Arch Pharm Res Vol 29, No 1, 96-101, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

High Performance Liquid Chromatographic Analysis of Isoflavones in Medicinal Herbs

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(Received July 7, 2005)

Phytoestrogens have been used as a food supplement to prevent osteoporosis. The isoflavones in the phytoestrogens are daidzein, genistein and formononetin which are present in various herbs. This study examined the quantity of isoflavones in medicinal herbs, which can be used as a phytoestrogen supplement; soybean. These isoflavones were quantified using high performance liquid chromatography (HPLC) with a UV/VIS detector. The concentration of daidzein in Puerariae Radix was 10,436.16±2,143.83 mg/kg of the dried herb, which was much higher than that extracted from soybeans, 341.47±18.96 mg/kg. The amount of genistein in Sophorae flavescentis Radix (336.09±50.89 mg/kg) was approximately 11 times higher than that extracted from soybean (30.03±7.17 mg/kg). The level of formononetin in Dalbergiae odoriferae Lignum, 2,189.14±136.46 mg/kg, was the highest among the herbs tested. The total isoflavone content of Puerariae Radix was approximately 30 times higher than that extracted from soybean. Therefore, plants from the family Leguminosae, particularly Puerariae Radix, can be a good source of phytoestrogens.

Key words: Isoflavone, Genistein, Daidzein, Formononetin, HPLC

INTRODUCTION

Phytoestrogens are plant-derived compounds with estrogen-like effects. Phytoestrogens, which were discovered through an examination of the cause of sterility in animals given particular animal feed stuffs (Franke *et al.*, 1995; Wang *et al.*, 1995), is found in hundreds of plants, such as alfalfa, peas, soybeans, oatmeal, Indian beans, rice, etc. (Saloniemmi *et al.*, 1995; Reinili and Block, 1996). The isoflavone class of phytoestrogens (diadzein, genistein, formononetin, etc.) is found in many plants of the family Leguminosae in large quantities. Plants belonging to the family Leguminosae are distributed worldwide, numbering some 36 genera and 92 species (Lee, 1996). Plants from the family Leguminosae have been used as food resources, particularly in East Asia; Korea, China, and Japan. In addition, they have been used as drugs.

Although daidzein, genistein, and formononetin have a lower affinity to the estrogen receptors (ER) than estrogen, they have attracted attention as a possible replacement for estrogen (Bickoff *et al.*, 1962; Miksicek, 1994; Pelissero *et al.*, 1991; Shyamala and Ferenczy, 1984; Welshons *et al.*, 1990). Formononetin has 1/10,000 fold lower toxicity on uterine adenocarcinoma cells than estrogen. This means that it would have a lower risk of adverse effects, such as metrauxe caused by estrogen (Wang *et al.*, 1995). Estrogen replacement therapy (ERT) has been widely used as a treatment for postmenopausal osteoporosis or aging. However, the NIH (National Institute of Health), released a report advising against long-term ERT on account of the adverse effects, such as breast cancer. A study on the use of phytoestrogens as a replacement for conventional ERT to prevent and treat osteoporosis is currently underway.

This study quantified the level of the isoflavone class of phytoestrogens, daidzein, genistein, and formononetin, which are found in various medicinal herbs reported in the Korea pharmacopeia, such as Puerariae Radix, Puerariae Flos, Glycyrrhizae Radix, Dalbergiae odoriferae Lignum, Cassiae Semen, Mucunae Caulis, Sophorae flavescentis Radix, Caraganae Radix, Sophorae Flos, Phaseoli Semen, Dolichoris Semen, Psoraleae Semen, Sophorae subprostratae Radix, Caesalpiniae Lignum, Gleditsiae Spina, Albizziae Cortex, Trigonellae Semen, and Astragali Radix from the family Leguminosae; Paeoniae Radix alba and

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Paeoniae Radix rubra from the family Ranunculaceae; and Carthami Semen from the family Compositae.

