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Quantitative Analysis of the Isoflavone Content in the Flower and the Root of *Pueraria thunbergiana* before and after Acid Hydrolysis

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Abstract – The contents of isoflavones and 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activities were determined in flowers (Puerariae Flos) and roots (Puerariae Radix) of *Pueraria thunbergiana* to differentiate both pharmacochemical or pharmacognostical usage of both oriental medicinal drugs. The tectorigenin contents in flower before and after acid hydrolysis were shown to be 17.10 μmole g^{-1} and 49.58 μmole g^{-1}, respectively, analyzed by HPLC. However, the root displayed much less content of tectorigenin even after acid hydrolysis (6.56 μmole g^{-1}) than in the flower. Acid hydrolysis made glycitin and tectoridin of the isoflavone glycosides almost disappear in the flower and root. DPPH assay results demonstrated that acid hydrolysis of both extracts or both glycosides should increase free radical-scavenging activities due to the increase of isoflavone contents. It is also suggested that the flower had much higher concentration of isoflavonoids than in the root.

Keywords – *Pueraria thunbergiana*, Leguminosae, isoflavone, tectorigenin, HPLC

Introduction

The *Pueraria thunbergiana* (Leguminosae) has been widely employed in oriental medicine. The flowers (Puerariae Flos) of this plant have been traditionally used to treat diabetes mellitus and alcoholic toxicity although the roots (Puerariae Radix) are much more widely used as antipyretics and analgesics for the treatment of the common cold (Kim, 1996).

It has been previously reported that the flower of *P. thunbergiana* protects mouse fibroblasts against immunologically stimulated hydrogen peroxide damage (Yoshikoshi *et al*., 1996), and that the root does against hepatocyte injury (Arao *et al*., 1997). The isoflavonoids such as kakkalide, irisolidone, genistein, and daidzein were isolated from the roots of *P. thunbergiana* (Kurihara *et al*., 1975; Kurihara *et al*., 1973; Kubo *et al*., 1975). We previously reported other isoflavones and their glycosides from the flower of *P. thunbergiana*: tectorigenin, glycitin, tectoridin, glycitein, 6"-O-xylosyltectoridin and 6"-O-xylosylglycitin etc. (Park *et al*., 1999). Tectorigenin (Fig. 1) is a component of the flower of *P. thunbergiana*, although it is contained mainly in a glycosidic form (isoflavone glycoside) rather than as an aglycone (isoflavone) (Park *et al*., 1999).

We also previously reported that the isoflavonoids in the flower of *P. thunbergiana* have shown several biological activities, including hypoglycemic (Lee *et al*., 2000), antimutagenic (Park *et al*., 2002), and in vitro antioxidant effects (Lee *et al*., 1999). Our previous biological assay results also revealed that tectorigenin functions as an inhibitor of diabetes mellitus in streptozotocin-induced diabetic rat (Choi *et al*., 2003). Since both isoflavone glycosides (tectoridin and glycitin) can be converted to the aglycone forms (tectorigenin and glycitein) in the gastrointestinal tract (Bae *et al*., 1999), total tectorigenin and glycitein as ingestive isoflavones should be measured after hydrolysis of the extract. In the present study, the isoflavonoid contents and DPPH (1,1-diphenyl-2-picryl hydrazyl) radical-scavenging activities in both flower and root were comparatively analyzed before and after acid hydrolysis. A significant correlation was found between the contents of isoflavones and free radical-scavenging activities in MeOH extract, and its acid-hydrolysate of isoflavones.

Experimental

Instruments and reagents – HPLC was performed on Varian HPLC system (Walnut Creek, CA, USA) that

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includes Prostar 210 solvent delivery module, Prostar 325 UV-Vis detector and 20 μL sample loop (Rheodyne, Rohnert Park, CA, USA). Separation was achieved on Shiseido (Chuoku, Tokyo, Japan) Capcell Pak C18 column (5 μL, 250 mm × 4.6 mm I.D.), and injection syringe was purchased from Hamilton (Reno, Nevada, USA). All the solvents used for analysis were the HPLC grade obtained from J.T Baker (Phillipsburg, NJ, USA).

