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Study of Substance Changes in Flowers of *Pueraria thunbergiana* Benth. During Storage

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Puerariae Flos is a traditional herbal medicine that has long been used as a treatment for colds, diabetes, and hangovers. The herbal medicine contains a wide variety of isoflavones such as kakkalide, tectoridin, and tectorigenin. This study demonstrates that the substances undergo a certain degree of change depending on the storage period by the method of HPLC and ^{13}C-NMR, and that the HPLC analysis can be used to determine the freshness of Puerariae Flos.

Key words: Puerariae Flos, Isoflavones, Substance change

INTRODUCTION

Puerariae Flos, the dried flowers of *Puerariae thunbergiana* Benth. (Leguminosae) for pharmacological uses were picked before it fully blooms, after removing the pods and leaves. The plant is traditional herbal medicine as a treatment for chills, fevers, dizziness, bloody sputum, and diabetes (Park *et al.*, 1999). The plant is particularly favored as a relief for hangovers due to its efficacy in stimulating alcohol catabolism (Kubo *et al.*, 1975; Kinjo *et al.*, 1987; Kinjo *et al.*, 1988). According to the *Flora Synensis* (Kim *et al.*, 1998), it is a cure for rectal ulcers and bleeding. Further, there are reports of its efficacy in protecting cells (Yoshikoshi *et al.*, 1996) and stimulating and protecting the liver (Arao *et al.*, 1997). The following compounds are known as the ingredients of Puerariae Flos: irisolidone, genistein, daidzein, glycitein, glycitin, 6"-*O*-xylosyl-tectoridin, 6"-*O*-xylosylglycitin, tectorigenin, tectoridin, kakkalide, kakkatin, kaikasaponon III, soyasaponin I, soyasaponin β g, soyasaponin Ab, glycyrrhizin, rutin, biocanin A, ononin, β-sitosterol, robinin, nicotiflorin, and quercetin, etc. (Kurihara *et al.*, 1973; Kurihara *et al.*, 1975; Kurihara *et al.*, 1976; Hwang *et al.*, 1998). This study investigated the effects of the storage period on the substances of Puerariae Flos at room temperature and presented the HPLC technique as a method for determining the level of freshness in Puerariae Flos.

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MATERIALS AND METHODS

Materials and extraction of herb

The experiment used dried Puerariae Flos (Koryo Oriental Medicine Distribution Co., Seoul, Korea) appraised by Lee Jae-hyun, a professor at the Department of Oriental Herbal Medicine, College of Pharmacy, Kyunghee University (Seoul, Korea). The samples are in storage at the Korea Institute of Oriental Medicine (Chinese origin: KIOM-99-3-0021 and -0022 for old one). The Anisaldehyde was purchased from Janssen Chimica (Japan) and methanol for the HPLC method was from Merck Co. (Darmstadt, Germany). All reagents used in the experiment were reagent-grade (Dongyang Chemical Co., Seoul, Korea).

To prepare the extracts, 1.5 kg of fresh Puerariae Flos (in storage for less than 5 years) were extracted in 9 L of 70% methanol for 7 days at room temperature. The herb was then extracted once, and filtered through a filter paper. Subsequently, the filtrate was evaporated using a vacuum rotary evaporator (Ratavapor R-114; Flawil, Schweiz) at 40°C and then freeze-dried. Then, 100 g of the total extract 275 g were dissolved in 700 mL of distilled water. The dissolved extracts were fractionated with ethylacetate, butanol, and water sequentially and

each fraction was concentrated and dried. Old Puerariae Flos (in storage for over 5 years) was extracted by the same procedures applied to fresh Puerariae Flos. Acidic hydrolysis of fresh Puerariae Flos was pursued with 1 g of fresh Puerariae Flos in 3 mL of 1 M HCl at 100°C for 2 hours. The hydrolysate was then neutralized with 10 N NaOH, and then treated in the same steps described above.

Isolation of compounds

Sample substances were dissolved from 2 mg of each extract in 1 mL of methanol and then centrifuged at 14,000 rpm for 10 min. Supernatant was recovered and filtered (0.45 μm, Minisart RC 4, Sartorius, Gottingen, Germany). Then 10 μL of the filtrate was injected into HPLC following the description in Table I. Silica gel column chromatography was performed on ethylacetate-fractionated extracts, which contained the largest amount of the key ingredients of Puerariae Flos. As a result, the fractionated extracts were separated into compound **(a)**: tectoridin and **(b)**: tectorigenin. Silica gel 60 column chromatography (2.5× 80 cm : 230~400 mesh ASTM, Merck Co.) was performed to separate compounds and the mobile phase was a mixture of chloroform, methanol, and distilled water. Initially, the solvent was mixed in the proportion of 10:1:0.1 (v:v:v). To increase the R_f values of the separated compounds, mobile phases of different combinations were continuously added (10:2:0.2 → 70:30:4).

