Mokchae Konghak 32(6) : 1~6, 2004

Quantification of Phytoestrogens in Woody Plants (Leguminosae) Using HPLC*1

Youngki Park*2†, Wi Young Lee*2, and Jin Kwon Ahn*2

ABSTRACT

Phytoestrogens are considered to exhibit biological activities in human and animal. There are few data on the contents of phytoestrogens in woody plants. This study was undertaken to examine phytoestrogen contents in five species (*Albizzia coreana, Albizzia julibrissin, Gleditsia japonica* var. koraiensis, *Maackia fauriei* and *Sophora japonica*) of leguminosae. An HPLC method was employed for the first time to analyze phytoestrogens in five species. The contents of daidzein and genistein were in the range of 2.9 ~170.5 μg/g and 1.3~118.4 μg/g, respectively. Daidzein and genistein were most abundantly present in the *Sophora japonica* among the samples examined.

Keywords : HPLC analysis, phytoestrogen, isoflavone, Leguminosae, biological activities

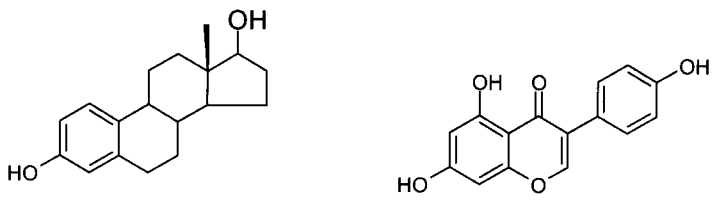
1. INTRODUCTION

Phytoestrogens, the natural products, are similar to estradiol in the molecular structure and function and have gained recognition as protective agents against wide range of human conditions, including breast, prostate, bowel and other cancers, brain function and menopausal symptoms (Fig. 1.) (Grace *et al.*, 2003). One important group of phytoestrogens is isoflavones like daidzein, genistein and biochanin A, which occur in soy and soy products. As important secondary metabolic compounds, isoflavones have been reported to play essential roles in preventing certain types of cancers and in reducing the risk of cardiovascular diseases. They are also known to reduce the activity of hemolysis and express estrogenic activities in animals.

In contrast with other groups of flavonoids, the distribution of isoflavonoids in plants is relatively sparse. According to Harborne (1994), 759 isoflavonoids have been isolated from leguminous plants, while another 80 isoflavonoids have been reported from non-leguminous plants. The family Leguminosae represents a very large family with 750 genera comprising more than 18,000 species. They are used as crop, forages and green manures. They also synthesize a wide range of natural products such as flavours, drugs, poisons and dyes. The family of Leguminosae is particularly rich in flavonoids and

*1 Received on February 4, 2004; accepted on March 26, 2004.

*2 Div. Biotechnology, Korea Forest Research Institute, Suwon 441-350, Korea
† Corresponding author : Youngki Park (wood0601@hanmail.net)



estrogen phytoestrogen

Fig. 1. Structures of estrogen (estradiol) and phytoestrogen (genistein).

related compounds; about 28% of all flavonoid and 95% of all isoflavonoid aglycone structures known from the plant kingdom are produced by legumes.

So far HPLC has been mainly used for the determination of isoflavones. Most studies have restricted their measurements to analytes like daidzein, genistein and their glycosides from soy and soybean foods. Most of them have analyzed only phytoestrogen content in soy and soybean food (Murphy *et al.*, 1999; Franke *et al.*, 1999). However, these methods for analysis of phytoestrogens were not suitable for woody plants (Leguminosae), because of the differences in the accompanying substances.

In the present study, we measured the content of phytoestrogens in five species of leguminosae, *Albizzia coreana, Albizzia julibrissin, Gleditsia japonica* var. koraiensis, *Maackia fauriei* and *Sophora japonica,* using a modified HPLC method.

2. MATERIALS and METHODS

2.1. Materials

The branch woods of *A. coreana, G. japonica* var. koraiensis, *M. fauriei* and *S. japonica* were collected from Chollipo arboretum, Taean, Chungnam and *A. julibrissin* was collected from Korea Forest Research Institute, Suwon, Korea. These samples were ground to fine powder (60~80 mesh) and stored at room temperature.

2.2. Chemicals

Methanol, acetic acid and dimethyl sulfoxide (DMSO) and all other solvents used were HPLC grade. Authentic standards of daidzein, genistein and biochanin A were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.3. Phytoestrogen Extraction

Approximately 200 mg of sample powders were extracted with 30 mℓ of methanol (acidified to pH 2 with trifluoroacetic acid) for 30 min under reflux at 80°C. In this study, we used trifluoroacetic acid instead of hydrochloric acid. Since trifluoroacetic acid is completely removable during evaporation, no additional neutralization step was necessary. After filtration and washing off the residue with 20 mℓ 80% methanol, the extract was dried off using a rotary evaporator. The extract was then dissolved in 2 mℓ dimethylsulfoxide (DMSO). An auto injector was used to inject 20 μℓ of the test solution into the HPLC system.

