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Quantitative Determination of Flavonoids from Stems of *Spatholobus suberectus*†

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Abstract – A simple reversed phase HPLC method was established for quantitative determination of liquiritigenin (**1**), genistein (**2**), isoliquiritigenin (**3**), and 7-hydroxyflavanone (**4**) from stems of *Spatholobus suberectus* Dunn (Leguminosae) using a binary gradient of H_2O and MeOH as a mobile phase with UV detection at 280 nm. All calibration curves showed good linear regression ($r^2 > 0.998$) within test ranges. The detection limits of the four compounds were 0.43 ~ 1.63 μg/mL. The contents of four flavonoids (**1 - 4**) from the stem of *S. suberectus* were 6.54 mg/g, 1.66 mg/g, 6.65 mg/g, and 1.93 mg/g, respectively.
Keywords – *Spatholobus suberectus*, Quantitative determination, Flavonooids

Introduction

Spatholobi Caulis is the stem of *Spatholobus suberectus* Dunn (Leguminosae), which is known in traditional Korean Medicine as 'Gye-hyeol-deung' and in traditional Chinese Medicine as 'Ji-xue-teng'. It has been used in Korean folk medicine for improvement of blood circulation and treatment of dysmenorrheal, anemia, paralysis, and arthralgia (Kim *et al.*, 1995). This herbal drug is mainly distributed throughout Korea and China.

The ethyl acetate fraction of Spatholobi Caulis has been reported to have an effect on tumor cell aggregation and migration (Kang *et al.*, 2003), and the methylene chloride fraction of Spatholobi Caulis has been reported to induce apoptosis *via* a caspase dependent pathway in U937 cells (Ha *et al.*, 2004). Previous studies have shown that the stem of this plant is composed mainly of flavonoids, sterols, and triterpenes (Cui *et al*., 2002; Lin *et al*., 1989; Zhu *et al*., 1988). Although several flavonoids have been reported, only a few quantitative studies of their chemical constituents have been conducted. However, no general studies on the variety of flavonoids have been conducted. Thus, we initiated a quantitative study of the various flavonoids. Among the compounds, liquiritigenin (**1**), genistein (**2**), isoliquiritigenin (**3**), and 7-hydroxyflavanone (**4**) were selected for the quantitative study because **1** is known to be a major compound in this species and **2 - 4** showed more potent 20S proteasome inhibition activity than other flavonoids in our previous study (Shim, 2011). Herein we report the contents of liquiritigenin (**1**), genistein (**2**), isoliquiritigenin (**3**), and 7-hydroxyflavanone (**4**) from the stems of *S. suberectus* by HPLC method using a reverse phase C-18 column. A chromatographic system was used for isolation of four flavonoids, and their chemical structures were determined by comparison of their spectroscopic parameters of 1D-NMR with those reported in the literature.

Experimental

General – The chromatographic system used for quantitative analysis consisted of an Agilent Series 1200 liquid chromatography, equipped with a G1322A vacuum degasser, G1311A quaternary pump, G1315D DAD detector, G1328B manual injector, and G1316A thermostatted column compartment, connected to Agilent ChemStation software. A phenomenex Luna C18 column (250 × 10 mm, 5 μm) was used for the analysis. Methanol (Fischer scientific) and water (Fisher scientific) used in this study were of HPLC grade and other reagents were of analytical grade.

Plant material – Stems of *S. suberectus* were purchased from the Daegu pharmacopoeia market in South Korea. Four flavonoids were isolated and purified in our laboratory.

Isolation of standard compounds – Air-dried stems of *Spatholobus suberectus* (1 kg) were finely cut and extracted three times (each 3 h) with refluxing methanol.

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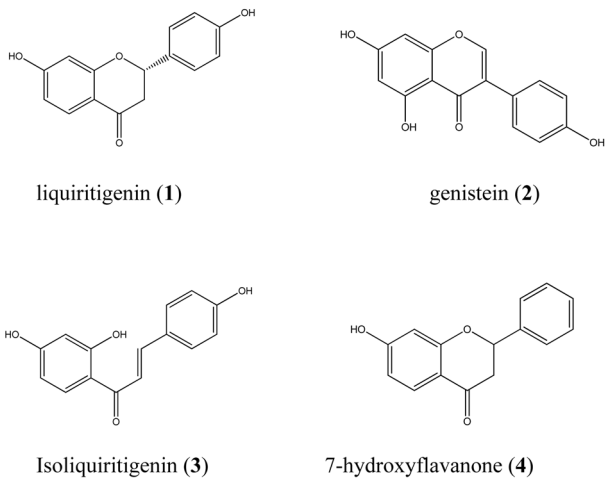

Fig. 1. Structures of liquiritigenin (**1**), genistein (**2**), isoliquiritigenin (**3**), and 7-hydroxyflavanone (**4**) isolated from *S. suberectus.*