Plant material – The root and flower of *Pueraria thunbergiana* (Leguminosae) were collected on September, 2007 in Mt. Chiak, Gangwon-do, Korea. The collected plant parts were dried and pulverized for HPLC analysis

Extraction – One gram of the pulverized plant materials were correctly weighed on a chemical balance. Each test plant material (1 g) added in the test tube was sonicated in MeOH (40 ml) at 40 °C for 2 h, and the extract was filtered and concentrated to dryness on a rotatory evaporator at 60 °C and finally a freeze dryer at –40 °C. The concentrated extract was dissolved in 80% aqueous MeOH and filtered through a 0.50 μm syringe filter, and the filtrate (20 μL) was injected into analyzer.

Acid hydrolysis – One gram of the pulverized plant material was sonicated in 50% aqueous MeOH (40 ml) at 40 °C for 2 h and was filtered. To the filtrate H_2SO_4 was added to a final concentration of 1% (w/v), and the mixture was stirred with magnetic bar for 40 min. The extract was concentrated to dryness on a rotatory evaporator, dissolved in 80% aqueous MeOH and filtered through a 0.50 μm syringe filter. The filtrate (20 μL) was loaded onto HPLC system.

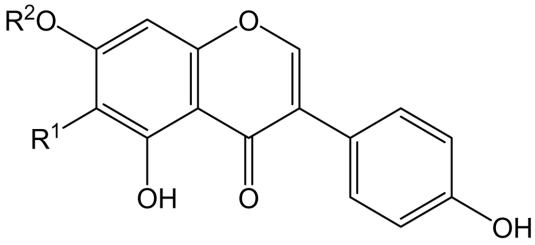
Standards and calibration curves – 1,1-Diphenyl-2-picryl hydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glycitin, tectoridin, glycitein, and tectorigenin were isolated from the flower of *P. thunbergiana* as previously reported (Park *et al.*, 1999). The isolated compounds were verified by comparison of the physicochemical data (mp, $[\alpha]_D$, ^{1}H-NMR and ^{13}C-NMR) with the published data (Park *et al.*, 1999). The purity of those compounds was approximately 90%. All standards were dissolved in 80% aqueous methanol. Standard curves were plotted using 5, 10, 25, and 50 μg g^{-1} with high linearities of $R^2 > 0.99$ (Table 1). The concentrations were calculated by comparing the peak areas of samples with those of the standards. The structures of standard compounds are shown in Fig. 1.

HPLC condition for isoflavones analysis – The samples and standard compounds were dissolved in 80% aqueous MeOH and were filtered through a 0.50 μm syringe before injection. The UV detector was fixed at 254 nm. The mobile phase was a mixed solvent of 0.05% phosphoric acid (solvent A) and acetonitrile (solvent B) in water. The gradient system was: 0 min, 95% A : 5% B; 0-20 min, 55% A : 45% B; 20 - 40 min, 20% A : 80% B; 40 - 50 min, 0% A : 100% B; 50 - 51 min, 95% A : 5% B; 51 - 60 min, 95% A : 5% B. Chromatography was performed at the flow rate of 1.00 mL min^{-1} in 50 min.

Table 1. Calibration curve equation of the four isoflavonoids used for HPLC analysis

Flavonoids	Retention time (min)	Regression Equation	
Glycitin	12.6	y = 592.4x + 2248.	R^2 = 0.997
Tectoridin	14.5	y = 836.4x + 3735.	R^2 = 0.997
Glycitein	18.6	y = 539.6x + 541.7	R^2 = 0.999
Tectorigenin	21.6	y = 839.9x + 4793.	R^2 = 0.996

y (area, μV), x (concentration, μg mL^{-1})



	R^1	R^2
Tectorigenin	OCH_3	H
Glycitein	H	H
Tectoridin	OCH_3	β-D-Glc(*p*)
Glycitin	H	β-D-Glc(*p*)

Fig. 1. Structure of isoflavonoids used as standard compounds for HPLC analysis.