MATERIALS AND METHODS

Reagents and Materials

The following plants from the family Leguminosae were purchased herbs at Kyung-dong herbal mart, Seoul, Korea: Puerariae Radix (Kimchon, Gyeongbuk, KIOM-3-0019), Puerariae Flos (China, KIOM-3-0021), Glycyrrhizae Radix (China, KIOM-3-0020), Dalbergiae odoriferae Lignum (China, KIOM-3-0011), Cassiae Semen (Gangjin, Chungnam, KIOM-3-0016), Mucunae Caulis (China, KIOM-3-0009), Sophorae flavescentis Radix (Umsong, Chungbuk, KIOM-3-0008), Caraganae Radix (China, KIOM-3-0017), Sophorae Flos (Shinan, KIOM-3-0010), Phaseoli Semen (China, KIOM-3-0013), Dolichoris Semen (China, KIOM-3-0015), Psoraleae Semen (China, KIOM-3-0007), Sophorae subprostratae Radix (China, KIOM-3-0004), Caesalpiniae Lignum (China, KIOM-3-0014), Gleditsiae Spina (China, KIOM-3-0012), Albizziae Cortex (China, KIOM-3-0003), Trigonellae Semen and Astragali Radix (Jeongseon, Gangweon-do, KIOM96-3-0001). Paeoniae Radix alba (Uiseong, Gyeongbuk, KIOM-3-0005) and Paeoniae Radix rubra (Korea, KIOM-3-0006) belonging to the family Ranunculaceae, and Carthami Semen belonging to the family Compositae (China, KIOM-3-0002). As standards, genistein and daidzein were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.), and formononetin was extracted and purified by Kim and Kim (1997) from Astragali Radix, and then examined by NMR (Nuclear Magentic resonance) spectroscopy and MS (Mass Spectrometry), prior to use. Aloe-emodin (Sigma Chem. Co., St. Louis, MO, U.S.A.) was used as an internal standard. Methanol (Merck CO., Darmstadt, Germany) was used as the HPLC solvent. The ethanol and methanol were obtained by distilling the first grade alcohol. All other reagents were purchased from the Sigma Chem. Co. (St. Louis, MO, U.S.A.).

HPLC analysis and Calibration curves

A spectra system P1000 (Thermo Separation Products, Fremont, CA, U.S.A.) as the HPLC system, and Spherex5 C18 (250×4.60 mm, 5 μ; Phenomenex Co., Torroance, CA, U.S.A.) and prodigy 5 μ ODS (30×4.60 mm; Phenomenex Co., Torroance, CA, U.S.A.) as columns were used to quantify the isoflavones. As defined in a previous report, a mixed solution of 5 mM NaH_2PO_4 solution (pH 4.6) and methanol (4:6) was used as the mobile phase, and the flow rate was 1 mL/min. Formononetin (0.5 μg/mL) was quantitatively analyzed at a wavelength of 260 nm at which the maximum absorbance was observed. Aloe-emodin was used as an internal standard by determining if the retention time and tailing of formononetin on the above mobile phase overlapped with those of the other substances in the test sample.

In order to make the standard calibration curves, daidzein (0.051 to 213.71 μg/mL), genistein (0.056 to 194.20 μg/mL), and formononetin (0.025 to 5.00 μg/mL) were diluted in methanol containing internal standard, aloe-emodin (4 μg/mL), was added and the supernatants (20 μL) were injected into HPLC to give individual chromatograms. The standard calibration curves were plotted by calculating the peak height ratios of the various concentrations of daidzein, genistein and formononetin to the internal standard. The calibration curve equations and correlation coefficients thereof were calculated using the SigmaPlot® 2000 program (SPSS Science Inc., Chicago, IL, U.S.A.).

Extraction and acid hydrolysis of isoflavones from medicinal herbs

The content of the aglycon-type isoflavones, which are sometimes called free isoflavones, were measured by finely pulverizing the dried medicinal herbs, adding the resulting pulverized herbs (each 50 g) to 70% methanol (500 mL), and extracting them by stirring at ambient temperature for 2 days. The extracts were concentrated under reduced pressure and freeze-dried. The total isoflavone, aglycone and glycoside content was quantified by adding 1 N HCl solution (1 mL) to each of the medicinal herbs (100 mg), and cooling them to room temperature after allowing them to hydrolyze at 100°C for 2 h. The cooled hydrolysate was neutralized by adding a 10 N NaOH solution, and freeze-drying the mixture.

Quantitative analysis of isoflavones contained in medicinal herbs

The contents of the aglycon-type or total-type isoflavones were measured by dissolving each of the dried 70% methanol extracts (10 mg) or hydrolysate (10 mg) in the mobile phase (600 μL), adding the internal standard (4 μg/mL), and centrifuging the resulting mixture at 14,000 rpm and 4°C for 20 min (Centrifuge 5402, Eppendorf, Hamburg, Germany). The supernatant was then filtered (0.45 μm, Minisart RC 4, Sartorius, Gottingen, Germany), and the filtrate was analyzed using the above HPLC conditions. The rate of recovery rate was calculated and adjusted by the reference standard of isoflavone. All samples were analyzed more than 3 times and the results are reported as a mean ± SE.