Methanolic extract was obtained by evaporation of the solvent under reduced pressure, followed by successive partitioning between H_2O and *n*-hexane, $CHCl_3$ and EtOAc. The $CHCl_3$-soluble extract (6.9 g) was submitted to open column chromatography on silica gel, using mixtures of hexane and ethyl acetate of increasing polarity as the mobile phase. This gave fractions 1 ~ 32. Recrystallization of fraction 18 (87.5 mg) in MeOH yielded 7-hydroxyflavanone (**4**, 3.6 mg) (Tranrisever *et al*., 1986). The ethyl acetate-soluble extract (25.5 g) was subjected to open column chromatography over silica gel (hexane - ethyl acetate with increasing polarity), yielding fractions 1 ~ 41. Among them, fraction 30 (22.8 mg) was subjected to semi-preparative reverse-phase HPLC (Perkin Elmer 200 series; Agilent ZORBAX Eclipse XDB-C18 9.4 × 250 mm 5-micron column; flow rate, 2 mL/min; 60% to 80% MeOH in H_2O for 20 min, followed by 80% to 100% MeOH in H_2O for 20 min; UV detection at 254 nm) to afford liquritigenin (**1**, 3.5 mg, t_R = 9.4 min) (Youssef *et al*., 1998), genistein (**2**, 2.5 mg, t_R = 13.2 min) (Yoon *et al*., 2004), and isoliquiritigenin (**3**, 1.5 mg, t_R = 15 min) (Markham *et al*., 1976) (Fig. 1).

Liquiritigenin (**1**) – yellow amorphous powder; ^{1}H-NMR (CD_3OD, 300 MHz): δ 7.73 (1H, d, *J* = 8.7 Hz, H-5), 7.33 (2H, dd, *J* = 7.2 , 0.3 Hz, H-2', 6'), 6.82 (2H, dd, *J* = 6.6, 2.1 Hz, H-3', 5'), 6.50 (1H, dd, *J* = 8.7, 2.1 Hz, H-6), 6.35 (1H, d, *J* = 2.1 Hz, H-8), 5.38 (1H, dd, *J* = 13.2, 3.0 Hz, H-2), 3.06 (1H, dd, *J* = 16.8, 12.9 Hz, H-3$_{ax}$), 2.69 (1H, dd, *J* = 16.8, 3.0 Hz, H-3$_{eq}$).

Genistein (**2**) – white amorphous powder; ^{1}H-NMR (CD_3OD, 300 MHz): δ 8.05 (1H, s, H-2), 7.36 (2H, d, *J* = 8.7 Hz, H-2', 6'), 6.84 (2H, d, *J* = 8.7 Hz, H-3', 5'), 6.32 (1H, d, *J* = 1.8 Hz, H-8), 6.20 (1H, d, *J* = 1.8 Hz, H-6).

Isoliquiritigenin (**3**) – yellow amorphous powder; ^{1}H-NMR (CD_3OD, 300 MHz): δ 7.97 (1H, d, *J* = 9.0 Hz, H-5), 7.79 (1H, d, *J* = 9.0 Hz, H-3), 7.62 (1H, d, *J* = 15.0 Hz, H-2), 7.62 (2H, d, *J* = 8.7 Hz, H-2', 6'), 6.84 (2H, d, *J* = 8.7 Hz, H-3', 5'), 6.84 (1H, d, *J* = 8.7 Hz, H-6), 6.28 (1H, d, *J* = 2.4 Hz, H-8).

7-Hydroxyflavanone (**4**) – white amorphous powder; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.84 (1H, d, *J* = 8.4 Hz, H-5), 7.36 – 7.47 (5H, m, H-2', 3', 4', 5', 6'), 6.52 (1H, d, *J* = 8.7 Hz, H-6), 6.45 (1H, d, *J* = 2.1 Hz, H-8), 5.45 (1H, dd, *J* = 13.0, 3.0 Hz, H-2), 3.02 (1H, dd, *J* = 17.1, 13.1 Hz, H-3$_{ax}$), 2.82 (1H, dd, *J* = 16.8, 3.3 Hz, H-3$_{eq}$).

Preparation of test sample – Air-dried stems of *S. suberectus* (10 g) were extracted three times for 3h with MeOH (100 mL) under reflux and evaporated *in vacuo*. Three mg of methanol extracts were diluted with 1mL H_2O-MeOH (1 : 1 v/v). The concentration of the sample was 3 μg/μL. A volume of 30 μL was injected into the HPLC system.

HPLC analysis – Extracts from stems of *S. suberectus* were analyzed in an Agilent Series 1200. The mobile phase was composed of water and methanol. Elution was initially performed with water-methanol (100 : 0), which was changed to linear gradient over a period of 50 min to water-methanol (0 : 100). The flow rate was 1.5 mL/min and 30 μL of samples were injected for analysis. UV detection was performed at 280 nm.