2.4. HPLC Analysis

The samples were analysed by HPLC (Thermo Separation Products, California, Inc.) using 5 μm LiChrospher 100 RP-18 (26×8.0 mm)

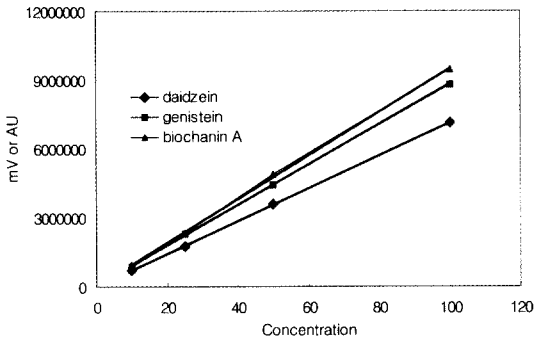


Fig. 2. Calibration curves for phytoestrogens analyzed by HPLC.

column. The chromatograms were monitored at 260 nm by UV detector (TSP, spectrum system UV 3000HR). For gradient elution two solvents were used: (A) 10% glacial acetic acid in H_2O, and (B) acetonitrile. Elution profile: 0~35 min, 10~50% B in A; 35~45 min, 50~100% B in A, 45~50 min 100% B; 50~51 min, 100~10 % B in A; 51~61 min, 10% B in A. The flow rate was 0.7 mℓ/min.

3. RESULTS and DISCUSSION

3.1. HPLC Analysis of Phytoestrogens

Daidzein, genistein and biochanin A from the samples were identified by comparing their retention times with those of authentic standards. Calibration was established using four-point calibration curves (Fig. 2). The concentrations of compounds were calculated on the basis of peak areas in the chromatogram. The chromatograms of daidzein, genistein and biochanin A (Fig. 3) are shown in Fig. 4. The retention times of these compounds were 20.4, 25.9 and 38.3 min at 260 nm, respectively.

3.2. Quantitation of Phytoestrogens

The contents of daidzein, genistein and biochanin A in five species woods of Leguminosae, *A. coreana*, *A. julibrissin*, *G. japonica* var. koraiensis, *M. fauriei* and *S. japonica*, were analyzed using HPLC method. The variation in the contents of phytoestrogens in the woods of these species is presented in Table 1. Daidzein and genistein concentrations were in the range of 2.9~170.5 μg/g and 1.3~118.4 μg/g, respectively. Relatively high concentration of daidzein and genistein was observed from the woods of *S. japonica*, while low concentration was detected from *A. coreana*, *A. julibrissin*, *G. japonica* var. koraiensis and *M. faurie*. From the woods of *M. fauriei* and *S. japonica*, biochanin A was observed, while no detectable amounts of biochanin A were found from the woods of *A. coreana*, *A. julibrissin* and *G. japonica* var. koraiensis.

From the woods of *S. japonica*, Takeda *et al.* (1977) isolated biochanin A, irisolidone, maackiain, pratensein, sissotrin, irisolidone-7-*O*-glucoside, rutin, 7-O-[β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl]-biochanin A and biochanin A 7-(xylosyl (β-1→6) glucoside). Park *et al.* (2001) also reported the isolation and identification of formononetin, irisolidone, dihydroformononetin and biochanin A from the woods of *S. japonica*. From the heartwood of *Maackia fauriei*, genistein, daidzin and other isoflavones were isolated (Hwang *et al.*, 1997; Ko *et al.*, 1999). However, in these studies, the contents of isoflavones were not examined.

According to Krenn *et al.* (2002), the content of daidzein, genistein and boichanin A in red clover were 110 μg/g, 100 μg/g and 2,040 μg/g, respectively. Soy is the most common food that have been analysed for phytoestrogens content since this is the richest dietary source for daidzein and genistein (Wilkinson *et al.*, 2002). Wang *et al.* (1994) reported that the total isoflavone contents of soybeans grown in different locations ranged from 1,176 to 1,749 μg/g. Choi *et al.* (1996) reported the total isoflavone contents from 458 to 2,317 μg/g from soybean.

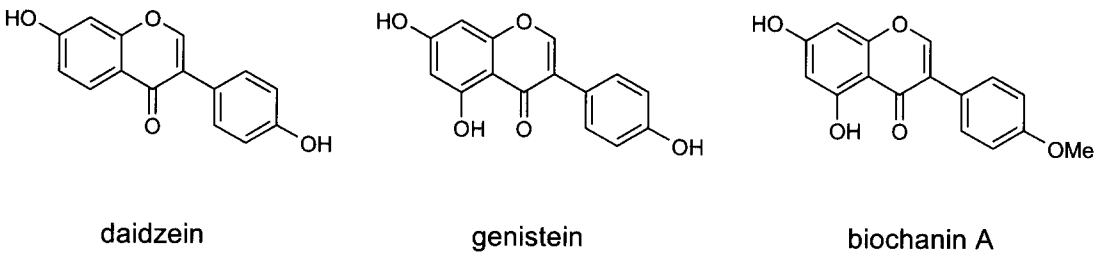


Fig. 3. Structures of the isoflavones.

