Natural Product Sciences
18(1) : 32-38 (2012)

High-Performance Liquid Chromatographic Quantification of Flavonol Glycosides in Orostachys Species

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Abstract – The herbs of *Orostachys japonicus* (Crassulaceae) have been used to treat gastric cancer, gastric ulcer or hemorrhage. Flavonoid glycosides, mainly kaempferol (Kp)- and quercetin (Qc) glycosides, have been isolated from *O. japonicus*; however, no quantitative information on those flavonol glycosides and no peroxynitrite-scavenging activity of the Orostachys extracts have been reported. In this study, Kp- and Qc glycosides were qualitatively and quantitatively analyzed by high-performance liquid chromatography (HPLC) in eight Orostachys and a Meterostachys species including *O. japonicas*, *O. margaritifolius*, *O. chongsunensis*, *O. minuta*, *O. ramosus*, *O. malacophylla*, *O. latiellipticus*, *O. iwarenge*, *O. iwarenge* for. *magnus*, and *Meterostachys sikokiana* distributed or cultivated in Korea. Distinctively, *O. margaritifolius* contained two flavonol 3,7-di-O-glycosides of Kp 3,7-di-O-glucoside and Kp 3-rhamnosyl-7-glucoside, but *O. japonicus* had two flavonol 3-O-rutinosides, Kp 3-rutinoside and Qc 3-rutinoside. The three species of *O. margaritifolius* (24.36 mg/g MeOH extract), *O. japonicus* (21.28 mg/g), and *O. minuta* (19.50 mg/g) showed relatively higher flavonoid contents. The flavonol glycosides were analyzed using eight standard compounds (Kp, Qc, Qc 3-O-rhamnoside, Qc 3-O-glucoside, Kp 3-O-rutinoside, Qc 3-O-rutinoside, Kp 3-O-rhamnosyl-7-O-glucoside, Kp 3,7-di-O-glucoside). The present HPLC method was validated to verify the linearity, precision, and accuracy. In addition, the peroxynitrite-scavenging activity was also discussed.
Keywords – *Orostachys japonicus*, Crassulaceae, flavonoids, flavonol, quantitative, HPLC

Introduction

Orostachys japonicus is a perennial herb that grows on mountain rocks or on the roofs of Korean traditional houses. In Korea, the herbs of this plant have been used to treat gastric cancer or gastric ulcer (Jung *et al.*, 1990). We previously reported the isolation of several compounds including flavonoids (Park *et al*., 1991), steroids and triterpenoids (Park *et al.*, 1991) and demonstrated that those flavonoids have anti-mutagenic activities (Park *et al.*, 1991). Flavonols or their glycosides from *O. japonicus* are: kaempferol (Kp), quercetin (Qc), afzelin, astragalin, isoquercitrin, cynaroside, Kp 3-O-rhamnosyl-7-O-glucoside (Kp-3-rha-7-glc), Kp 3,7-di-O-glucoside (Kp-3,7-di-glc) (Park *et al.*, 1991), herbacetin 8-O-α-D-ribopyranoside (Je Ma *et al.*, 2009), and gossypetin 8-O-α-D-lyxopyranoside (Sung *et al.*, 2002). Four catechin derivatives including (−)-epicatechin, (−)-epicatechin 3,5-digallate, (−)-epicatechin 3-gallate, (−)-epicatechin 5-gallate were also reported (Kim *et al.*, 2009).

Recently, we reported that the flavonoid-rich fraction from the extract of *O. japonicus* prevents the gastric ulcer induced by HCl/EtOH or indomethacin/bethanechol in mice (Jung *et al*., 2007); however, the content of the flavonoids has not been reported. From *O. japonicus*, several biological activities have been reported: human immunodeficiency virus type 1 protease inhibitory activity (Park *et al.*, 2000), anti-apoptotic activity in H_2O_2-induced cells (Yoon *et al*., 2000), antioxidant and anti-proliferative activity (Kim *et al.*, 2003), and hepatoprotective effects (Park *et al.*, 2005); however the active compounds have not been identified.