Calibration curve – Stock solutions (1 mg/mL) of liquiritigenin (**1**), genistein (**2**), isoliquiritigenin (**3**), and 7-hydroxyflavanone (**4**) isolated from stems of *S.suberectus* were prepared individually in water-methanol (1 : 1), and different concentrations (0.2, 0.1, 0.05, 0.025, 0.0125 mg/mL) of these compounds were loaded onto an HPLC for preparation of the calibration functions. The calibration functions of compounds **1 - 4** were calculated with peak area (*y* axis), concentration (*x* axis, mg/mL), and mean valued (n = 3) ± standard deviation.

Limit of detection (LOD) and limit of quantification (LOQ) – LOD = 3.3 × (SD of the response/slope of calibration curve). LOQ = 10 × (SD of the response/slope of calibration curve)

Recovery test – To evaluate the accuracy, a recovery test was performed by adding three different concentrations of known standard (high, medium, and low) to samples. Recovery (%) = (total amount after spiking original amount in sample)/spiked amount × 100

Results and Discussion

For simultaneous determination of four bioactive

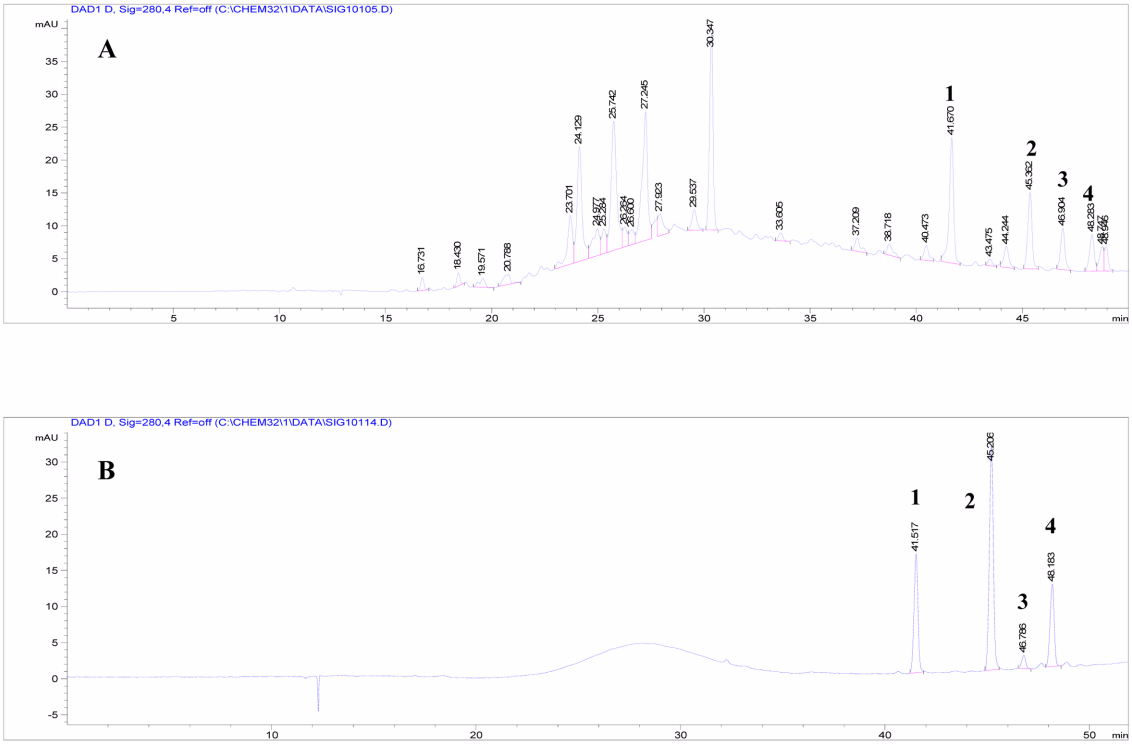

Fig. 2. HPLC chromatograms of the extract of *S. suberectus* (A) and standard mixture (B).

flavonoids in the stems of *S. suberectus*, the optimal chromatographic conditions were investigated. The optimal mobile phase composition for analysis of liquiritigenin (**1**), genistein (**2**), isoliquiritigenin (**3**), and 7-hydroxyflavanone (**4**) from the MeOH extracts of the stems of *S. suberectus* was selected by performance of several HPLC runs with various concentrations of methanol in water as a mobile phase. A solution of the initial 100% water, which was changed gradually over a period of 50 min to 100% methanol, was selected as the mobile phase.

