Natural Product Sciences
18(1) : 39-46 (2012)

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

Agung Nugroho[1,2], Myung-Hoe Kim[1], Chan Mi Lee[3], Jae Sue Choi[3], Sanghyun Lee[4], and Hee-Juhn Park[5,*]

[1]Department of Applied Plant Sciences, Graduate School, University, Wonju 220-702, Korea
[2]Department of Agro-industrial Technology, Faculty of Agriculture, Lambung Mangkurat University, Indonesia 70712, Indonesia
[3]Department of Food Science and Nutrition, Pukyong National University, Busan 607-737, Korea
[4]Department of Integrative Plant Science, Chung-Ang University, Anseong 456-756, Korea
[5]Department of Pharmaceutical Engineering, Sangji University,Wonju 220-702, Korea

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 (ONO_2^-)-scavenging assay, and the present study was performed to qualitatively and quantitatively identify the active compounds from *S. brachyotus* and validate the present high-permormance liquid chromatography (HPLC) coupled with ultraviolet absorption detection method based on accuracy, precision and repeatability. Five phenolic substances including the main compound, luteolin 7-*O*-*β*-D-glucuronopyranoside, as well as chlorogenic acid, luteolin 7-*O*-rutinoside, luteolin 7-*O*-*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-*O*-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 (IC_{50}, 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-*O*-*β*-D-glucuronopyranoside, peroxynitrite, HPLC, validation

Introduction

Peroxynitrite ($ONOO^-$) is generated in the living system by the reaction between superoxide ($•O_2^-$) and nitric oxide (•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*-glucuronide, 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*-glucuronide and 7-*O*-rutinoside of luteolin were considerably higher than those of luteolin and its 7-*O*-

*Author for correspondence
Tel: +82-33-730-0564; E-mail: hjpark@sangji.ac.kr

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 Sinlim-nyeon, 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 ^{1}H- and ^{13}C-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 °C 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

was maintained at 40 °C using a temperature controller. Analysis was performed at a flow rate of 1.00 ml/min with detection wavelength fixed at 254 nm.

Method Validation of Quantitative Analysis – According to the International Conference on Harmonization guidelines (ICH,2005), the analytical method was validated by determination of the linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, repeatability, and accuracy of each analyte.

Linearity of Standard Compounds – Using standard solutions, linearity was examined. Standard compounds were dissolved in methanol to prepare standard solutions. Six different concentrations of standard solutions dissolved in methanol were prepared in the range of 1.25 - 55.00 μg/ml by serial dilution method. The linearity of the calibration curves was determined by plotting the mean peak area (y axis) versus concentration (x axis) for each analyte in that range.

LOD and LOQ – After injecting an aliquot (20 μL) of the serial dilutions of six individual standard solutions, LODs and LOQs under the present HPLC method were determined at signal to noise (S/N) ratios of 3 and 10, respectively.

Precision, Repeatability, and Accuracy Studies – Intra- and inter-day variabilities of the *S. brachyotus* extract were measured to validate the precision. The intra-day variability was determined by analyzing that sample within 24 h. The solutions were injected five times, and the relative standard deviation (RSD) value was calculated for the concentration of each analyte in the extract and considered to be a measure of precision. Each sample was injected five times a day on three consecutive days to assess the intra-day variability. To validate the repeatability, five different working sample solutions were analyzed and variations were expressed as relative standard deviation (RSD). Accuracy was evaluated in a recovery test by calculating the mean recovery (%) of standard compounds from a spiked extract solution versus a non-spiked extract sample.

Assay for Peroxynitrite-Scavenging Activity – Peroxynitrite (ONO_2^-) scavenging activity was assessed by a modified Kooy's method (Kooy *et al*., 1994) that involved the monitoring of fluorescent produced from non-fluorescent dihydrorhodamine 123 (DHR 123) in the presence of peroxynitrite. In brief, the rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM diethylene triamine pentaacetic acid (DTPA). The final DHR 123 concentration was 5 μM. The buffer in this assay was prepared prior to use and placed on ice. The plant extracts were dissolved in 10% dimethyl sulfoxide (f.c. 5 μg/ml). The background and final fluorescence intensities were measured 5 min after treatment with and without the addition of authentic peroxynitrite (10 μM) dissolved in 0.3 N sodium hydroxide. The fluorescence intensity of the oxidized DHR 123 was evaluated using a microplate fluorescence reader BioTek® FL 500 (BioTek Instruments Inc., Winooski, VT, USA) at excitation and emission wavelengths of 480 and 530 nm, respectively. Peroxynitrite-scavenging values were calculated as the final fluorescence intensity minus the background fluorescence via the detection of DHR 123 oxidation and expressed as mean ± SD. L-Penicillamine was used as a positive control.