Since several species belonging to the genus Orostachys are frequently cultivated for ornamental use in Korea, those plants were also employed for quantification in the present study. The plant species used for analysis were: *O. japonicus* (Maxim) A. Berger, *O. margaritifolius*

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Y. N. Lee (Korean name, jinjubawisol), *O. chongsunensis* Y. N. Lee (jungsunbawisol), *O. minuta* (Kom.) A. Berger (jombawisol), *O. ramosus* Y. N. Lee (gajibawisol), *O. malacophylla* (Pall.) Fisch (dunggeunbawisol), *O. latiellipticus* Y. N. Lee (Pocheon bawisol), *O. iwarenge* (Makino) Hara (yeonhwabawisol), *O. iwarenge* for. *magnus* Y. N. Lee (ulleungbawisol), and *Meterostachys sikokiana* (Makino) Nakai (nanjaengibawisol). In the present study, the flavonoids of these Orostachys species were qualitatively and quantitatively compared. To verify the present HPLC method, validation of linearity, precision, and accuracy was also performed according to the ICH (International Conference on Harmonization) guidelines. In addition, the assay for peroxynitrite-scavenging activity was also performed on Orostachys species containing such flavonol glycosides.

Experimental

Instruments and Reagents – The HPLC used for the analysis was a Varian HPLC system (Walnut Creek, CA, USA) consisted of Prostar 210 solvent delivery module and Prostar 325 UV-Vis detector. A Shiseido (Chuoku, Tokyo, Japan) Capcell Pak C18 column (5 μm, 250 mm × 4.6 mm, i.d.) was used for analysis. Solvents used as mobile phases were HPLC grade (J.T.Baker, Phillipsburg, NJ, USA). Reagents for the peroxynitrite-scavenging assay were: diethylenetriaminepentaacetic acid (DTPA, Sigma Chemical Co., St. Louis, MO, USA), dihydrorhodhamine 123 (Molecular Probes, Eugene, OR, USA), and peroxynitrite (Cayman Chemicals Co., Ann Arbor, MI, USA).

Plant Material – A commercially available *O. japonicus* was purchased from Chun-Il Medicinal Herb Co. (Woosan-dong, Wonju, Korea) and used for extraction after being identified by Professor Sang-Cheol Lim (Department of Landscape and Horticulture, Sangji University, Korea). Other plant materials purchased from Simpol Co. (Beomchun-dong, Busan, Korea) were: *O. margaritifolius* Y. N. Lee (jinjubawisol), *O. chongsunensis* Y. N. Lee (jungsunbawisol), *O. minuta* (Kom.) A. Berger (jombawisol), *O. ramosus* Y. N. Lee (gajibawisol), *O. malacophylla* (Pall.) Fisch (dunggeunbawisol), *O. latiellipticus* Y. N. Lee (Pocheon bawisol), *O. iwarenge* (Makino) Hara (yeonhwabawisol), *O. iwarenge* for. *magnus* Y. N. Lee (ulleungbawisol), and *Meterostachys sikokiana* (Makino) Nakai (nanjaengibawisol). The Korean plant names were noted in the parentheses for reference. These were dried and pulverized for extraction.

Standard Compounds – Five standard compounds of kaempferol (Kp), quercetin (Qc), quercetin 3-O-α-L-rhamnopyranoside (Qc-3-rha), quercetin 3-O-β-D-glucopyranoside (Qc-3-glc), quercetin 3-O-β-rutinoside (Qc-3-rut) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA) and three standard compounds of kaempferol 3-O-β-rutinoside (Kp-3-rut), kaempferol 3-α-L-rhamnopyranosyl-7-O-β-D-glucopyranoside (Kp-3-rha-7-glc), kaempferol 3,7-di-O-β-D-glucopyranoside (Kp-3,7-di-glc) were provided by Professor Jae Sue Choi (Department of Food Science and Nutrition, Pukyong National University, Korea). Each was dissolved in MeOH and filtered through 0.50-μm syringe filter before injection.

Sample Solutions – To prepare the extract, 10.0 g of each plant material were weighed precisely and 200 ml MeOH were added to the flask and extracted at 50 °C for 6 h using ultrasonicator. The extracted solution was concentrated to dryness on a rotatory evaporator under reduced pressure, and further lyophilized on a freeze-dryer to give each extract. Sample solutions (2,000 μg/ml) were prepared by dissolving each extract in MeOH.

