High-Performance Liquid Chromatographic Quantification and Validation of Luteolin Glycosides from *Sonchus brachyotus* and Their Peroxynitrite-Scavenging Activity

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Abstract – In Korea, the leaves of *Sonchus brachyotus* (Compositae), an edible mountainous vegetable, are traditionally used to treat hepatitis and hemorrhage and are known to have diuretic action. The aqueous ethanolic extract of this plant was selected in our screening experiment using the peroxynitrite (ONOO¹⁻) -scavenging assay, and the present study was performed to qualitatively and quantitatively identify the active compounds from *S*. *brachyotus* and validate the present high-performance liquid chromatography (HPLC) coupled with ultraviolet absorption detection method based on accuracy, precision and repeatability. Five phenolic substances including the main compound, luteolin 7-β-D-glucuronopyranoside, as well as chlorogenic acid, luteolin 7-β-D-rutinoside, luteolin 7-β-D-glucopyranoside, and luteolin, were found in the aqueous ethanolic extract of *S. brachyotus*. In the HPLC validation experiment, the linearity of the four compounds was established by \( R^2 \) values of more than 0.999 within the test ranges, and the recovery rate ranged from 98.2 - 105.3%. Luteolin 7-β-glucuronide was a predominant compound (143 mg/g of extract and 18.3 mg/g of the dry weight of plant material) with a potent peroxynitrite-scavenging effect (IC₅₀, 1.02 ± 0.08 µM). Luteolin and its three glycosides together with chlorogenic acid were qualitatively and quantitatively determined using an HPLC method validated in the present study.

Keywords – *Sonchus brachyotus*, Compositae, luteolin 7-β-D-glucuronopyranoside, peroxynitrite, HPLC, validation

Introduction

Peroxynitrite (ONOO¹⁻) is generated in the living system by the reaction between superoxide (\( \cdot \text{O}_2⁻ \)) and nitric oxide (\( \cdot \text{NO} \)) (Radi et al., 1991). Its over production causes aging-related disease such as obesity, diabetes mellitus and atherosclerosis (Patcher et al., 2005; Drel et al., 2007; Korda et al., 2008). Therefore, we have phytochemically studied Korean mountainous vegetables that have ability to scavenge peroxynitrite (Nugroho et al., 2009, 2010). In the course of that study, a variety of polyphenols including caffeoylquinic acids or flavonoids were often detected or quantified by high-performance liquid chromatography (HPLC) coupled with ultraviolet absorption detection. There are many well-known polyphenols in foods that are responsible for peroxynitrite-scavenging: catechin, epicatechin and hydroxycinnamates among wine flavonoids (Boveris et al., 2002), epigallocatechin among green tea polyphenols (Tipce et al., 2007), and caffeoylquinic acids among chwinamul polyphenols (Nugroho et al., 2009).

During our ongoing studies on the peroxynitrite-scavenging effects of mountainous vegetables belonging to the family Compositae, the potent activity of the *Sonchus brachyotus* extract was observed. Therefore, this study was performed for the qualitative and quantitative identification of active compounds in this plant.

In the present study, luteolin and its three glycosides (7-O-glucoside, 7-O-glucuronic acid, and 7-O-rutinoside of luteolin) were used for analysis. It has been reported that light/water stress (Yaginuma et al., 2002) or salinity stress (Agati et al., 2011) increase the biosynthesis of luteolin and its 7-O-glucoside in certain plants. In our HPLC method validated in the present study, the contents of 7-O-glucuronicid and 7-O-rutinoside of luteolin were considerably higher than those of luteolin and its 7-O-
glucoside.

*S. brachyotus* is a perennial herb belonging to the family Compositae. It is 30 - 100 cm high and its basal leaves disappear in the flowering period from August to October in Korea. Young leaves of *S. brachyotus* are used as an edible mountainous vegetable, as diuretics, and to treat hepatitis and hemorrhage in Korea (Moon *et al.*, 1984; Kim *et al.*, 1994). However, *S. oleraceus* and *S. asper*, which have strong thorns at the margin of the leaf, are taxonomically distinguished from *S. brachyotus* without thorns (Moon *et al.*, 1984). Although anxiolytic (Vilela *et al.*, 2009), anti-inflammatory (Vilela *et al.*, 2010) and anti-nephrotoxic (Khan *et al.*, 2010) effects of *S. asper* have been reported, the constituents and biological activities of *S. brachyotus* are unknown.