Results and Discussion

Isolation Method and Standard Compounds – We isolated the main compound on a larger scale by extracting the plant materials with aqueous ethanol (30% ethanol). The lyophilized n-butanol extract prepared by solvent fractionation was further fractionated using a Diaion HP-20 column. The fraction showing a major peak in HPLC chromatogram was chosen and further purified by MPLC to give the major compound. This compound was identified as luteolin 7-*O*-*β*-D-glucuronopyranoside by comparison of ^{1}H- and ^{13}C-NMR spectroscopic data with the literature (Gulluce *et al*., 2010) and used as a standard compound. Three other compounds, chlorogenic acid, luteolin 7-*O*-*β*-D-glucopyranoside, and luteolin (Sigma, USA), and luteolin 7-*O*-rutinoside (offered by Prof. Sang-Hyun Lee) were also used, since those compounds were also identified in the extract of *S. brachyotus*. Those five compounds have not previously been found in *S. brachyotus*. The chemical structures are shown in Fig. 1.

Optimization of HPLC Condition – Column, mobile phases and detection wavelength were considered in the HPLC chromatographic resolution. A Capcell Pak C18 column (5 μm, 250 mm × 4.6 mm) was chosen because it produced more selective and sharper peaks. The two solvents in the mobile phase, 0.05% TFA (trifluoroacetic acid) in water (A solvent) and 0.05% TFA in methanol-acetonitrile (60:40) (B solvent), were chosen and run according to the programmed gradient elution; this solvent system produced better peak shapes and resolution than the methanol-water or methanol-acetonitrile system. Addition of TFA improved the peak shapes by protecting the phenolic substances from ionization.