HPLC Condition – To prepare standard solutions, standard compounds were dissolved in MeOH using a vortex mixer and an ultrasonicator. After filtering the solutions through a syringe filter, they were injected onto the HPLC system. Two mobile phases consisted of A and B solutions, respectively. Solution A was 0.05% trifluoroacetic (TFA) acid aqueous solution and solution B was 0.05% TFA in MeOH-CH_3CN (60 : 40). Gradient elution was programmed as follows: (A)/(B) = 85/15 (0 min) → 35/65 (35 min, hold for 10 min) → 0/100 (47 min; hold for 4 min to wash the column) → 85/15 (54 min; hold for 6 min to equilibrate the column condition). Recording of the chromatogram was also programmed from 0 min to 37 min. The column temperature was 40 °C, and the flow rate was 1.00 ml/min. The wavelength (254 nm) for detection was chosen after our preliminary experiment, because that wavelength displayed good sensitivity for detection of the flavonol compounds.

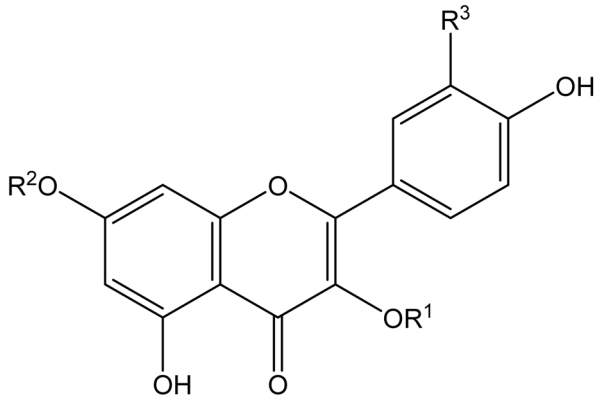
Linearity of Standard Compounds – Standard solutions were used to measure HPLC chromatograms and to establish the linearity of calibration curve equations. Eight standard solutions in the range of 1.56 - 50.00 μg/ml were prepared by serial-dilution method and injected onto the HPLC system. Linearity was assessed by the r^2 value of the regression equation plotting each peak area (y axis) versus each concentration (x axis).

LOD and LOQ – An aliquot (20 μl) of the serial dilution of the eight individual standard solutions was injected and then LOD (limit of detection) and LOQ (limit of quantification) values were determined at signal-

to-noise (S/N) ratios of 3 and 10, respectively.

Precision and Accuracy Studies – The two extracts (*O. margaritifolius* and *O. japonicus*) were analyzed by HPLC to observe intra-day and inter-day variabilities. The intra-day variability was determined by measuring contents within a day. Each extract was analyzed five times a day and the RSD values were considered a measure of accuracy. For inter-day variability, each sample was measured five times for 4 consecutive days. Accuracy was assessed by performing recovery test. The recovery rate was determined 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-scavenging activity was measured by modifying the method described by Kooy *et al.* (1994). This method monitors the fluorescence of rhodamine 123 rapidly formed from non-fluorescent DHR 123 in the presence of peroxynitrite. Rhodamine buffer solution (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 DTPA. The final concentration of DHR 123 was 5 μM. In this assay, the buffer solution was prepared before use and then stood on ice. Sample extracts were dissolved in 10% DMSO to prepare sample solutions. The intensity of final fluorescence was measured in the presence and in the absence of 10 μM peroxynitrite in 0.3N NaOH. The fluorescece intensity of oxidized DHR 123 was measured using a microplate fluorescence reader FL 500 (Bio-Tek Instruments Inc., Winooki, VT, USA) at excitation and emission wavelengths of 480 and 530 nm, respectively. Peroxynitrite-scavenging activity was determined by calculating the final fluorescence minus the background fluorescence via detection of DHR 123 oxidation and expressed as mean ± SD. L-Penicillamine was employed as a positive control.



