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Contents of Certain Isoflavones in *Glycine dolichocarpa*, *G. tabacina* and *G. tomentella* Collected in Taiwan

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ABSTRACT

Glycine tabacina, *G. tomentella* and *G. dolichocarpa* are three wild perennial soybean species native to Taiwan. *Glycine tabacina* and *G. tomentella* are the source of I-Tiao-Gung, a Chinese herb. This study was commissioned to measure the presence of isoflavones in each and qualify/quantify isoflavone content using an appropriately configured multi-component HPLC method.

Isoflavone in these soybean species was most heavily concentrated in the root systems. Daidzein was the predominant isoflavone found throughout plant structures, with the exception of *G. tomentella* seeds. Isoflavone concentrations were highest in the roots and stems of *G. tabacina*, in the leaves of *G. tomentella*, and in the seeds of *G. dolichocarpa*. Significant differences in isoflavone levels exist between species and structural components. The results of this study can help set selection and qualification parameters for *Glycine germplasm* and serve as reference when confirming genetic markers in Chinese herbs.

Key words: wild soybean, *Glycine*, isoflavone, quantitative analysis, species variation

INTRODUCTION

Three perennial species of *Glycine* are native to Taiwan. These include *G. tabacina*, *G. tomentella* and *G. dolichocarpa*. Their distribution is limited to the Penghu archipelago and Kinmen Island (for *G. tabacina*), the Dadu mountains of Taichung, Pingtung and Kinmen Island (for *G. tomentella*), and Taitung (for *G. dolichocarpa*). Significant variations in morphological and RAPD characteristics exist among Taiwan's wild soybean species[1-4].

Isoflavones, naturally occurring plant compounds found almost exclusively in soybeans and other leguminous plants, have been intensively studied in recent years with regard to their substantial health benefits. Isoflavones belong to a group of plant compounds with potential anti-carcinogenic properties. Acting as antioxidants and tyrosine protein kinase inhibitors, they may lower the risk of cardiovascular disease and breast cancers[5-9].

Significant variations in soybean isoflavone content due to varietal and environmental conditions have been reported[10] and isoflavone content has been found to be significantly lower in soybean seeds gestated at high temperatures than in seeds gestated at low temperatures[11]. Genotype, genotype × year, genotype × location, and genotype × year × location interactions were identified as significant variables influencing total and individual isoflavone content values in soybean seeds. The possibility of breeding in a cultivar development program for isoflavone content as a quantitative trait has been reported[12]. Weak but significant correlation has been found to exist between agronomic characteristics and isoflavone content in soybeans (*G. max*)[13]. A study done in Korea found that wild soybean (*G. soja*) seeds had a generally higher isoflavone content than the seeds of cultivars or landraces (*G. max*)[14]. Isoflavones have also been isolated from the leaves and roots of soybean plants[15,16].

G. tomentella is grown as a commercial crop in Kinmen, with their roots being used as the source of the herbal drug, I-Tiao-Gung, which is used as an important component of many herbal health products. Wild *G. tabacina* roots have also been used for many years as an herbal drug, although little work has been done to identify its constituent compounds. The objective of this study was to develop a reliable analytical method by which the isoflavone contents in different structural components of Taiwan's three wild soybean species could be compared.

MATERIALS AND METHODS

I. *Materials*

Seeds of the three perennial subgenus *Glycine* species used in this experiment were collected from Taiwan, Penghu and Kinmen and germinated at a farm run by the National Pintung University of Science and Technology in 2001. Photographs of the plants and seeds are shown in Figures 1 and 2. The plants harvested after being allowed to grow in the field for one full year. Component plant structures, including roots, stems, leaves and seeds, were separated for use in this analysis.

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G. tabacina

G. tomentella

G. dolichocarpa

Figure 1. The plant of three wild soybean species.

II. *Chemicals and Reagents*

Figure 3 shows marker substance structures. Genistin and genistein were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Daidzin, daidzein, and the internal standard cinnamaldehyde were purchased from Fluka Chemie AG (Switzerland).

The 95% ethanol solution used in our research was purchased from the Taiwan Tobacco and Wine Bureau (ROC). Acetonitrile and methanol (HPLC grade) were obtained from Mallinckrodt, Inc. (USA) and phosphoric acid (analytical-reagent grade) was purchased from Kanto Chemical (Japan). Ultra-pure distilled water with a resistivity greater than 18 MΩ was prepared with a mini-Q system (Millipore, Bedford, MA, USA). Samples prepared for high-performance liquid chromatography (HPLC) were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA). All other reagents used met analytical grade quality standards.