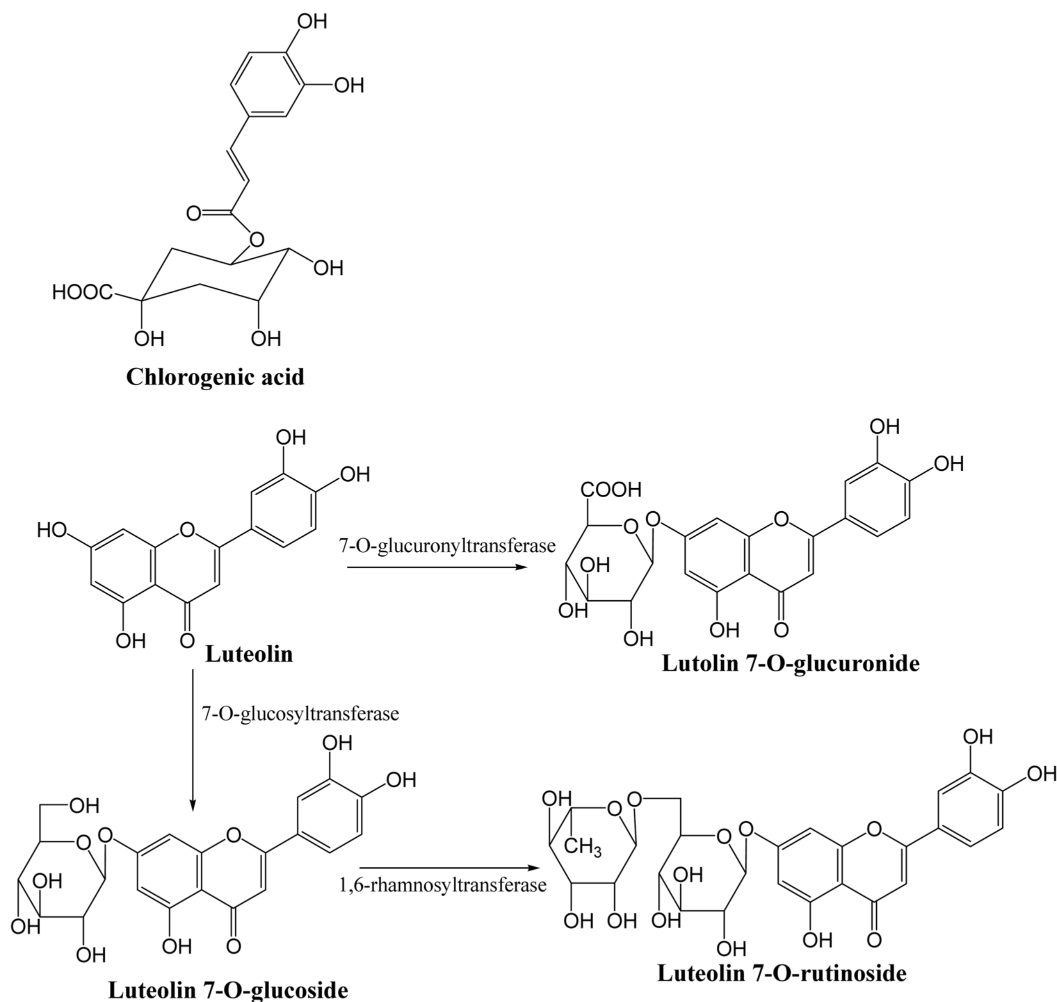

Fig. 1. Structure of chlorogenic acid and luteolin derivatives, and presumed pathway for biosynthesis of luteolin glycosides.

A wavelength 254 nm for detection was chosen during our consideration of the four ones, 254, 280, 300 and 360 nm, because it was the most sensitive. That means LOD and LOQ of the reference compounds can be achieved in a smaller concentration. Since of that, compounds contained in the extract, which present in very low concentration, were able to be detected and quantified accurately. In addition, that wavelength was also sensitive enough to detect the two types of phenolics including caffeoylquinic acids and flavones simultaneously. The following HPLC conditions were thus chosen: column temperature, 40 °C; flow rate, 1.00 ml/min; detection wavelength, 254 nm. The HPLC chromatograms of standard compound mixtures and plant extract are shown in Fig. 2.

Method Validation of Quantitative Analysis – HPLC experiments on linearity, precision and repeatability were performed to ensure that the present method was sensitive, selective, precise and accurate. Serially-diluted solutions of the four compounds prepared in the range of 1.25 - 40.00 μg/ml for chlorogenic acid and luteolin 7-*O*-glucoside, 1.72 - 55.00 μg/ml for luteolin 7-*O*-glucuronide, and 1.56 - 50.00 μg/ml for luteolin and its rutinoside, respectively, were injected onto HPLC, and calibration curve equations were calculated. As shown in Table 1, the linearity and sensitivity of the four compounds were established based on R^2 values > 0.999 and LOD and LOQ values < 2.83 μg/ml, where LOD and LOQ represent limit of detection and limit of quantification, respectively.

Intra-day and inter-day variabilities are used to validate the precision of the HPLC method. To assess the repeatability, sample solutions were injected five times onto the HPLC system, and relative standard deviation (RSD) values were calculated: As shown in Table 2, the