RESULTS AND DISCUSSION

Calibration curves for daidzein, genistein and formononetin

The retention times of daidzein, genistein, formononetin

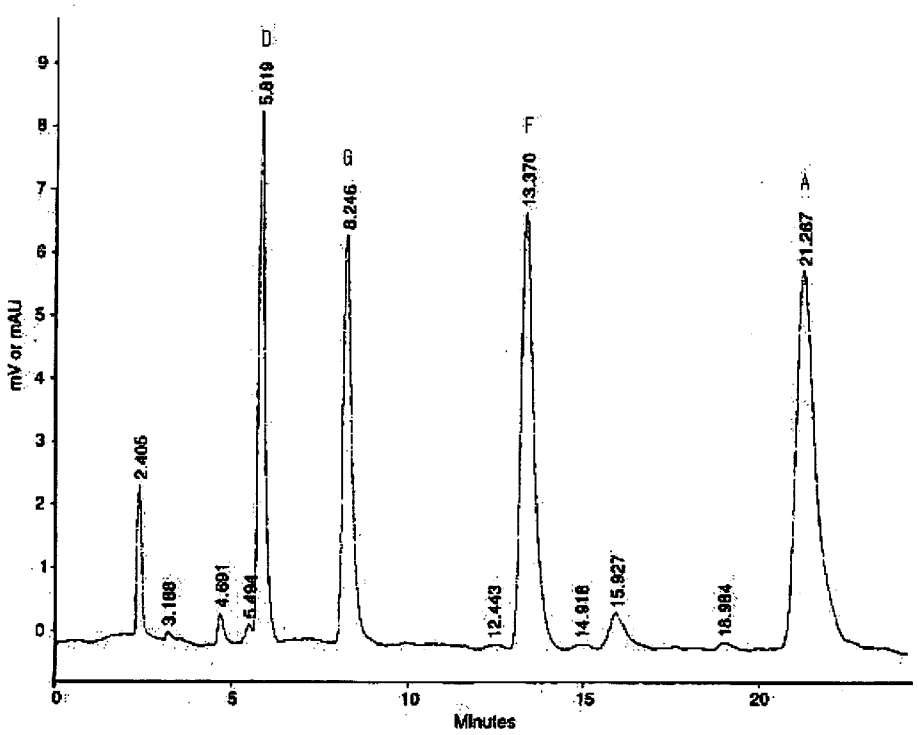

Fig. 1. A HPLC chromatogram of daidzein (D), genistein (G), formononetin (F), and aloe-emodin (A)

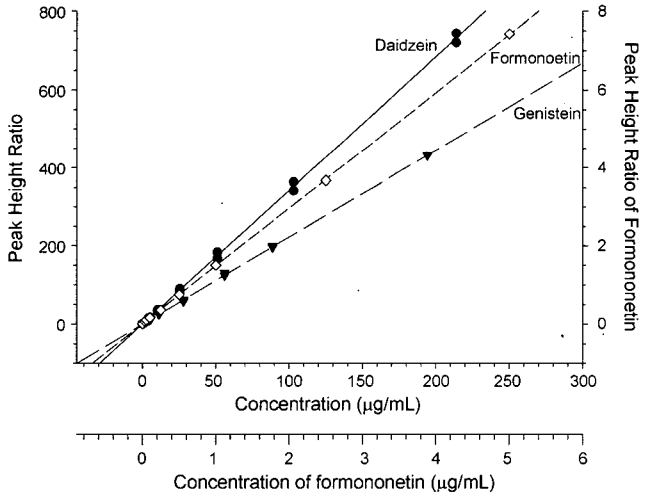

Fig. 2. Standard curves for the isoflavones. The Y axis represents the peak height ratio of isoflavone to the internal standard. The X axis indicates the concentration of isoflavone: daidzein (●), genistein (▼), formononetin (◇) and aloe-emodin (4 μg/mL). These fitting equations are daidzein (●); y = 3.4257x - 0.1689 (r^2=0.9994), genistein (▼); y = 2.2317x - 0.5067 (r^2=0.9998), and formononetin (◇); y = 1.4795x + 0.0098 (r^2=1.0000).

and the internal standard, aloe-emodin, were 5.82 min, 8.25 min, 13.37 min, and 21.27 min, respectively (Fig. 1). The HPLC peaks of the isoflavones contained in each sample were verified using the standard reference material. Each peak height in quantitative HPLC analysis was