Kp-3,7-di-glc (**1**) : R^1 = β-D-glc(*p*), R^2 = β-D-glc(*p*), R^3 = H

Kp-3-rha-7-glc (**2**) : R^1 = α-L-rha(*p*), R^2 = β-D-glc(*p*), R^3 = H

Qc-3-rut (**3**) : R^1 = β-rutinose, R^2 = H, R^3 = OH

Qc-3-glc (**4**) : R^1 = β-rutinose, R^2 = H, R^3 = OH

Kp-3-rut (**5**) : R^1 = β-rutinose, R^2 = R^3 = H

Qc-3-rha (**6**) : R^1 = α-L-rha(*p*), R^2 = H, R^3 = OH

Qc (**7**) : R^1 = R^2 = H, R^3 = OH

Kp (**8**) : R^1 = R^2 = R^3 = H

Fig. 1. Structure of eight flavonoids used for analysis of Orostachys species.

Results and Discussion

Validation of HPLC Method – In the present study, the HPLC quantification and its validation of *O. japonicus* and *O. margaritifolius* and the peroxynitrite-scavenging activity of the extracts of nine Orostachys species were pursued, considering a wide spectrum of pharmacological activities of flavonoids distributed in

Table 1. Calibration curves, detection limits and quantification limits of the analytes under UV wavelength 254 nm

Analyte	Calibration equation (linear model)[a]	Linear range (μg/ml)	R^2 [b]	LOD[c] (μg/ml)	LOQ[d] (μg/ml)
Kp-3,7-diglc	y = 175.145x + 20.07	1.56-50.00	0.9998	0.38	1.27
Kp-3-rha-7-glc	y = 249.012x + 24.29	1.56-50.00	0.9998	0.25	0.82
Qc-3-rut	y = 143.532x + 15.18	1.56-50.00	0.9999	0.42	1.34
Qc-3-glc	y = 472.749x + 75.62	1.56-50.00	0.9999	0.11	0.36
Kp-3-rut	y = 289.936x + 34.05	1.56-50.00	0.9999	0.19	0.62
Qc-3-rha	y = 458.110x + 62.59	1.56-50.00	0.9998	0.12	0.39
Qc	y = 659.275x + 87.33	1.88-60.00	0.9999	0.09	0.23
Kp	y = 456.719x + 56.76	1.56-50.00	0.9998	0.13	0.43

[a]y, peak area at 254 nm; 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).

Table 2. Analytical results of intra-day (n = 5) and inter-day variabilities (4 days, n = 5 in each day)

Analyte	Intra-day variability				Inter-day variability			
	Content (mg/g)	RSD (%)	RRT[a]	RSD[b] (%)	Content (mg/g)	RSD (%)	RRT[a]	RSD[b] (%)
Kp-3,7-diglc[1]	3.29	1.50	0.73	0.11	3.28	2.72	0.74	0.14
Kp-3-rha-7-glc[1]	3.81	1.35	0.81	0.12	3.79	2.15	0.81	0.15
Qc-3-rut[2]	6.57	1.13	0.98	0.10	6.58	1.87	0.98	0.12
Qc-3-glc[2]	3.28	1.55	1.00	0.09	3.30	2.31	1.00	0.12
Kp-3-rut[2]	6.07	0.83	1.08	0.12	6.07	1.45	1.08	0.16
Qc-3-rha[2]	2.74	1.89	1.10	0.12	2.75	2.49	1.11	0.17
Qc[2]	1.10	1.71	1.31	0.15	1.11	2.57	1.31	0.19
Kp[2]	1.52	1.61	1.47	0.16	1.52	2.46	1.48	0.19

[a]RRT: relative retention time, [b]RSD of retention time of the analyte, [1]Quantified in MeOH extract of *O. margaritifolius*; [2]Quantified in MeOH extract *O. japonicus*.