Analysis of compounds

The 70% methanol extracts of fresh and old Puerariae Flos and hydrolysate of fresh Puerariae Flos were dissolved in 100% methanol. Then, HPLC analysis was performed as described above. From each of the fractions separated with columns, two key compounds were found using the same solvent used in the column separation and TLC (50 TLC plates: silica gel 60 F_{254}; Merck Co.). Coloring reagent was anisaldehyde reagent (anisaldehyde:acetic acid: methanol:sulfuric acid = 0.5:10:85:5 (v:v:v:v)). Further, FAB mass, ^{13}C-NMR, ^{1}H-NMR, HMBC, HSQC, D_2O exchange, and DEPT were performed to identify the structures of the compounds at our request (the National Center for Inter-University Research Facilities, Seoul National University, Seoul, Korea).

RESULTS AND DISCUSSION

Fresh Puerariae Flos (in storage for less than 5 years) and old Puerariae Flos (stored for over 5 years) yielded different HPLC chromatogram (Fig. 1). This is considered

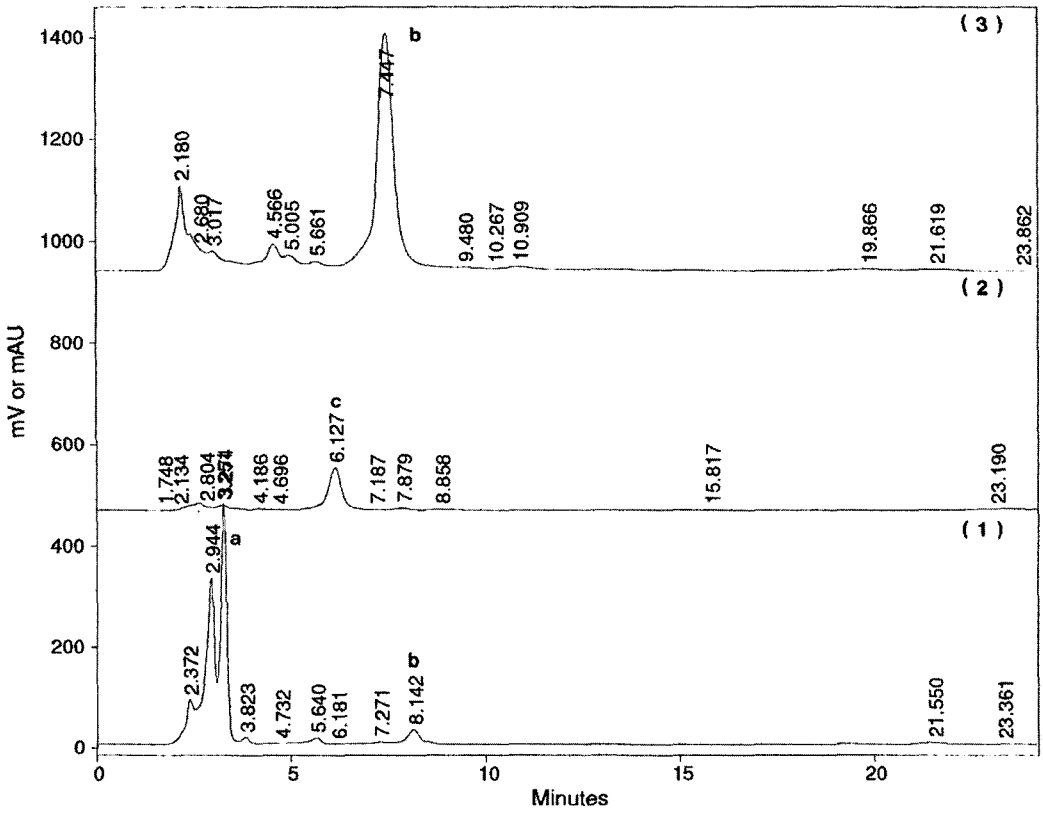
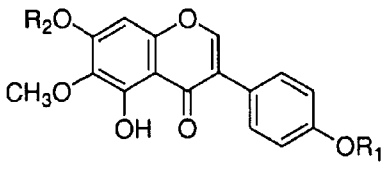


Fig. 1. The representative HPLC chromatograms of Puerariae Flos : (1) Fresh Puerariae Flos, (2) Old Puerariae Flos, and (3) hydrolized fresh Puerariae Flos.