A variety of polyphenolic substances and their pharmacological activities have been reported from the leaves of mountainous vegetables. HPLC analysis and fingerprint of the phenolic substances of the mountainous herbs will lead to their predictable and reasonable utilization for biomedical food or medicinal therapeutics. Based on the International Conference on Harmonization guidelines (ICH, 2005), validation of the HPLC analytical method should be performed to ensure that it is precise, accurate, and reproducible. In this research, the phenolic substances were isolated, and HPLC quantification and validation and peroxynitrite-scavenging assays were performed.

**Experimental**

**Instruments and Reagents** – HPLC was performed using a Varian HPLC system (Varian, Inc., Walnut Creek, CA, USA) consisting of a Prostar 210 solvent delivery module and Prostar 325 UV-Vis detector. A Shiseido (Tokyo, Japan) Capcell Pak C18 column (5 µm, 250 mm × 4.6 mm I.D.) was used for separation. All solvents used in the analysis were HPLC grade purchased from J.T. Baker® (Phillipsburg, NJ, USA).

**Plant Material** – The aerial parts of *S. brachyotus* (Compositae) were collected from a field in Sinlimnyeon, Wonju-si, Gangwon-do, Korea. This plant was identified by Professor Sang-Cheol Lim (Department of Horticulture and Landscape Architecture, Sangji University, Korea). A voucher specimen was deposited in the laboratory of Natural Product Chemistry, Department of Pharmaceutical Engineering, Sangji University, Korea. The collected plant was air-dried at room temperature and pulverized with a blender prior to extraction.

**Standard Compounds** – The dried plant material (350 g) was extracted with aqueous ethanol (30% ethanol) under reflux for 6 hours for three times. The extracted solution was filtered, evaporated, and freeze-dried to give a powdery extract (32 g). The lyophilized extract (30 g) was fractionated into diethyl ether- and n-butanol fractions. The latter fraction (6.8 g) was fractionated using a Diaion HP-20 column (410 g, 6.0 × 35.0 cm) with sequential eluents [water (1.5 L) → 50% methanol (1.5 L) → methanol (1.0 L)]; eight fractions (each 500 mL) were obtained. Fractions 4 and 5 were combined, evaporated, and chromatographed by Medium Pressure Liquid Chromatography (Eyela ceramic pump, Tokyo, Japan) on two combined silica gel closed-columns (silica gel 40 µm, 5.2 × 23.0 cm, 200 g, Yamazen Co., Japan) with a flow rate of 3 mL/min to yield 126 fractions (each 10 mL). Fractions 86 - 108 were combined, evaporated, and recrystallized to produce luteolin 7-O-β-D-glucurono-pyranoside (4), a yellowish amorphous powder (1.26 g), which was identified by comparison of its 1H- and 13C-NMR spectroscopic data with literature (Gulluce *et al.*, 2010). Three other compounds, chlorogenic acid (1), luteolin 7-O-β-D-glucopyranoside (3), and luteolin (5), were purchased from Sigma Chemical Co. (St. Louis, MO, USA) for HPLC analysis. Luteolin 7-O-β-rutinoside (2) was offered from Prof. Sang-Hyun Lee (Chung-Ang University, Anseong, Korea).

**Sample Solutions** – Ten grams of the air-dried and pulverized aerial part of *S. brachyotus* was accurately weighed and soaked in 200 ml of the aqueous ethanolic extract in a 500-ml Erlenmeyer flask. Using an ultrasonicator, the sample was extracted at 50 ℃ for 6 hours; the extract solution was then filtered and dried on a rotary vacuum evaporator under reduced pressure. The viscous extract was freeze-dried for 12 hours to produce a powdery extract (yield: 12.8%) that was used to prepare sample solution. A part of the aqueous ethanolic extract was weighed and diluted to produce five sample solutions with different concentrations for HPLC injection (precision, recovery, and repeatability tests).

**HPLC Conditions** – Standard compounds were dissolved in methanol using a vortex mixer and ultrasonicator to prepare standard solutions, which were then filtered through 0.50-µm syringe filters before injection. The mobile phase was a mixed solvent of 0.05% trifluoroacetic acid (TFA) in water (solvent A) and 0.05% TFA in methanol-acetonitrile (60 : 40) (solvent B). The gradient elution system was as follows: (A)/(B) = 68/32 (0 min; hold for 10 min) → 40/60 (30 min) → 0/100 (32 min; hold for 4 min) → 68/32 (36 min; hold for 8 min to equilibrate the column condition). Column temperature