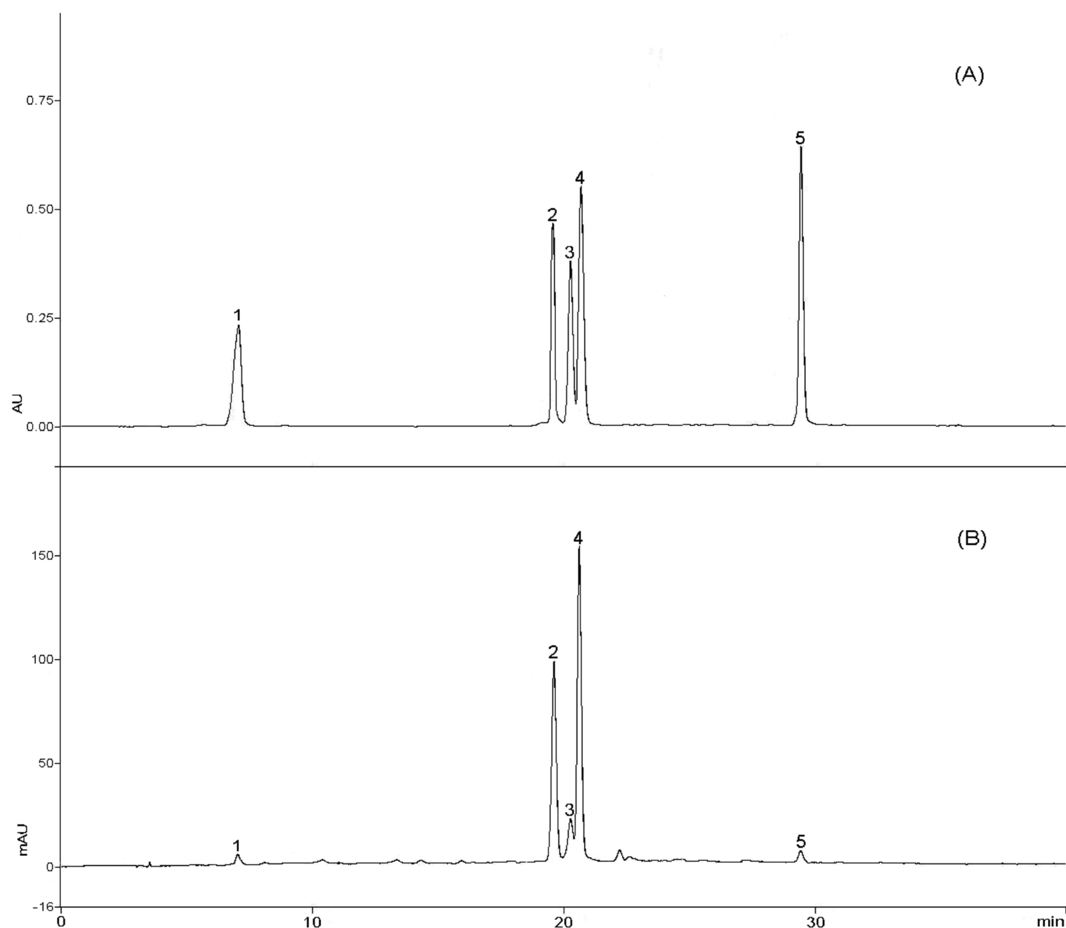

Fig. 2. (A) Chromatogram of mixed standard compounds: (**1**) chlorogenic acid; (**2**) luteolin 7-O-rutinoside; (**3**) luteolin 7-*O*-glucoside; (**4**) luteolin 7-O-glucuronide; (**5**) luteolin. (B) Chromatogram of aqueous ethanol extract of *S. brachyotus*.

Table 1. Calibration curves, detection limits and quantification limits of the analytes

Analyte	Calibration equation (linear model)[a]	Linear range (μg/ml)	R^2 [b]	LOD[c] (μg/ml)	LOQ[d] (μg/ml)
Chlorogenic acid	y = 85.929x + 11.292	1.25-40.00	0.9998	0.74	2.47
Luteolin 7-*O*-rutinoside	y = 175.245 + 34.547	1.56-50.00	0.9999	0.38	1.26
Luteolin 7-*O*-glucoside	y = 200.82x + 40.731	1.25-40.00	0.9999	0.34	1.13
Luteolin-7-*O*-glucuronide	y = 48.813x + 95.759	1.72-55.00	0.9997	0.85	2.83
Luteolin	y = 399.22x + 164.85	1.56-50.00	0.9997	0.25	0.83

[a]y, peak area at 254nm; x, concentration of the standard (μg/ml); [b] R^2, correlation coefficient for 6 data points in the calibration curves (n = 3); [c] LOD, limit of detection (S/N = 3); [d] LOQ, limit of quantification (S/N = 10).

RSD values in intra-day variability and inter-day variability were < 2.95% and < 3.57%, respectively, indicating that the present method is precise. The RSD values in the repeatability experiment were < 3.81%.

A recovery test was performed to determine accuracy. The aqueous ethanolic extract was spiked with standard compounds to observe changes in the recovery rate (%). Accuracy was evaluated by measuring the mean recovery (%) of standard compounds from the spiked extract solution versus the non-spiked extract sample. Average recovery rates ranged from 98.2% - 105.3%, and the RSDs were between 2.4% - 3.1%, as shown in Table 3. Therefore, the present HPLC method was established by validation of the precision, accuracy and sensitivity for

Table 2. Analytical results of intra-day (n = 5) and inter-day variabilities and repeatability of analytes in aqueous ethanol extract