	R_1	R_2
(a) : Tectoridin	H	Glu
(b) : Tectorigenin	H	H
(c) : Kakkalide	CH_3	Glu-Xyl

Fig. 2. The structures of **(a)** Tectoridin and **(b)** Tectorigenin isolated from fresh Puerariae Flos, and **(c)** Kakkalide from old Puerariae Flos.

to be the function of changes in substances caused by the duration of storage. As shown in Fig. 1, chromatogram (1) of fresh Puerariae Flos showed retention time peaking at 3.274 minutes **(a)** and 8.142 minutes **(b)**. In chromatogram (2) of old Puerariae Flos, new peak **(c)** appeared at 6.127 minutes. As for the chromatogram (3) of hydrolysate of fresh Puerariae Flos, peak **(a)** showed a distinctive decrease while peak **(b)** increased at (3). This is considered and indentifited by HPLC to be the result of hydrolysis of peak **(a)** into peak **(b)**. Meanwhile, LC-mass was performed to identify the compounds of the peaks that appeared in the chromatograms. The molecular weight of peak **(c)** in the chromatogram (2) of old Puerariae Flos was 608, while the weight of peak **(b)** in chromatogram (3) was 300. Based on an analysis of the compounds of Puerariae Flos (Kurahara *et al.*, 1975), the compound is considered to be kakkalide (FW: 608). FAB-mass, ^{13}C-NMR, ^{1}H-NMR, HMBC, HSQC, D_2O exchange, and DEPT were performed on **(a)** and **(b)** for compound identification.

Compound **(a):** Tectoridin : Pale yellow needles (MeOH). FAB-MS: *m/z* 463[M$^+$H]$^+$. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz): Table II (DMSO-d_6).

Compound **(b):** Tectorigenin : Yellow needles (MeOH). FAB-MS: *m/z* 301[M$^+$H]$^+$. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz): Table II (Aceton-d_6).

Separation of the compounds **(a)** and **(b)** from fresh Puerariae Flos was performed under the conditions described below, and various methods were used for structural analysis of compounds. Based on comparisons with NMR data in the papers (Lee *et al.*, 1999; Li *et al.*, 1986), compound **(a)** was confirmed to be tectoridin (FW: 462), and compound **(b)** tectorigenin (FW: 300) which was the aglycone of tectoridin. Therefore, it is conjectured that when Puerariae Flos is stored for prolonged periods (over 5 years), tectoridin in the plant combines with methyl and xylose and converts into kakkalide. Further, as indicated in Fig. 1, peak **(b)** that appeared in the chromatrogram (1) of fresh Puerariae Flos was identical to peak **(b)** in the chromatogram (3) of hydrolized Puerariae Flos, and peak **(a)** was hydrolized into peak **(b)**. This work, which was confirmed by the HPLC method, shows that prolonged storage (over 5 years) causes changes in the compounds of Puerariae Flos. The level of freshness of Puerariae Flos which had been stored for various periods of time was easily determined by the HPLC method. Therefore, it is believed that tectorigenin joins with a methyl and a xylose with the passage of time and converts into kakkalide.

Table I. HPLC conditions used to determine fractionated extracts from Puerariae Flos

Column	LUNA 5 μ, C18(2), 250×4.6 mm (Phenomenex, U.S.A.)
Detector	OD_{260} with UV detector
Mobile phase	5 mM NaH_2PO_4 (pH 4.6) : MeOH = 40 : 60 (v:v)
Flow Rate	1 μL/min.
Injection volume	10 μL

Table II. ^{13}C-NMR and ^{1}H-NMR spectral data of compounds **(a)**: tectoridin (DMSO-d_6) and **(b)**: tectorigenin (Aceton-d_6) from fresh Puerariae Flos

Position	^{13}C-NMR(125 MHz)		^{1}H-NMR(500 MHz)	
	a	b	a	b
2	155.6(CH)	154.6(C)	8.5(s)	8.2(s)
3	122.9(C)	123.1(C)		
4	181.6(C)	182.1(C)		
5	153.8(C)	154.5(C)		
6	133.3(C)	132.3(C)		
		60.7(OCH3)	3.8(s)	3.9(s)
7	157.5(C)	157.9(C)		
8	94.9(CH)	94.5(C)	6.9(s)	6.5(s)
9	153.3(C)	154.6(C)		
10	107.3(C)	106.6(C)		
1'	121.9(C)	123.6(C)		
2', 6'	131.1(CH)	131.3(C)	7.5(d, 8.4)	7.5(d, 8.7)
3', 5'	116.1(CH)	116.0(C)	6.9(d, 8.4)	6.9(d, 8.7)
4'	158.3(C)	158.5(C)		
Glc-1	101.0(CH)		5.2(d, 7.1)	
2	74.0(CH)		3.4(m)	
3	77.5(CH)		3.4(m)	
4	70.5(CH)		3.2(m)	
5	78.2(CH)		3.5(m)	
6			3.5(m)	
			3.8(m)	

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