The standard compounds were used for verification of HPLC peaks of liquiritigenin (**1**), genistein (**2**), isoliquiritigenin (**3**), and 7-hydroxyflavanone (**4**) contained in each stem of *S. suberectus*. Confirmation was provided by the spiking test. The retention times of liquiritigenin (**1**), genistein (**2**), isoliquiritigenin (**3**), and 7-hydroxyflavanone (**4**) were 41.67, 45.36, 46.90, and 48.28 min in the analytical HPLC system, respectively (Fig. 2). All measurements were performed in triplicate. The peak area (y) of analytes was measured and plotted against the concentration (x) of analytes after HPLC analysis. The regression equations of each calibration curve of the compounds (**1** ~ **4**) were as follows: $y = 4291.6094x - 1.3005$, $y = 8994.7061x - 3.2392$, $y = 1228.3270x + 0.4378$, and $y = 3275.6307x - 32.4879$, respectively (Fig. 3). The correlation coefficients of each calibration curve were

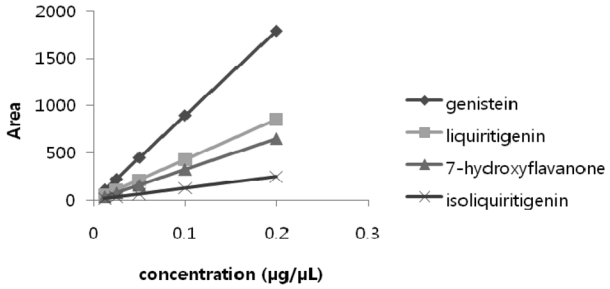

Fig. 3. Calibration curves of liquiritigenin, genistein, isoliquiritigenin, and 7-hydroxy flavanone.

0.99986, 0.99990, 0.99971, and 0.99958, respectively. Good linearity was achieved between peak areas, and concentrations of the four flavonoids ranged from 5.0 to 50.0 μg/mL .

Under the above HPLC conditions, the detection limits (LOD) of liquiritigenin (**1**), genistein (**2**), isoliquiritigenin (**3**), and 7-hydroxyflavanone (**4**) were 1.28, 0.43,1.63, and 1.27 μg/mL (UV_{280} nm), respectively, and the quantification limits (LOQ) were 3.88, 1.32, 4.93, and 3.85 μg/mL (UV_{280} nm), respectively. The extraction recovery test was performed by extracting a known amount of compounds 1 - 4 from Spatholobi Caulis sample. The % recovery of standard ranged from 95.3 to 110.0%, and the RSD was less than 1.0% (Table 2).

Table 1. Calibration graphs, linear ranges, LOD, LOQ, and content of compounds **1** ~ **4**

Compounds	Linear range (μg/mL)	Slope (a)	Intercept (b)	Correlation Coefficient (r^2)	LOD (μg/mL)	LOQ (μg/mL)	Content (mg/g)
1	5 ~ 50	4291.6904	−1.3005	0.99986	1.28	3.88	6.54 ± 0.59
2	5 ~ 50	8994.7061	−3.2392	0.99990	0.43	1.32	1.66 ± 0.26
3	5 ~ 50	1228.3270	+0.4378	0.99971	1.63	4.93	6.65 ± 1.18
4	5 ~ 50	3275.6307	−2.4879	0.99958	1.27	3.85	1.93 ± 0.42

Table 2. Recovery of compounds **1 - 4** through standard addition

Compound	Spiked concentration (μg/ml)	Detected concentration (μg/ml)	Recovery (%)	RSD (%)
liquiritigenin (**1**)	12.5	13.75	110.0	0.3
	6.25	6.06	97.0	1.0
	3.13	2.98	95.3	0.5
genistein (**2**)	50	52.7	105.4	0.5
	25	26.07	104.3	0.6
	12.5	12.44	99.5	0.3
isoliquiritigenin (**3**)	100	108.6	108.6	0.5
	50	52.3	104.6	1.0
	25	23.85	95.4	0.9
7-hydroxyflavanone (**4**)	50	49.85	99.7	0.8
	6.25	6.36	101.8	0.6
	3.13	3.10	99.2	0.3

Results are summarized in Tables 1 and 2. Contents of the four flavonoids (**1** ~ **4**) in the stems of *S. suberectus* were 6.54 ± 0.59, 1.66 ± 0.26, 6.65 ± 1.18, and 1.93 ± 0.42 mg/g, respectively.

A rapid and optimized chromatographic method with UV detection was designed for use in monitoring of the quality of flavonoids from the stems of *S. suberectus*. Validation data indicated that the developed analytical methods are suitable to measure the concentration of four flavonoids in Spatholobi Caulis. The most abundant compound was isoliquiritigenin. The developed HPLC/UVD method for quantitative analysis of major flavonoids provides a promising prospect for comprehensive quality control of Spatholobi Caulis and its related herbal medicines.

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