Table 3. Recovery of each analyte by addition of standard compounds to sample solutions (n = 3)

Analyte	Initial concentration (μg/ml)	Amount added (μg)	Concentration after addition (μg/ml)		Recovery (%)	RSD (%)
			Expected	Measured		
Kp-3,7-diglc[1]	12.25	12.50	24.75	23.87	96.44	1.48
Kp-3-rha-7-glc[1]	14.18	12.50	26.68	26.13	97.94	1.26
Qc-3-rut[2]	8.48	6.25	14.73	15.03	102.04	1.16
Qc-3-glc[2]	4.23	3.13	7.36	7.55	102.58	1.09
Kp-3-rut[2]	7.84	6.25	14.09	13.78	97.80	1.24
Qc-3-rha[2]	3.53	3.13	6.66	6.85	102.85	1.21
Qc[2]	1.42	1.88	3.30	3.47	105.15	1.05
Kp[2]	1.97	1.56	3.53	3.68	104.25	1.11

[1]Quantified in MeOH extract of *O. margaritifolius*; [2]Quantified in MeOH extract *O. japonicus*.

Table 4. Content of analytes in MeOH extract of samples

Sample	Content of analyte in MeOH extract of plant material (mg/g)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Total**
O. japonica	-	-	6.57(1.13)	3.28(1.55)	6.07(0.83)	2.74(1.89)	1.10(1.71)	1.52(1.61)	21.28
O. margaritifolius	3.29(1.50)	3.81(1.35)	-	5.12(1.20)	-	10.85(0.73)	0.50(1.26)	0.79(2.59)	24.36
O. chongsunensis	-	-	2.51(1.26)	0.72(2.04)	0.79(1.86)	0.12(3.96)	0.05(3.92)	-	4.19
O. minuta	-	-	1.93(1.26)	10.84(0.86)	-	4.66(0.93)	1.62(1.72)	0.45(2.77)	19.50
O. ramosus	-	-	1.92(2.30)	0.36(3.01)	0.95(2.09)	-	0.17(3.34)	0.24(3.15)	3.64
O. malacophylla	-	-	7.37(0.90)	0.55(2.85)	-	-	0.25(3.17)	-	8.17
O. latiellipticus	-	-	-	2.39(1.26)	1.43(2.18)	-	-	-	3.82
O. iwarenge	-	-	2.13(1.33)	-	-	-	-	-	2.13
O. iwarenge magnus	-	-	1.03(1.71)	-	2.24(1.56)	-	-	0.63(1.98)	3.90
M. sikokiana	-	2.21(1.66)	-	1.83(1.51)	-	-	-	-	4.04

Value in parentheses are RSD (%) for n = 3; (-) not identified in the extract.

numerous plants. In particular, the present quantification experiment was performed because of the highly reported anticancer- (Sreelatha *et al.*, 2011) and anti-ulcerogenic (Gurbuz *et al.*, 2009) activities of flavonoids. Eight Orostachys species were simultaneously analyzed including *O. japonicus* and one *Meterostachys sikokiana*, since they are taxonomically related with *O. japonicus*.