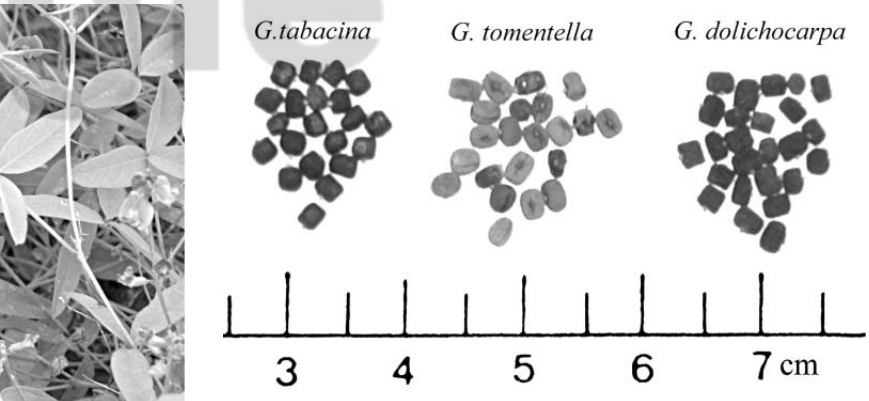
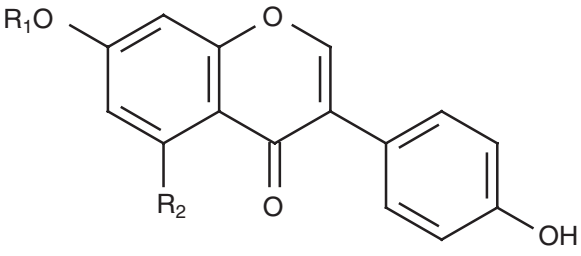


Figure 2. The seeds of three wild soybean species.



	R_1	R_2
Daidzin	Glu	H
Daidzein	H	H
Genistin	Glu	OH
Genistein	H	OH

Figure 3. Structures of the studied compounds.

III. *HPLC Instruments and Conditions*

HPLC was conducted using a Hitachi system equipped with a degasser DG-2410, Pump L-7100, UV/Vis Detector L-7420 and Autosampler L-7200. Peak areas were calculated with D-7000 HSM software.

We used a Cosmosil 5C18-AR-II (4.6 mm I.D. × 250 mm) packed column. The mobile phase are shown in Table 1.

We injected 20 μL of each sample solution prepared as described above into the HPLC column for analysis. The results were quantified by interpolating results onto the linear regression plot based on the standard solution.

IV. *Preparation of Standard and Internal Standard Solutions*

(I) *Standard Solution Preparation*

Standard solutions were prepared by dissolving 18.0 mg, 4.0 mg, 10.0 mg, and 1.6 mg, of daidzin, genistin, and daidzein genistein in 70% methanol to give sequential concentrations of 360.0 μg/mL, 80.0 μg/mL, 200.0 μg/mL, and 32.0 μg/mL, respectively.

(II) *Internal Standard Solution Preparation*

The internal standard was prepared by dissolving 25.3 mg of cinnamaldehyde in 70% methanol to achieve a final volume of 250 mL (101.2 μg/mL).

V. *Extraction Conditions*

Ground samples of one gram each were extracted with 100 mL of neat methanol, 80% and 50% methanol, respectively, and boiled for 2 hr. Solutions were filtered, evaporated, and adjusted to 5 mL with the addition of 70% methanol. The internal standard, cinnamaldehyde, was then added to each solution to a concentration of 50.6 μg/mL.

We calculated isoflavone extraction ratios after filtering extracts through 0.45 μm membrane filters.

VI. *Preparation of Sample Solutions*

All samples were cut into pieces and dried at 60°C for 24 hr. One gram from each sample was accurately weighed and extracted with 100 mL of methanol at 80°C for 2 hr. The solution was filtered, evaporated, and adjusted to 5 mL by the addition of 70% methanol solution. The internal standard was added as described above. This solution was the test used for subsequent HPLC analysis after 0.45 μm membrane filter filtration.