Analyte	Intra-day variability		Inter-days variability		Repeatability (n = 5)	
	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)
Chlorogenic acid	2.55	2.15	2.61	2.73	2.53	2.84
Luteolin 7-*O*-rutinoside	26.7	1.05	26.7	1.35	26.7	1.77
Luteolin 7-*O*-glucoside	1.63	2.83	1.59	3.41	1.64	3.68
Luteolin 7-*O*-glucuronide	143	0.37	143	0.54	143	0.72
Luteolin	1.03	2.95	1.06	3.57	1.05	3.81

Table 3. Recovery of each analyte as determined by standard addition method (n = 3)

Analyte	Initial concentration (μg/ml)	Amount added (μg)	Concentration after addition (μg/ml)		Recovery (%)	RSD (%)
			Expected	Measured		
Chlorogenic acid	3.04	2.50	5.54	5.79	104.5	3.1
Luteolin 7-*O*-rutinoside	31.8	25.0	56.8	58.6	103.2	2.6
Luteolin 7-*O*-glucoside	1.97	2.50	4.47	4.70	105.2	2.9
Luteolin 7-*O*-glucuronide	42.9	55.0	97.9	96.1	98.20	2.7
Luteolin	1.26	1.56	2.82	2.97	105.3	2.4

Table 4. Content of analytes in aqueous ethanol extract and the dry weight of plant material

Analyte	Content of analytes (mg/g)		
	of extract	of DW[a]	RSD[b]
Chlorogenic acid	2.53	0.32	2.84
Luteolin 7-*O*-rutinoside	26.7	3.40	1.77
Luteolin 7-*O*-glucoside	1.64	0.21	3.68
Luteolin 7-*O*-glucuronide	143	18.3	0.72
Luteolin	1.05	0.13	3.81

[a]DW, dry weight of plant material; [b]RSD (%) for n = 5; [c]RC, relative concentration.

Table 5. Relative retention time

Analyte	RRT	RSD (%)
Chlorogenic acid	0.32	0.12
Luteolin 7-*O*-rutinoside	0.93	0.15
Luteolin 7-*O*-glucoside	0.98	0.15
Luteolin 7-*O*-glucuronide	1.00	0.16
Luteolin	1.42	0.21

simultaneous determination of the five compounds in *S. brachyotus*.

Sample Analysis and Presumed Biosynthesis – The peak area of luteolin 7-*O*-glucuronide was considerably larger than those of other peaks. The concentration of that compound was 143 mg/g extract, which is equivalent to 18.3 mg/g dry weight (DW) of plant material and that of luteolin 7-*O*-rutinoside was 26.7 mg/g extract (3.40 mg/g DW). Compared to the content of luteolin 7-*O*-glucuronide and 7-*O*-rutinoside, the level of luteolin 7-*O*-glucoside was much lower as 1.64 mg/g extract (equivalent to 0.21 mg/g dry weight). As shown in Table 4, the levels of chlorogenic acid and luteolin were very low compared with that of luteolin 7-*O*-glucuronide and 7-*O*-rutinoside. These results indicated that luteolin was contained predominantly in the glucuronide or rutinoside forms rather than as glucosides or a free form. The two peaks of 7-*O*-glucoside and 7-*O*-glucuronide of luteolin, which were close to each other, were confirmed by a recovery test using standard compounds. As shown in Fig. 2, the identified five peaks were successfully separated in HPLC chromatogram. Relative retention times (RRTs) of the isolates were determined with a reference of luteolin 7-glucuronide and may be useful for identification, as shown in Table 5.

The enzyme UDP-glucose : luteolin 7-*O*-glucosyltransferase is known to catalyze the attachment of D-glucose to luteolin to produce luteolin 7-*O*-glucoside. Further, 1,6-rhamnosyltransferase catalyze the linkage of L-rhamnose to 6-*O*-position of D-glucose to produce rutinose (α-L-rhamnopyranosyl (1 → 6)-β-D-glucopyranose), although the biosynthesis of luteolin 7-*O*-rutinoside has not been known. In addition, another enzyme UDP-glucuronide : luteolin 7-*O*-glucuronyltransferase plays a role in the attachment of D-glucuronic acid to luteolin to produce luteolin 7-*O*-glucuronide (Bowles *et al*., 2005; Grotewold, 2008). In Table 4, the contents of

Table 6. Peroxynitrite-scavenging effect (%) of aqueous ethanol extract of *S. brachyotus* and its constituents