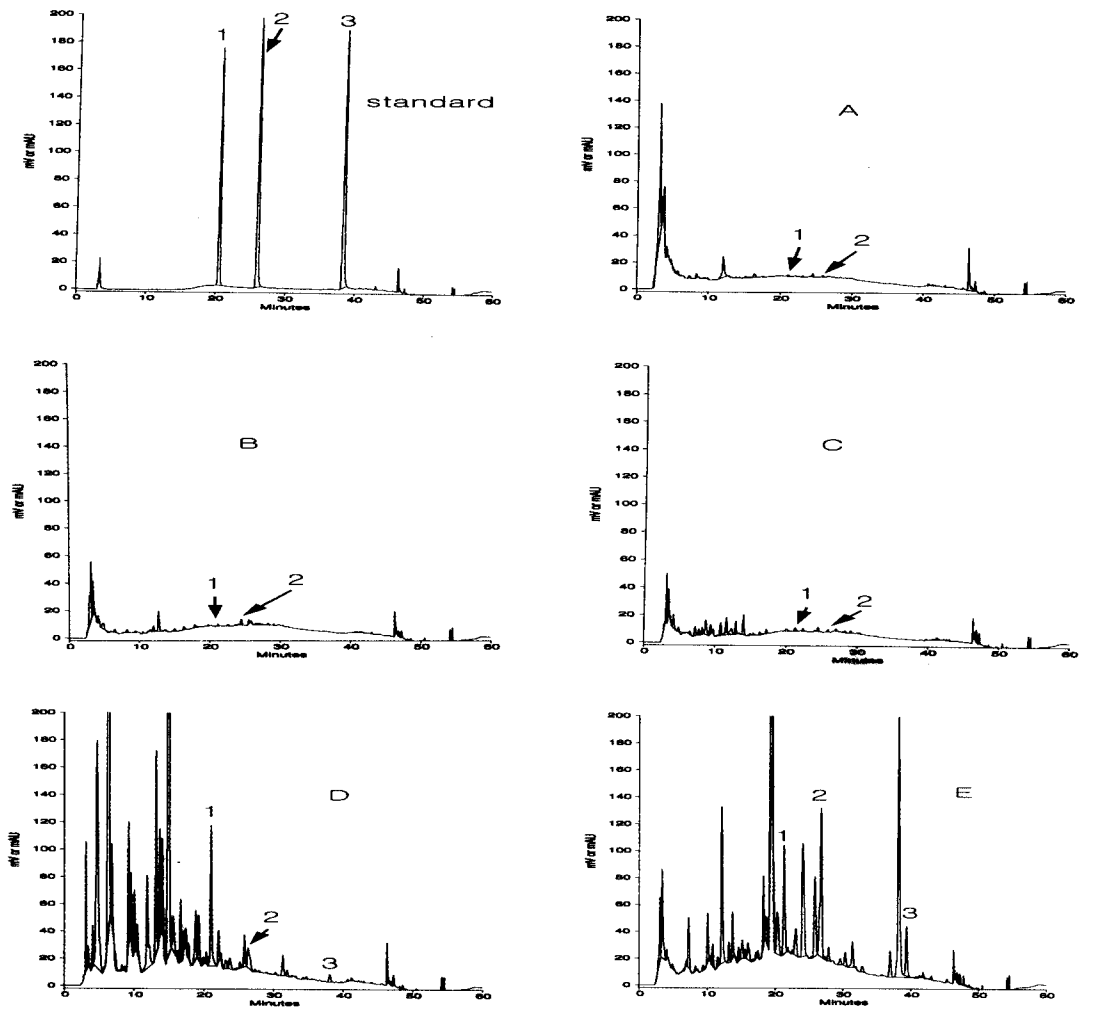


Fig. 4. The HPLC chromatogram of phytoestrogen in standard, A, *A. coreana*; B, *A. julibrissin*; C, *G. japonica* var. koraiensis; D, *M. fauriei* and E, *S. japonica*. Peak identification: 1, daidzein; 2, genistein; and 3, biochanin A.

Table 1. The contents of phytoestrogens in the woods of five species

Species	Phytoestrogen content (μg/g)		
	daidzein	genistein	biochanin A
Albizzia coreana	3.1	1.6	-
Albizzia julibrissin	2.9	1.3	-
Gleditsia japonica var. koraiensis	6.4	2.6	-
Maackia fauriei	14.6	35.8	10.0
Sophora japonica	170.5	118.4	64.3

- : not detected

From these results, it can be concluded that the contents of daidzein and genistein from the woods of *S. japonica* are similar to those of red clover. However, the total isoflavone contents of these five species of leguminose were lower than those of soybean.

4. CONCLUSION

Phytoestrogens contents of Leguninosae were analysed for the first time by HPLC. The contents of daidzein and genistein in Leguminosae ranged from 2.93 to 170.53 μg/g and from 1.34 to 118.48 μg/g, respectively. Among five species tested, the wood parts of *S. japonica* had the highest amounts of phytoestrogens. The contents of daidzein and genistein from the woods of *S. japonica* are comparable to those of red clover. The method developed in this study may be useful for the quantification of daidzein, genistein and biochanin A in woody plants. The advantages of this method over the conventional methods may be its requirement of small samples, simplicity in sample preparation and short analysis time.

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