VII. *Calibration Method*

The standard solutions of each marker substance were diluted with 80% methanol to give sequential concentrations of daidzin -- 11.25, 22.5, 45.0, 90.0, 180.0, 360.0 μg/mL; genistin -- 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 μg/mL; daidzein -- 6.25, 12.5, 25.0, 50.0, 100.0, 200.0 μg/mL; genistein -- 1.0, 2.0, 4.0, 8.0, 16.0, 32.0 μg/mL.

Each dilution contained 50.6 μg/mL of cinnamaldehyde (the internal standard). After filtration through a 0.45 μm membrane filter, 20 μL of each concentrate was injected into the HPLC column for analysis. The calibration curve was plotted using the ratio of peak areas corresponding to each standard solution and the internal standard solution on the Y-axis, versus each concentration on the X-axis. Linear regression was used to evaluate the parameters of y = ax + b and the correlation coefficient.

Table 1. The mobile phase were mixtures of 10% acetonitrile (A), 50% acetonitrile (B) and 100% acetonitrile (C) (adjusted with phosphoric acid to pH 2.8) with a linear gradient elution

Time (min)	A	B	C
0	95	5	0
25	95	5	0
45	85	15	0
55	55	45	0
70	55	45	0
80	0	40	60
95	95	5	0

HPLC conditions: flow rate: 1.0 mL/min; detection wavelength: UV 240 nm; column temperature: 30°C.

VIII. *Validation*

(I) *Precision*

Standards of daidzin (22.5, 90.0, 360.0 μg/mL), genistin (1.25, 10.00, 80.00 μg/mL), daidzein (0.13, 1.00, 6.00 μg/mL), and genistein (2.0, 8.0, 32.0 μg/mL) were injected both at intra-day (injecting each concentration three times during 24 hr) and inter-day (injecting each concentration four times during 7 days with each injection separated by at least 24 hr) intervals to check reproducibility. Standard deviation (S.D.) and coefficients of variation (C.V. %) were then calculated.

(II) *Accuracy*

A series of various concentrations of daidzin (22.5, 90.0, 360.0 μg/mL), genistin (1.25, 10.00, 80.00 μg/mL), daidzein (0.13, 1.00, 6.00 μg/mL), and genistein (2.0, 8.0, 32.0 μg/mL) were spiked into a sample solution of *G. tabacina* root, then extracted to test solutions as described in section VI. The internal standard was added to each solution to reach a concentration of 50.6 μg/mL. All samples were filtered through a 0.45 μm membrane filter and subjected to HPLC analysis and the recovery calculation.

RESULTS AND DISCUSSION

I. *HPLC Separation of Marker Substances*

Only four of the 12 known types of isoflavones (daidzin, genistin, daidzein and genistein) found in edible soybeans (*G. amx*) were found in the three wild species analyzed for our study. While not detected in the course of our research, other isoflavone types may be present in these