Treatment	Concentration (μg/ml)				IC_{50}
	10.0	2.0	0.40	0.080	
Aqueous ethanol ext.	78.20 ± 6.45[a]	59.12 ± 4.89	41.27 ± 2.77	42.64 ± 4.47	1.20 ± 0.05 μg/ml
Luteolin	96.56 ± 5.71	97.15 ± 0.27	85.85 ± 3.70	26.80 ± 9.65	0.71 ± 0.10M
Luteolin 7-*O*-glucoside	97.16 ± 0.71	88.80 ± 1.27	54.34 ± 3.41	41.01 ± 3.50	0.67 ± 0.11M
Luteolin 7-*O*-glucuronide	96.19 ± 2.17	83.36 ± 0.31	48.39 ± 1.57	11.48 ± 2.33	1.02 ± 0.08 M
Penicillamine	90.12 ± 1.02	74.45 ± 6.08	48.39 ± 1.57	11.48 ± 2.33	3.41 ± 0.42M

the two compounds, 7-*O*-glucuronide and 7-*O*-rutinoside of luteolin, were considerably higher than luteolin and its 7-*O*-glucoside, suggesting that the former two compounds may be the final products from the precursors of the latter two compounds in biosynthetic pathways. In our experiment, the two compounds, 7-*O*-glucoside and 7-*O*-glucuronide of luteolin, were successfully separated.

Peroxynitrite-Scavenging Activity – As shown in Table 6, the aqueous ethanolic extract had strong peroxynitrite-scavenging activity with an IC_{50} value of 1.20 μg/ml. The IC_{50}s of luteolin and its 7-*O*-glucoside and its 7-*O*-glucuronide were 0.71 μM, 0.67 μM and 1.02 μM, respectively, suggesting that those components in the extract individually contribute to the potent peroxynitrite-scavenging effect of the extract. However, luteolin 7-*O*-rutinoside was not tested in this experiment.

The present results indicated that luteolin 7-*O*-glucuronide, which the extract contains in high proportion, may largely contribute to the peroxynitrite-scavenging activity of the extract. Levels of luteolin, its 7-*O*-glucoside and even chlorogenic acid were considerably lower than that of luteolin 7-*O*-glucuronide and 7-*O*-rutinoside. Shimoi *et al*. (1999) reported that luteolin and its 7-*O*-glucoside are absorbed after being converted into luteolin-glucuronide and luteolin during passage through the intestinal mucosa, and are thereby present in the serum in free form or as the conjugate (mono-glucuronide). Schneider *et al*. (2000) reported that luteolin 7-*O*-glucoside can be hydrolyzed by an anaerobic bacterium, *Eubacterium ramulus*. There are many reports on the pharmacological actions of luteolin 7-*O*-glucoside: inhibition of superoxide generation in neutrophils (Lu *et al*., 2002), inhibition of protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) in lipopolysaccharide-induced macrophage RAW 264.7 cells (Hu *et al*., 2004), anti-asthmatic activity through down-regulation of helper 2 cytokine (Jin *et al*., 2009) and hepatoprotective activity (Qiusheng *et al*., 2004). Significant pharmacological activities of luteolin have also been reported: inhibition of iNOS and COX expression (Hu *et al*., 2004); anxiolytic activity (Vilela *et al*., 2009); gastroprotective activity due to inhibition of H^+-K^+-ATPase (Freitas *et al*., 2008); and inhibitory activities on α-glucosidase (Kim *et al*., 2000), monoamine oxidase (Han *et al*., 2007), and low density lipoprotein (LDL) oxidation (Brown *et al*., 1998). As for luteolin 7-glucuronide, preventive effects against reflux esophagitis and gastritis (Min *et al*., 2006), antimutagenic activity (Nagy *et al*., 2009) and antidepressant activity (Vilela *et al*., 2010) were reported. Based on the literatures cited above, luteolin and its 7-*O*-glucuronide may represent the pharmacological actions of luteolin glycosides.

The aqueous ethanolic extract possessing a potent peroxynitrite-scavenging effect could be also used in the treatment of aging-related diseases, especially those associated with overproduction of peroxynitrite, such as obesity, diabetes mellitus, and atherosclerosis. In conclusion, the aqueous ethanolic extract of *S.brachyotus* has potent peroxynitrite-scavenging effects, mainly due to its high content of luteolin 7-*O*-glucuronide. In addition, this verified HPLC analytical method could be useful for quality control of *S. brachyotus*.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (# 20100021039).