The structure of the eight standard compounds used are

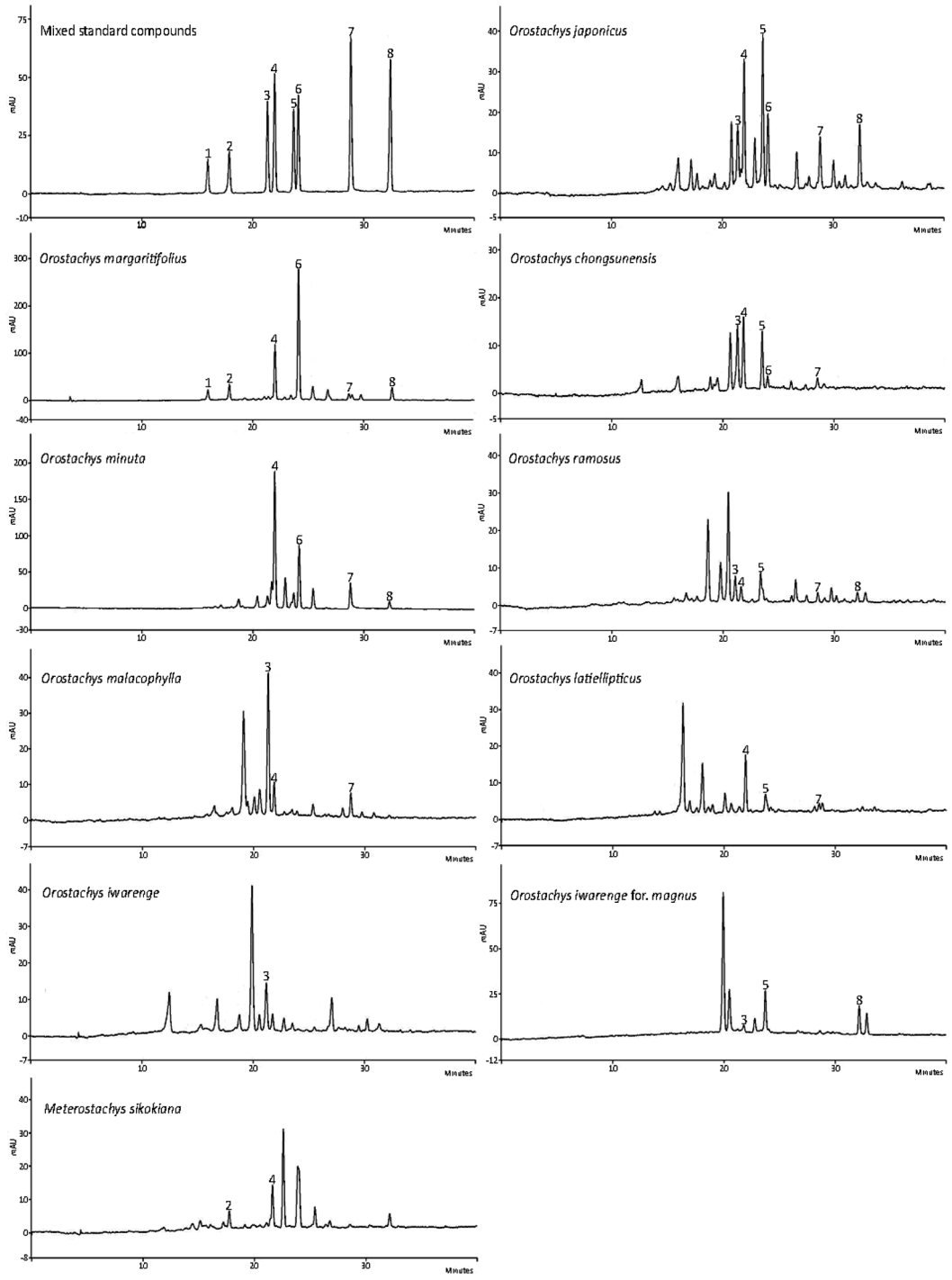


Fig. 2. HPLC Chromatograms of a mixed standard solution and sample solutions of Orostachys extracts. (**1**) Kp-3,7-di-glc; (**2**) Kp-3-rha-7-glc; (**3**) Qc-3-rut; (**4**) Qc-3-glc; (**5**) Kp-3-rut; (**6**) Qc-3-rha; (**7**) Qc; (**8**) Kp.

shown in Fig. 1. Linearity of calibration curve equations of standard compounds were established in the range of 1.56 - 50.00 μg/ml based on the r^2 values more than 0.999 (Table 1). The present HPLC method was evaluated as sensitive for detection and quantification from the LOD values < 0.38 μg/ml and LOQ values < 1.34 μg/ml.

The intra-day and inter-day variabilities are shown in Table 2. The variabilities of Kp-3,7-di-Glc and Kp-3-rha-7-glc were analyzed in the *O. margaritifolius* extract and those of the other six compounds were analyzed in the *O.*

japonicus extract. The precision was verified from the RSD value of intra-day variability over 0.83 - 1.89% and that of inter-day variability over 0.12 - 0.19%. The results of recovery test are shown in Table 3. The accuracy of the method was also verified from the recovery rate ranging from 96.44 - 105.15%.