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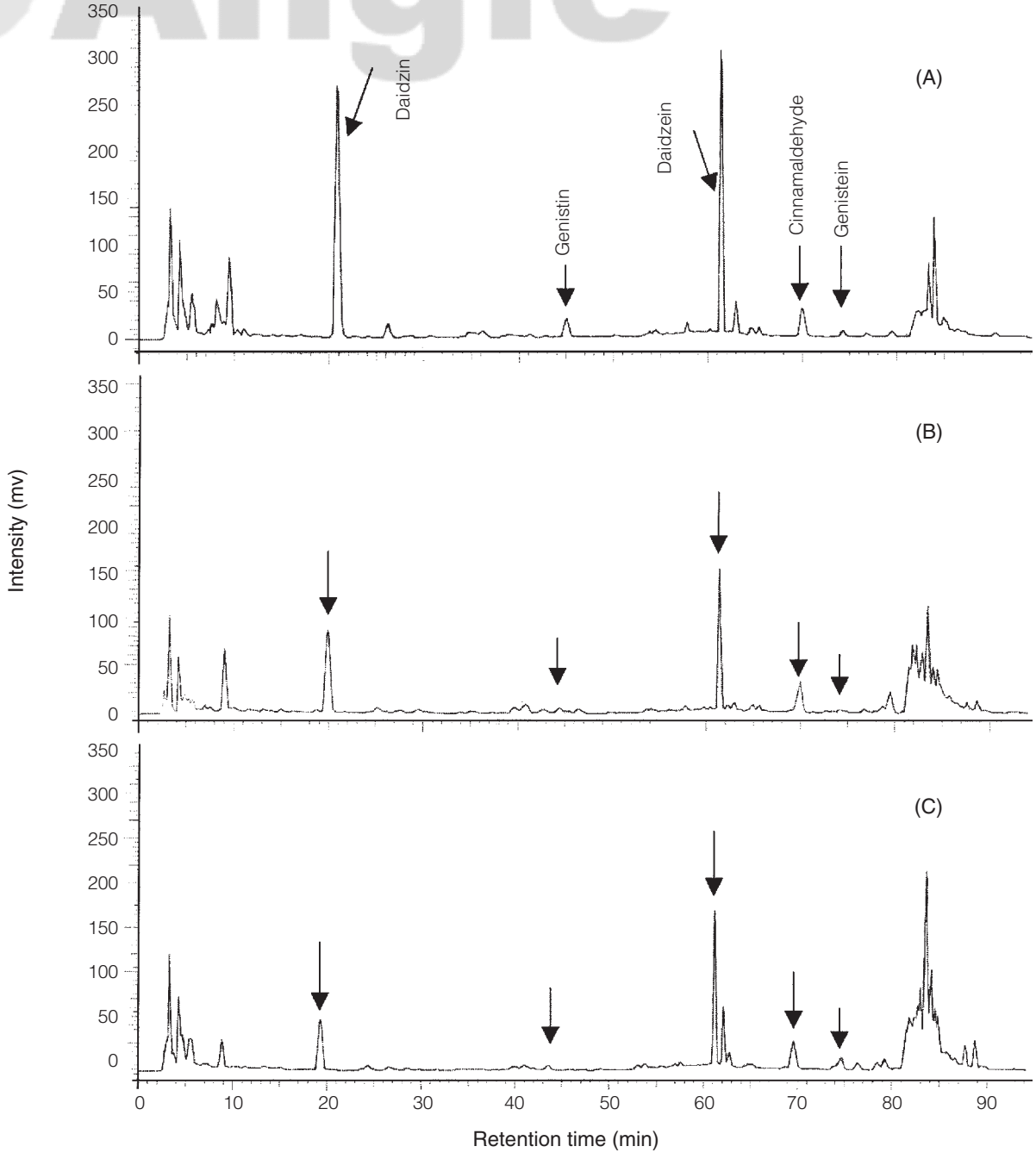

Figure 4. HPLC chromatograms of the MeOH extract in the root of *G. tabacina* (A), *G. tomentella* (B) and *G. dolichocarpa* (C).

soybean varieties in concentrations below the ability of our instruments to detect.

HPLC chromatograms of methanol obtained from the *G. tabacina*, *G. tomentella* and *G. dolichocarpa* root extracts are shown in Figure 4. The chromatogram shows that daidzin, genistin, daidzein, genistein peaks are easily distinguished from the internal standard (cinnamaldehyde). Their respective retention times are: 20 min for daidzin, 47 min for genistin, 61 min for daidzein, 76 min for genistein, and 70 min for the cinnamaldehyde. Apparently, there was no interaction between *Glycine* plant components. Therefore, the above conditions can be used for marker substance quantification.

While HPLC analysis retention time was longer than that reported in other studies[10-14,17], the authors believed it worth while to lengthen the retention time to obtain improved separation between the internal standard and marker components.

II. *Calibration Line*

Linear regression equations, correlation coefficients

and calibration line concentration range for marker substances are listed in Table 2. All calibration curves for the 6 ingredients were in good linear correlation (C.V. = 0.9998-0.9999).

III. *Extraction Conditions*

There were no significant differences in isoflavone content among the three different extract concentrations (Table 3). Therefore, we extracted all samples with neat methanol in the interests of convenience and preparation efficiency. Although this extraction method is quite different from that used in other reported research (which used 80% methanol, ethanol or HCl)[10-14], our method is similar to that employed by Kawamura *et al.*[17].

IV. *Validation*

The results of the inter-day and intra-day tests on the four isoflavones indicates very good reproducibility, with coefficients of variation of the inter-day and intra-day test ranging between 0.14-2.03% and 1.38-2.09%, respectively (Table 4).

Also, good recovery from the analysis were obtained as follows: 97.80-110.92% for daidzin; 92.70-112.80% for genistin; 98.00-112.00% for daidzein; and 92.30-106.28% for genistein, respectively (Table 5).