References

Agati, G., Biricolti, S., Guidic, L., Ferrini, F., Fini, A., and Tattini, M., The biosynthesis of flavonoids is enhanced similarly by UV radiation and root zone salinity in *L. vulgare* leaves. *J. Physiol.* **168**, 204-212 (2011).

Boveris, A., Validez, L., and Alvarez, S., Inhibition by wine polyphenols of peroxynitrite-initiated chemiluminescence and NADH oxidation. *Ann. N. Y. Acad. Sci.* **957**, 90-102 (2002).

Bowles, D., Isayenkova, J., Lim, E.K., and Proppenberger, B., Glycosyltransferases: managers of small molecules. *Curr. Opin. Plant*

Biol. **8**, 254-263 (2005).

Brown, J.E. and Rice-Evans, C.A., Luteolin-rich artichoke extract protects low density lipoprotein from oxidation *in vitro*. *Free. Radic. Res.* **29**, 247-255 (1998).

Coleta, M., Campos, M.G., Cotrim, M.D., Lima, T.C.M., and Cunha, A.P., Assessment of luteolin (3,4,5,7-tetrahydroxyflavone) neuropharmacological activity. *Behav. Brain. Res.* **189**, 75-82 (2008).

Drel, V.R., Patcher, P., Vareniuk, I., Pavlov, I., Lyzogulbov, V.V., Grovez, J.T., and Obrosova, I.G., A peroxynitrite decomposition catalyst counteracts sensory neuropathy in streptozotocin-diabetic mice. *Eup. J. Pharmacol.* **569**, 48-58 (2007).

Freitas, C.S., Baggio, C.H., Finau, J., Anginoni, M., Pizzolatti, M.G., Santos, A.R., and Marques, M.C., Inhibition of H^+-K^+ ATPase in the gastroprotective effect of *Baccharis illinita* DC. *J. Pharm. Pharmacol.* **60**, 1105-1110 (2008).

Gulluce, M., Karadayi, M., Guvenalp, Z., Ozbek, H., Arasoglu, T., and Baris, O., Isolation of some active compounds from *Origanum vulgare* L. ssp. *Vulgare* and determination of their genotoxic potentials. *Food Chem.* **130**, 248-253 (2010).

Han, X.H., Hong, S.S., Hwang, J.S., Lee, M.K., Hwang, B.Y., and Ro, J.S., Monoamine oxidase inhibitory components from *Cayratia japonica*. *Arch. Pharm. Res.* **30**, 12-17 (2007).

Hu, C. and Kitts, D.D., Luteolin and luteolin 7-O-glucoside from dandelion flower suppress iNOS and COX-2 in RAW 264.7 cells. *Mol. Cell. Biochem.* **265**, 107-113 (2004).

ICH. *Guideline Q2 (R1) – Validation of Analytical Procedures: Text and Methodology.* ICH: Geneva; pp. 1-13, 2005.

Jin, M., Yang, J.H., Lee, E.K., Lu, Y., Kwon, S., Son, K.H., Son, J.K., and Chang, H.W., Antiasthmatic activity of luteolin 7-*O*-glucoside from *Ailanthus altissima* through the down regulation of T helper 2 cytokine expression and inhibition of prostaglandin E_2 production in an ovalbumin-induced asthma model. *Biol. Pharm. Bull.* **32**, 1500-1503 (2009).

Khan, R.A., Khan, M.R., Sahreen, S., and Bokhari, J., Prevention of CCl_4-induced nephrotoxicity with *Sonchus asper* in rat. *Food Chem. Toxicol.* **48**, 2469-2476 (2010).

Kim, J.S., Kwon, C.S., and Son, K.H., Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. *Biosci. Biotechnol. Biochem.* **64**, 2458-2461 (2000).

Kim, T.J., *Korean Plant Resources*, Vol IV. Publishing Department of Seoul National University, Seoul, pp. 319-320 (1996).

Kooy, N.W., Royall, J.A., Ischiropoulos, H., and Beckman, J.S., Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radic. Biol. Med.* **16**, 149-156 (1994).

Korda, M., Kubant, R., Patton, S., and Malinski, T., Leptin-induced endothelial disfunction in obesity. *Am. J. Physiol. Heart. Circ. Physiol.* **295**, 1514-1521 (2008).