Measurement of radical-scavenging activity by DPPH – One gram of freeze-dried pulverized sample was mixed with 100 mL of 80% aqueous methanol and was stirred for 24 h at 24 °C. The extract was filtered through Whatman No. 42 paper, evaporated at below 50 °C, and freeze-dried at –40 °C. The residues were dissolved in DMSO to make 1% solution (w/v) and filtered through a 0.45 μm membrane filter (Nylon, TITAN). The free radical scavenging activity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the method of Oh *et al* (2005). Each 0.25 mL of sample solution was mixed with 2.5 mL of 0.35 mM DPPH in 50% ethanol, and the mixtures were left for 30 min at room temperature in the dark. The DPPH value was measured at 517 nm with an UV-VIS spectrophotometer (Hitachi, Tokyo, Japan). DPPH activity was calculated as an inhibition

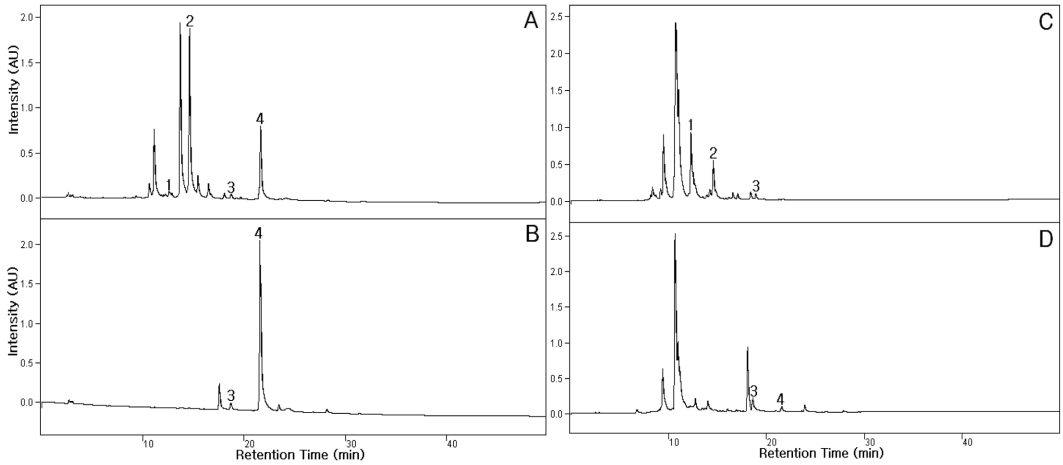


Fig. 2. HPLC chromatograms of the MeOH extract of *P. thunbergiana* flower (A) and its acid hydrolysate (B) and of the MeOH extract of *P. thunbergiana* root (C) and its acid hydrolysate (D).
1 (Glycitin), **2** (Tectoridin), **3** (Glycitein), and **4** (Tectorigenin).

Table 2. Isoflavonoid contents in the MeOH extract of the flower and root of *Pueraria thunbergiana*

Plant Tissue	Type of Extract	Isoflavone			
		Glycitin	Tectoridin	Glycitein	Tectorigenin
Flower	MeOH extract	0.75 ± 0.14[a,b]	25.27 ± 3.58	2.07 ± 1.32	17.10 ± 0.67
	Acid hydrolysate	nd[c]	nd	3.84 ± 0.14	49.58 ± 3.51
Root	MeOH extract	5.27 ± 0.45	6.41 ± 0.40	3.36 ± 0.50	nd
	Acid hydrolysate	nd	nd	9.96 ± 0.90	6.56 ± 0.51

[a] Values represent mean ± S.D. based on three experiments, [b] Reported in μmol g^{-1}, [c] nd : not detected.

percentage based on the following equation:

$$\text{Free radical scavenging activity (\%)} = (1 - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100$$