V. *Sample Content Analysis*

Significant differences in individual and total isoflavone contents were observed between different species and plant structures (Table 6). The root was found to have the highest concentration of isoflavones, followed by the stem. With the exception of *G. tomentella* seeds, daidzein was the predominant isoflavone found throughout all plant structures. Daidzein was the major isoflavone constituent in roots, representing 78-90% of the total isoflavones, followed by daidzin. *G. tabacina* had the highest isoflavone concentration of all three species in roots and stems. *G. tomentella* had the highest isoflavone concentration of the three species in leaves. *G. dolichocarpa* had the highest isoflavone concentration of the three species in the seeds. Significant differences in isoflavone content exist between species as well as plant structures, with the most significant difference found in the levels of isoflavones in roots and stems.

Differing from the findings of Wang and Murphy[13], the level of isoflavone content found in seeds was very low, despite high daidzein and daidzin levels in roots (Table 6). This discrepancy in findings may be attributable to differences in materials and analysis methods.

Isoflavone distribution patterns differ significantly between wild and domesticated soybean (*G. max*) species. According to Choi *et al.*[14], the highest content of genistein is found in certain landraces of *G. max* and daidzein content is highest the wild soybean species *G. soja*, found in Korea.

Table 2. Calibration curves of the marker substances (n = 5)

Compound	Concentration range (μg/mL)	Regression equation	r^2
Daidzin	11.25-360.0	y = 0.538x – 0.123	0.9999
Genistin	1.25-80.0	y = 10.535x – 0.1134	0.9998
Daidein	6.25-200.0	y = 0.0862x + 0.04	0.9999
Genistein	1.00-32.0	y = 103.36x – 0.5621	0.9999

Table 3. Relative contents of isoflavones in the root extracted with different concentrations of methanol

Component	100% MeOH	80% MeOH	50% MeOH
Daidzin (%)	100.00	93.36	95.08
Genistin (%)	100.00	100.00	90.00
Daidein (%)	99.05	94.66	100.00
Genistein (%)	100.00	75.00	75.00

Table 4. Reproducibility of intra-day and inter-day analysis of *G. tabacina*

Compound	Concentration (μg/mL)	Mean ± S.D. (RSD%)	
		Intra-day (n = 5)	Inter-day (n = 4)
Daidzin	22.50	23.12 ± 0.30 (1.28)	22.92 ± 0.47 (2.05)
	90.00	91.81 ± 1.16 (1.26)	89.55 ± 1.80 (2.01)
	360.00	363.54 ± 2.73 (0.75)	361.77 ± 4.99 (1.38)
Genistin	1.25	1.20 ± 0.01 (0.85)	1.29 ± 0.02 (1.67)
	10.00	10.67 ± 0.06 (0.60)	9.94 ± 0.17 (1.71)
	80.00	80.57 ± 0.11 (0.14)	82.05 ± 1.28 (1.56)
Daidzein	0.13	0.12 ± 0.002 (2.03)	0.14 ± 0.002 (1.67)
	1.00	0.96 ± 0.01 (1.04)	1.08 ± 0.02 (1.56)
	6.00	6.14 ± 0.03 (0.42)	6.05 ± 0.12 (1.98)
Genistein	2.00	2.07 ± 0.04 (1.86)	2.11 ± 0.04 (1.93)
	8.00	8.09 ± 0.07 (0.84)	8.14 ± 0.17 (2.09)
	32.00	32.56 ± 0.14 (0.43)	31.82 ± 0.63 (1.98)

Table 5. Recovery of daidzin, genistin, daidzein and genistein in *G. tabacina* (n = 5)

Compound	Concentration (μg/mL)	Recovery (%) Mean ± S.D. (RSD%)
Daidzin	22.50	107.40 ± 1.04 (0.97)
	90.00	110.92 ± 0.64 (0.58)
	360.00	97.80 ± 0.66 (0.67)
Genistin	1.25	112.80 ± 1.08 (0.96)
	10.00	92.70 ± 0.50 (0.54)
	80.00	97.93 ± 0.63 (0.64)
Daidzein	0.13	112.00 ± 0.09 (0.08)
	1.00	98.00 ± 0.39 (0.40)
	6.00	103.66 ± 0.76 (0.73)
Genistein	2.00	94.50 ± 1.95 (2.06)
	8.00	92.30 ± 1.59 (1.72)
	32.00	106.28 ± 0.83 (0.78)

They also demonstrated that significant differences in isoflavone content exist between different species of soybean. Isoflavone distribution may provide a useful tool by which familial relationships within the *Glycine* species can be traced and defined. More extensive studies would be worthy to further determine the interrelationships.