Lu, J., Feng, X., Sun, Q., Lu, H., Manabe, M., Sugahara, K., Ma, D., Sagara, Y., and Kodama, H., Effect of six flavonoid compounds from *Ixeris sonchifolia* on stimulus-induced superoxide generation and tyrosyl phosphorylation in human neutrophils. *Clin. Chim. Acta.* **316**, 95-99 (2002).

Min, Y.S., Bai, K.L., Yim, S.H., Lee, Y.J., Song, H.J., Kim, J.H., Ham, I.H., Whang, W.K., and Sohn, U.D., The effect of luteolin 7-O-β-D-glucuronopyranoside on gastritis and esophagitis in rats. *Arch. Pharm. Res.* **29**, 484-489 (2006).

Moon, K.S., *Constituents and Utilization of Medicinal Herbs*. Science Encyclopedia Publishing Co., Pyeongyang, 1984.

Nagy, M., Krizkova, Z., Sersen, F., and Krajcovic, J., Antimutagenic activity and radical scavenging activity of water infusions and phenolics from ligustrum plants leaves. *Molecules* **14**, 509-518 (2009).

Nugroho, A., Kim, K.H., Lee, K.R., Alam, M.B., Choi, J.S., Kim, W.B., and Park, H.J., Qualitative and quantitative determination of the caffeoylquinic acids on the Korean mountainous vegetables used for chwinamul and and their peroxynitrite-scavenging effect. *Arch. Pharm. Res.* **32**, 1361-1367 (2009).

Nugroho, A., Choi, J., Lee, K.R., Bachri, M.S., Choi, J.S., Kim, W.B., Lee, K.T., and Park, H.J., Anti-ulcerogenic effect of the caffeoylquinic acid-rich extract from *Ligularia stenocephala* and HPLC analysis. *Biol. Pharm. Bull.* **33**, 493-497 (2010).

Patcher, P., Obrosova, I.G., Mabley, J.G., and Szabo. C., Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. *Curr. Med. Chem.* **12**, 267-275 (2005).

Qiusheng, Z., Xubo, X.S., Meng, S., and Changhai, W., Protective effects of luteolin-7-glucoside against liver injury caused by carbon tetrachloride in rats. *Pharmazie* **59**, 286-289 (2004).

Radi, R., Beckman, J.S., Bush, K.M., and Freeman, B.A., Peroxynitrite oxidation of sulfhydryls, the cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266**, 4244-4250 (1991).

Schneider, H. and Blaut, M., Anaerobic degradation of flavonoids by *Eubacterium ramulus*. *Arch. Microbiol.* **173**, 71-75 (2000).

Shimoi, K., Okada, H., Furugori, M., Goda, T., Takase, S., Suzuki, M., Hara, Y., Yamamoto, H., and Kinae, N., Intestinal absorption of luteolin and luteolin 7-O-L-glucoside in rats and humans. *FEBS Letters* **438**, 220-224 (1998).

Tipce, G.L., Leung, T.M., Hung, M.W., and Fung, M.L., Green tea polyphenols as an anti-oxidant and anti-inflammatory agent for cardiovascular protection. *Cardivasc. Hematol. Disord. Drug Targets* **7**, 135-144 (2007).

Vilela, F.C., Soncini, R., and Giusti-Paiva, A., Anxiolytic-like effect of *Sonchus oleraceus* L. in mice. *J. Ethnopharmacol*. **124**, 325-327 (2009).

Vilela, F.C., Bitencourt, A.D., Cabral, L.D., Franqui, L.S., Soncini, R., and Giusti-Paiva, A., Anti-inflammatory and antipyretic effects of *Sonchus oleraceus* in rats. *J. Ethnopharmacol.* **127**, 737-741 (2010).

Vilela, F.C., Padilha-Mde, M., Alves-da-Silva, G., Soncini, R., and Giusti-Paiva, A., Antidepressant-like activity of *Sonchus oleraceus* in mouse models of immobility tests. *J. Med. Food* **13**, 219-222 (2010).

Winkel, B.S.J., The biosynthesis of flavonoids. In *The Science of Flavonoids*. Grotewold (Ed.). Springer, New York, pp. 71-95, 2008.

Yaginuma, S., Shiraishi, T., Ohya, H., Igarashi, K., Polyphenol increases in safflower and cucumber seedlings exposed to strong visible light with limited water. *Biosci. Biotechnol. Biochem.* **66**, 65-72 (2002).

Received February 8, 2012
Revised March 5, 2012
Accepted March 7, 2012