel (50 μm, 120 g) eluting with a hexane–EtOAc gradient (20–50% EtoAc, 10 ml/min) to give 60 subfractions. Repeated silica gel CC of subfractions 16–20 (830 mg) yielded **7** (38 mg), **8** (32 mg) and **11** (13 mg). Further purification of subfractions 21–26 (340 mg) by silica gel (50 μm) CC gave **5** (27 mg) and a mixture (140 mg) of **9** and **10**. The mixture of **9** and **10** was purified by preparative TLC (hexane–EtOAc, 2:1) to give **9** (31 mg) and **10** (23 mg). Fractions 30–47 (8.5 g) were fractionated over silica gel (50 μm, 120 g) eluting with $CHCl_3$−acetone (0–15% acetone, 10 ml/min) to give 60 subfractions. Repeated silica gel (50 μm, 50 g) CC of subfractions 12–17 (960 mg) yielded **2** (140 mg), **3** (36 mg) and **4** (12 mg). Subfractions 21–24 (630 mg) were purified by preparative TLC ($CHCl_3$–acetone, 20:1) to give **1** (210 mg) and **6** (19 mg). All isolated compounds were identified by comparison to previously reported spectroscopic data (see Supplementary data).

2.4. Preparation of standard solutions

All standard compounds were isolated from root barks of *A. keiskei* by chromatography. Two mixed standard stock solutions consisting of coumarins (**1**–**11**) or chalcones (**12**–**16**) were prepared in methanol because detection by MS required different ionisation modes; positive mode for coumarin and negative for chalcone. The working standard solutions were prepared by diluting the mixed standard solution with methanol to a series of concentrations within the ranges: 0.16–10.5 μg/g for coumarins and 0.006–0.39 μg/g for chalcones. The standard stock and working solutions were all stored at 4 °C until use and filtered through a 0.45 μm membrane prior to injection.

2.5. Preparation of sample solutions

Whole *A. keiskei* plants were divided into four different parts (leaves, stems, root bark and root cores) and pulverized into powder. Each sample (1.0 g) was extracted in 25 ml of methanol for 6 h at room temperature with shaking. The supernatant was centrifuged at 3000*g* for 5 min and then filtered through a 0.45 μm syringed filter prior to analysis. 10 μl of filtrate was injected into the HPLC for quantitative analysis. Three replicates of the extraction process were carried out on independent samples.

2.6. HPLC with MS detection

Chromatographic separation was performed on an Eclipse XDB-C18, 4.6 × 150 mm, 5 mm column (Agilent Technologies, USA). Column temperature was 25 °C. The mobile phase (A) was acetic acid/water (0.1/100, v/v), and the mobile phase (B) was acetic acid/methanol (0.1/100, v/v). The linear gradient solvent system was as follows: 0–3 min, 10% B; 3–8 min, 40% B; 8–13 min, 60% B; 13–33 min, 100% B; 33–38 min, 100% B. The flow rate was 1 ml/min. The column was equilibrated for 10 min between injections. UV spectra were recorded over a range of 200–400 nm and chromatograms were acquired at 254 and 310 nm. The LC elution was introduced directly into the ESI interface without splitting. Standard compounds were analysed in positive ion mode. The nebulizer pressure was 60 psi; dry gas flow was 8 l/min; dry temperature was 550 °C; and capillary voltage was 5.5 kV/−4.5 kV. The analysis was carried out using scans from *m/z* 100 to 500.

2.7. Mass spectrometry

Identification of peaks was performed using a 3200 QTRAP system from Applied Biosystems/MDS Sciex (Applied Biosystems, Foster City, CA, USA), a triple quadrupole linear ion trap mass spectrometer equipped with Turbo V sources and TurboIonspray interface. The instrument was operated using electrospray ionisation source in positive and negative mode simultaneously. The ion spray voltage was set to 5500 and −4500 V for ESI^+ and ESI^-, respectively. The turbo spray temperature was maintained at 500 °C. Nebulizer gas (gas 1) and heater gas (gas 2) were set at 40 and 50 psi, respectively. The curtain gas was kept at 25 psi and interface heater was on. Nitrogen was used in all cases. Multiple-reaction monitoring (MRM) mode was employed for