Table I. The recovery yields of 70% methanol extraction

	Yield (% of dried sample)
Leguminosae	
Soybean	9.82±0.90
Albizziae Cortex	11.16±0.20
Caesalpiniae Lignum	8.44±0.10
Caraganae Radix	7.69±0.36
Cassiae Semen	8.98±0.18
Dalbergiae odoriferae Lignum	30.63±1.30
Dolichoris Semen	6.25±0.20
Gleditsiae Spina	3.01±0.06
Glycyrrhizae Radix	14.78±0.29
Mucunae Caulis	11.63±0.29
Phaseoli radiate Semen	6.07±0.28
Psoraleae Semen	13.54±0.23
Puerariae Flos	17.28±0.51
Puerariae Radix	10.32±1.00
Sophorae flavescentis Radix	7.33±0.48
Sophorae Flos	13.64±0.40
Sophorae subprostatae Radix	17.36±0.56
Trigonellae Semen	9.81±0.44
Ranunculaceae	
Paeoniae Radix alba	11.29±0.18
Paeoniae Radix rubra	25.42±1.47
Compositae	
Cartami Semen	4.99±0.19

determined by calculated the valley-to-valley as the baseline. The calibration curves were plotted using the peak height ratio, which has a relatively smaller error than that of the peak area ratio under the influence of the adjacent peaks.

Correlation coefficients of each calibration curve for daidzein, genistein, and formononetin were r^2=0.9994, r^2=0.9998, and r^2=1.0000, respectively (Fig. 2). Under the above HPLC condition the detection limits of both daidzein and genistein were 50 ng/mL (UV_{260nm}), and that of formononetin was 25 ng/mL (UV_{260nm}).

Quantitative analysis of isoflavones contained in medicinal herbs

Table I shows the recovery rates of each 70% methanol extract of medicinal herbs. The isoflavone class of phytoestrogens (daidzein, genistein, formononetin, etc.) contained in legumina and processed soy food was quantified using the method reported elsewhere (Kim *et al.*, 2000). In this study, the isoflavones contained in medicinal herbs belonging to the Leguminosae family and others were quantified. Daidzein, genistein, biochanin A, formononetin, coumestrol etc are known as the isoflavone class of phytoesterognes, and it was reported that the major isoflavones are of the glycoside-type (Bickoff *et al.*, 1962; Franke *et al.*, 1994; Reinli and Block, 1996). Therefore, this study quantified the aglycon-type isoflavones from the 70% methanol extracts of the samples by hydrolyzing the samples and the measuring the total content of both glycoside- and aglycon-type isoflavones. It was reported that most phytoestrogens and glycosides can be extracted with 70% methanol. However, it is very difficult to extract the whole quantity of components contained in a natural product. Therefore, the medicinal herb samples provided a minimum 3 to 6 of their respective extracts, and each of the extracts was analyzed 3-4 times to give a mean in order to minimize the error. The quantity of isoflavones contained in the dried medicinal herbs is represented by isoflavones mg/kg dried herbs (Table II).

Daidzein was detected in following order of abundance: Puerariae Radix > Puerariae Flos > Psoraleae Semen > Soybean > Albizziae Cortex > Mucunae Caulis > Sophorae

Table II. Isoflavone content in medicinal herbs

	Daidzein (mg/kg)		Genistein (mg/kg)		Formononetin (mg/kg)	
	Free	Total	Free	Total	Free	Total
Leguminosae						
Soybean	14.52± 0.58	341.47± 18.96	22.49± 0.93	30.03± 7.17	0.06± 0.01	0.16± 0.05
Albizziae Cortex	11.15± 1.87	331.87± 103.32	ND	ND	0.38± 0.07	3.89± 1.78
Astragali Radix	ND	ND	ND	ND	9.63± 1.62	43.53± 8.21
Caesalpiniae Lignum	ND	ND	ND	ND	ND	ND
Caraganae Radix	ND	ND	ND	ND	ND	ND
Cassiae Semen	ND	ND	ND	ND	ND	ND
Dalbergiae odoriferae Lignum	ND	ND	ND	ND	1802.04±200.82	2189.14±136.46
Dolichoris Semen	ND	ND	ND	ND	ND	ND
Gleditsiae Spina	ND	ND	ND	ND	ND	ND
Glycyrrhizae Radix	ND	ND	10.65± 1.88	22.60± 0.93	10.51± 2.26	233.93± 22.70
Mucunae Caulis	1.41± 1.31	171.01± 15.54	17.06±14.48	ND	6.75± 0.83	19.74± 4.72
Phaseoli radiate Semen	ND	ND	ND	ND	ND	ND
Psoraleae Semen	92.78±36.55	633.35± 27.81	ND	ND	ND	ND
Puerariae Flos	21.13± 3.50	1002.53± 206.41	ND	ND	ND	ND
Puerariae Radix	303.49±51.73	10436.16±2143.83	30.62± 2.72	232.08±83.30	24.28± 1.84	308.94± 60.80
Sophorae flavescentis Radix	11.95± 5.67	trace	101.26±38.50	336.09±50.89	30.81± 11.03	342.74± 30.63
Sophorae Flos	ND	ND	9.27± 2.59	248.44±52.16	0.50± 0.07	ND
Sophorae subprostatae Radix	ND	ND	106.33± 5.62	62.82± 9.52	64.19± 3.05	51.76± 7.24
Trigonellae Semen	ND	ND	ND	ND	ND	N D
Ranunculaceae						
Paeoniae Radix alba	ND	ND	ND	ND	ND	ND
Paeoniae Radix rubra	ND	ND	ND	ND	ND	ND
Compositae						
Cartami Semen	ND	ND	ND	ND	ND	ND