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Table 6. The isoflavone contents in different organs of three wild soybean species

Component	*G. tabacina* (μg/g DW)	*G. tomentella* (μg/g DW)	*G. dolichocarpa* (μg/g DW)
Root			
Daidzin	69.10 ± 8.53[a]	22.74 ± 0.87	12.81 ± 0.33
Genistin	0.32 ± 0.05	0.13 ± 0.01	0.06 ± 0.00
Daidzein	249.10 ± 26.24	143.76 ± 11.18	118.37 ± 4.65
Genistein	0.04 ± 0.00	0.03 ± 0.00	0.05 ± 0.00
Total	318.56 ± 34.81	166.66 ± 11.09	131.29 ± 4.92
Stem			
Daidzin	41.74 ± 9.08	18.35 ± 0.33	20.42 ± 6.12
Genistin	0.76 ± 0.14	0.46 ± 0.01	0.06 ± 0.00
Daidzein	76.81 ± 13.50	83.00 ± 1.68	40.05 ± 12.67
Genistein	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Total	119.35 ± 22.14	101.84 ± 1.99	60.56 ± 18.75
Leaf			
Daidzin	6.97 ± 0.41	21.28 ± 1.13	12.93 ± 0.86
Genistin	0.46 ± 0.02	0.12 ± 0.00	0.14 ± 0.00
Daidzein	12.99 ± 4.49	31.73 ± 5.29	14.62 ± 1.38
Genistein	0.06 ± 0.00	0.07 ± 0.00	0.07 ± 0.00
Total	20.47 ± 4.21	53.20 ± 5.96	27.76 ± 1.95
Seed			
Daidzin	2.26 ± 0.17	8.18 ± 0.23	9.83 ± 0.20
Genistin	0.14 ± 0.01	0.12 ± 0.00	0.15 ± 0.00
Daidzein	2.52 ± 0.55	0.23 ± 0.39	28.96 ± 1.02
Genistein	0.03 ± 0.00	ND[b]	0.03 ± 0.00
Total	4.94 ± 0.70	8.54 ± 0.45	38.97 ± 1.11

[a]Values are presented as means ± S.D., n = 5. [b]Not detected.

CONCLUSIONS

The multi-component HPLC method developed in this study proved suitable for the simultaneous quantification of four marker substances in *G. tabacina*, *G. tomentella* and *G. dolichocarpa*. Significant differences in the isoflavone contents were identified between species. While the roots of *G. tomentella* and *G. tabacina* have been employed as raw material for herbal medicines for many years, we found *G. tabacina* isoflavones to be in concentrations greater that those found in *G. tomentella*. This was especially true of the isoflavones daidzein and daidzin.

Plant compositions can vary in terms of seasons and growing conditions. In our present study, environmental variables were minimized by growing the plants at the same farm, using the same methodology, and same harvest schedule. As a result, isoflavone content identified in our research should reflect only differences resulting from genotype. Therefore, study results are suitable to serve as selection and quality parameters for *Glycine germplasm* collection programs and as reference when confirming genetic markers in Chinese herbs.

In terms of wild soybean distribution, Taiwan is in a unique geographical situation. The island's location at the southernmost part of the *Soja* subgenus growing region and the northernmost part of the *Glycine* subgenus growing region makes it a place rich in wild soybean species. Previous research on wild soybeans have focused attention on classification and utilization of useful genes, while ignoring their current use in medicinal preparations. *G. tabacina* and *G. tomentella* roots have been used in Chinese medicines in Taiwan for many years. The present study confirms that the root systems of these three species contains the highest concentration of isoflavones of any plant structure. Nevertheless, the amount of isoflavone in the seeds of the wild species is far less than that found in domesticated varieties. In addition to the effects of different materials and different analysis methods, observed differences may be attributed to the distant relationship between wild and domesticated soybeans, with the amount of isoflavone having gradually decreased in the root and increased in the seed during the process of selective breeding. This hypothesis deserves further study.

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