HPLC Quantification – The contents of the tested compounds in Orostachys species are shown in Table 4. The two flavonol 3,7-di-O-glycosides (bisdesmosides) (Kp-3,7-di-Glc and Kp-3-rha-7-glc) were not observed in *O. japonicus* but the other six flavonoids were present; however, the two flavonol 3,7-di-O-glycosides were present in *O. margaritifolius*. On the other hand, the two flavonol 3-O-rutinosides (monodesmosides) of Kp-3-rut and Qc-3-rut were found in *O. japonicus* but not in *O. margaritifolius*. The moiety of 3-O-rutinoside fully refers to 3-O-α-L-rhamnopyranosyl (1 → 6)-β-D-glucopyranoside. By calculating the sum of the flavonoid contents, three extracts containing relatively high quantity of flavonoids are: *O. margaritifolius* (24.36 mg/g), *O. japonicus* (21.28 mg/g), and *O. minuta* (19.50 mg/g). Other extracts contained considerably smaller quantities of flavonoids than those three extracts. The flavonoids contained in high quantity were: Qc-3-rha (10.85 mg/g) in *O. margaritifolius* and Qc-3-glc (10.84 mg/g) in *O. minuta*. The contents (mg/g) in dry weight including the extract yield are shown in Table 5. HPLC chromatograms of the nine extracts are also shown in Fig. 2 for the comparison of HPLC chromatographic patterns.

Peroxynitrite-Scavenging Effect -Results of the assay for peroxynitrite-scavenging activity of the MeOH extracts are shown in Table 6. The IC_{50} values were higher than the IC_{50} of the positive control (L-penicillamine). Relatively higher activities were observed in *O. japonicus* (IC_{50}, 5.49 μg/ml), *O. margaritifolius* (7.96 μg/ml) and *O. minuta* (5.56 μg/ml). Overproduction of peroxynitrite (ONO_2^-) formed from the reaction between superoxide anion radical and nitric oxide (Radi *et al.*, 1991) causes aging-related disease such as obesity, diabetes mellitus and atherosclerosis (Korda *et al.*, 2008; Patcher *et al.*, 2005; Drel *et al*., 2007). There is an example in which Kp and Qc have been identified from the plant extracts with anticancer activity. A wide spectrum of pharmacological activities of Kp and Qc have been reported. The *in vivo* anti-ulcerogenic activity of a flavonol glycoside isolated from *Equisetum palustre* was reported (Gurbuz *et al.*, 2009). Under the assumption that flavonol glycosides are hydrolyzed in the gastrointestinal tract or hepatic tissue, the traditional use of *O. japonicus* for gastric cancer or gastric ulcer would partly depends on

Table 5. Content of analytes in the dry weight of plant materials

Sample	Extract yield (%)	Content of analyte in the dry weight of plant material (mg/g)								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Total**
O. japonica	7.05	-	-	0.46	0.23	0.43	0.19	0.08	0.11	1.50
O. margaritifolius	14.32	0.47	0.55	-	0.73	-	1.56	0.07	0.11	3.49
O. chongsunensis	40.81	-	-	1.02	0.30	0.32	0.05	0.02	-	1.71
O. minuta	16.67	-	-	0.32	1.81	-	0.78	0.27	0.07	3.25
O. ramosus	29.67	-	-	0.57	0.11	0.28	-	0.05	0.07	1.08
O. malacophylla	26.32	-	-	1.94	0.14	-	-	0.07	-	2.15
O. latiellipticus	9.16	-	-	-	0.22	0.13	-	-	-	0.35
O. iwarenge	35.68	-	-	0.76	-	-	-	-	-	0.76
O. iwarenge magnus	17.95	-	-	0.19	-	0.40	-	-	0.11	0.70
M. sikokiana	18.07	-	0.40	-	0.33	-	-	-	-	0.73

RSD values are same with the data of content of analytes in MeOH extracts (Table IV).

Table 6. Peroxynitrite-scavenging effect (%) of the MeOH extract of Orostachys species

Extract	IC_{50} (μg/ml)	Extract	IC_{50} (μg/ml)
O. japonicus	5.49 ± 0.38	*O. malacophylla*	21.48 ± 1.56
O. chongsunensis	16.00 ± 1.17	*O. iwarenge*	32.07 ± 0.48
O. minuta	5.56 ± 0.27	*O. iwarenge for. magnus*	25.70 ± 0.62
O. margaratifolius	7.96 ± 0.23	Penicillamine	0.83 ± 0.08
O. ramosus	24.93 ± 2.19		

the content of these flavonol glycosides in the extract.

Acknowledgements

This research was supported by the Sangji University Research Fund, 2010.

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