ND: Not detected

flavescentis Radix. Puerariae Radix had the highest daidzein content (10,436.16±2,143.83 mg/kg). However, daidzein was not detected in the other medicinal herbs (Table II).

Genistein was detected in following order of abundance: Sophorae flavescentis Radix > Sophorae Flos > Puerariae Radix > Sophorae subprostratae Radix > Soybeans > Glycyrrhizae Radix > Mucunae Caulis. Sophorae flavescentis Radix had the highest genistein content (336.09 ± 50.89 mg/kg). However, genistein was not detected in other medicinal herbs. The content of the aglycon-type genistein in Mucunae Caulis was 17.06 ± 14.48 mg/kg. However, no aglycon-type genistein was detected after acid-hydrolysis. The content of aglycon-type genistein in Sophorae subprostratae Radix was 106.33 ± 5.62 mg/kg, and a larger amount of aglycon-type genistein was detected after acid-hydrolysis (Table II).

Formononetin was detected in following order of abundance: Dalbergiae odoriferae Lignum > Sophorae flavescentis Radix > Puerariae Radix > Glycyrrhizae Radix > Sophorae subprostratae Radix > Astragali Radix > Mucunae Caulis > Albizziae Cortex > Soybean. Dalbergiae odoriferae Lignum had the highest formononetin content (2,189.14 ± 136.46 mg/kg) but formononetin was not detected in the other medicinal herbs.

The total content of isoflavones was the highest in Puerariae Radix. The order of abundance was Puerariae Radix > Dalbergiae odoriferae Lignum > Puerariae Flos > Sophorae Flos > Psoraleae Semen > Soybeans > Albizziae Cortex > Sophorae Flos > Mucunae Caulis > Sophorae subprostratae Radix. However, among the plants from the family Leguminosae examined, no isoflavones were detected in Cassiae Semen, Caraganae Radix, Phaseoli Semen, Dolichoris Semen, Caesalpiniae Lignum, Gleditsiae Spina and Trigonellae Semen, (Table II). In a previous report, formononetin was extracted, separated, purified, and verified from Astragali Radix, and used as a reference material (Kim and Kim, 1997; Kim *et al.*, 2000). Generally, Astragali Radix has a higher formononetin content than the other legumina and processed soy foods. However, in this study, the formononetin content in Dalbergiae odoriferae Lignum was 50 times higher than that of Astragali Radix. Therefore, Dalbergiae odoriferae Lignum is believed to be a good source of formononetin.

No isoflavones were detected in Paeoniae Radix alba and Paeoniae Radix rubra from the family Ranunculaceae, and Carthami Semen from the family Compositae (Table II).

When hydrolyzing medicinal herbs, aglycon-type genistein was the most commonly detected isoflavones, while daidzein and formononetin mainly occurred in the type of glycoside bond (Table II). The rates of daidzein and formononetin recovery by the hydrolysis of the medicinal herbs were 94 ± 7.0% and 94 ± 0.3%, respectively. On the other hand, genistein was almost completely decomposed during hydrolysis resulting in a low recovery rate (67 ± 2.6%).

In a previous study (Kim *et al.*, 2002), Puerariae Radix, which has a high isoflavone content, inhibited the bone loss in ovariectomized rats (Kim *et al.*, 2002). It is believed that medicinal herbs with a high phytoestrogen content can contribute to the prevention and treatment of postmenopausal osteoporosis.

CONCLUSION

Dalbergiae odoriferae Lignum had the highest formononetin content, while Sophorae flavescentis Radix and Puerariae Radix had the highest genistein and daidzein content, respectively. Dalbergiae odoriferae Lignum had the highest total isoflavone content but Puerariae Radix had a generally high concentration of daidzein, genistein and formononetin.

ACKNOLEDGEMENT

This research was supported by grants, #01-PJ2-PG6-01NA01-0002 and #03-PJ9-PG6-SO01-0002 from Health technology planning & evaluation board (HPEB).

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