Results and Discussion

Quantification of isoflavonoids in MeOH extract and its acid-hydrolysate – The HPLC patterns of isoflavonoids in the flower extract (A) and its acid-hydrolysate (B) and in the root extract (C) and its acid-hydrolysate (D) are shown in Fig. 2. The contents of four isoflavonoids quantitatively determined by HPLC are listed in Table 2. The flower contained all four types of isoflavonoid, among which tectoridin was accumulated at the highest level (25.27 μmol g^{-1}). However, tectorigenin level (49.58 μmol g^{-1}) was highest in acid hydrolysate, and this value was higher than the sum (42.37 μmol g^{-1}) of tectoridin and tectorigenin before acid hydrolysate extract, which suggests that other glycosides were also contained. It was also observed that the isoflavone glycosides (glycitin and tectoridin) were disappeared in both acid hydrolysate due to the chemical conversion. The tectoridin content in the root of *P. thunbergiana* was 6.41 μmol g^{-1} before hydrolysis whereas tectorigenin content was 6.56 μmol g^{-1} in its acid hydrolysate. The tectoridin was not detectable after acid hydrolysis but detectable before hydrolysis. However, glycitein, which was detectable before and after hydrolysis, was quatitatively increased to 9.96 μmol g^{-1} by hydrolysis. High level of glycitein in the acid hydrolysate was due to the hydorlysate of gycitein glycosides (e.g., glycitin).

We previously reported that tectoridin and glycitin in the flower of *P. thunbergiana* could be transformed to tectorigenin and glycitein, respectively, by intestinal microbial flora (Bae *et al.*, 1999). Therefore, the four isoflavonoids, glycitein, tectorigenin, glycitin and tectoridin, were chosen for HPLC analysis of the flower and root extracts and their acid hydrolysates. Our data suggest that the variant proportions of the isoflavonoids in the flower (Puerariae Radix) and root (Puerariae Flos) reflect the traditionally different usage for the oriental medication. Since tectorigenin is known as an isoflavone with potent biological activities, the quantity of this compound may become a marker for the quality evaluation of Puerariae

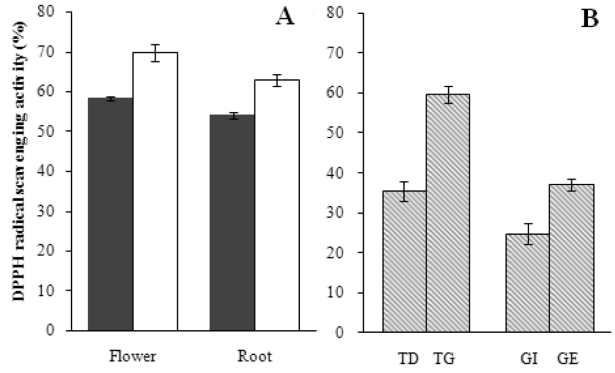


Fig. 3. DPPH radical scavenging activities of flowers and roots of *P. thunbergiana* (A) and four isoflavonoids (B).
(A) ■: DPPH activity of MeOH extract; □: DPPH activity of acid hydrolysate.
(B) TD, Tectoridin; TG, Tectorigenin; GI, Glycitin; GE, Glycitein.

Flos rather than for Puerariae Radix. In particular, it will be desirable that the quality of Puerariae Flos should be evaluated by the tectorigenin content in the acid hydrolysate of the extract or by the sum of tectorigenin and tectoridin levels in the non-hydrolyzed extract.

DPPH radical scavenging activities – The DPPH radical scavenging activities of the test materials (MeOH extracts and the acid-hydrolysates of flowers and roots of *P. thunbergiana* and the four isoflavonoids, tectoridin, tectorigenin, glycitin and glycitein) are shown in Fig. 3. The free radical-scavenging activities of the MeOH extracts of flowers and roots were shown to be 58.30% and 54.01%, respectively. Acid hydrolysis increased the activities by almost 10% in both flower and root. The DPPH radical scavenging activity of acid hydrolysate from Puerariae Flos containing higher level of tectorigenin was highest among the extracts tested.

Free radical-scavenging activities of both isoflavone glycosides, glycitin (24.75%) and tectoridin (35.53%) did not reach the values of the isoflavones, glycitein and tectorigenin, respectively. These results are consistent with the previous reports that *Pueraria* species have antioxidant properties and that their major isoflavonoids contents significantly contribute to antioxidant activity (Guerra *et al*., 2000; Jiang *et al*., 2005; Pandey *et al*., 2007; Cherdshewasarta *et al*., 2008). In conclusion, it is suggested that Puerariae Flos could be differentiated from Puerariae Radix in terms of high level of tectorigenin and its glycosides. In addition, acid hydrolysates of the MeOH extract of Puerariae Flos may more efficiently represent its pharmacochemical or pharmacognostical usage.

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