Original Article
Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.5327/fst.00006

Validation study of isoflavone derivatives and simultaneous analysis of *Puerariae Flos*

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Abstract

Using LC/MS, isoflavones (glycitin, genistin, tectoridin, glycitein, genistein, and tectorigenin) known as *Puerariae Flos* marker compounds were identified. The simultaneous analysis and validation of six marker compounds were performed using the HPLC-DAD method. To validate the analysis method of six marker compounds 1–6, the linearity was showed in the calibration curve as coefficient of correlation (R^2) range of 0.9997–1.000. The limit of detection (LOD) and limit of quantitation (LOQ) of six marker compounds were measured in the ranges of 0.2720–0.7884 μg/mL and 0.8243–2.3890 μg/mL, respectively. Intermediate precision, repeatability, and accuracy evaluations according to the Ministry of Food and Drug Safety (MFDS) guidelines showed that the relative standard deviation (RSD) less than 1% and the stability less than 2%, respectively. Also, the simultaneous validation method is attributed to analysis of the different isoflavones Flos ne compounds in Puerariae extract. It could be potentially used as sources of pharmaceutical and natural antioxidant ingredients.

Keywords: *Puerariae Flos*; Leguminosae; tectoridin; genistein; isoflavones.

Practical Application: The functionality of *Puerariae Flos* as a natural antioxidant has been confirmed, and through simultaneous analysis and validation of *Puerariae Flos* marker compound, it is intended to be provided as basic data for research on drugs and foods.

1 INTRODUCTION

Puerariae Flos is a perennial vine from the bean family. It is a plant native to East Asia, such as Korea, Japan, and China, with uneven long oval- or bell-shaped flowers of 0.5–1.5 cm length, 0.2–0.6 cm wide, and 0.2–0.3 cm thickness. It is purple or pale red in color with the bottom part of the petal stained yellow. It also has a unique scent flavor with slightly sweet taste, and due to its cool temperament it has been used in oriental medicine to treat headache, fever, thirst, loss of appetite, bloated stomach, and vomiting due to excessive drinking. According to a recent study, physiological activities such as diabetes control, liver protection, lipid improvement, cancer cell death, anti-oxidative effect, and whitening effect of *Puerariae Flos* have been reported (Hsu et al., 2003; Hwang et al., 2007; Hwang et al., 2018; Lee & Im, 2012; Park et al., 2009).

The reported main components of *Puerariae Flos* are isoflavones (tectoridin, genistein, daidzein, tectorigenin, and puerarin), flavonoids (quercetin), saponins (kaikasaponin I and kaikasaponin III), etc (1, 2, 6–10). Among these flavonoids, isoflavones have been reported to show anti-estrogenic, anti-oxidative, anti-aging, anti-inflammatory, anticancer, hyperlipidemic, cytoprotective, low cholesterolemic, hepatoprotective, and anti-HIV effects (Lee et al., 1999; Lee et al., 2005; Lee et al., 2009; Lee et al., 2019; Park & Park, 2018).

Although research results on various physiological activities and component analysis of *Puerariae Flos* extract have been reported, there are insignificant studies on quantitative testing methods or content analysis of isoflavone components showing various physiologically active effects. Therefore, this study established a simultaneous analysis method of the six surface components separated from the *Puerariae Flos* using HPLC-DAD. Also, to verify their validity, analysis methods such as linearity, accuracy, detection limits, and calibration limits were performed in accordance with the guidelines of the Ministry of Food and Drug Safety (MFDS).

2 MATERIALS AND METHODS

2.1 Material

The *Puerariae Flos* sample extracted from 1.2 kg of brown flower was purchased from Donggwang General Trading Co., Ltd. (Seoul, Korea) in December 2019 and was identified by Professor Kim Hyeon-jeong of Mokpo University. The samples were stored and used in the drug analysis laboratory of Dongshin University College of Oriental Medicine.

2.2 Reagents and instruments

Glycitin, glycitein, genistein, and tectorigenin used as indicator ingredients were purchased from Avention (Incheon, Korea); genistin was purchased from LC Laboratories (Boston, USA); and finally, tectoridin was purchased from Aladdin (Shanghai, China). The mobile phase acetonitrile solvent used

Received 5 May, 2022.
Accepted 3 Aug., 2023.
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This work was supported by the National Research Foundation of Korea(NRF), grant funded by the Korean government (MSIT) (No. 2022R1F1A1076398) and "Leaders in Industry-university Cooperation 3.0" Project.

in HPLC analysis was of HPLC grade and was purchased from Daejung Chemical (Siheung, Korea). For the column, C18 YMC (ODS-A 5 μm, 4.6 × 250 mm) was used. The equipment used for the test was a grinder (NFM-3561SN, NUC Co., Daegu, Korea), a reflux extractor (MS-DM, MISUNG Co., Seoul, Korea), and a lyophilizer (HyperCOOL HC3110, Hanil Scientific Inc., Gimpo, Korea). The equipment used for HPLC-DAD analysis was Agilent 1100 Technologies (Agilent Technologies, California, USA) equipped with autosampler and DAD, and for LC/MS, MS-8050 (Shimadzu, Kyoto, Japan) linked with LC-30A HPLC (Shimadzu, Kyoto, Japan) was used.

2.3 Preparation of analytical samples

After grinding using a grinder (NFM-3561SN, NUC Co., Daegu, Korea), distilled water, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, and 100% ethanol were added to 10 g of *Puerariae Flos* sample. Each was added in 100 mL at a time and the solution was extracted under reflux at 100 °C for 1 h. The filtered sample was then concentrated under reduced pressure using 185 mm Whatman filter paper (GE Healthcare Life Sciences, California, USA) and was powdered with a freeze-dryer (HyperCOOL HC3110, Hanil Scientific Inc., Gimpo, Korea). Finally, after dissolving 1 mL of MeOH in 1 mg of ground powder, it was filtered with a 0.45 μm syringe filter to prepare a sample for analysis.

2.4 Standard solution preparation

The standard compounds used in this study, glycitin (1), genistin (2), tectoridin (3), glycitein (4), genistein (5), and tectorigenin (6), were dissolved in 100% methanol at a concentration of 250 μg/mL. Then, they were diluted into the concentrations of each 10 μg/mL, 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, and 100 μg/mL to be filtered down into a standard solution of the indicator component using a 0.45 μm syringe filter.

2.5 Separation of indicator components

MS-8050 (Shimadzu, Kyoto, Japan) linked with HPLC (LC-30A, Shimadzu, Kyoto, Japan) and triple quadrupole mass spectroscopy (MS) were used to measure mass spectrometry of the six peaks of major components. Analysis was then performed by ionizing the sample with an electrospray ionization (ESI) device under detailed conditions such as scan range of 100–1,000 m/z, interface voltage at 3 kV, drying gas flow at 10 L/min, and temperature of 350°C.

2.6 HPLC analysis conditions

For HPLC analysis of six indicator components of isoflavone of *Puerariae Flos*, A (0.1% formic acid in water) and B (acetonitrile) were used as mobile phases, and then mobile phases were flowed under the A:B ratios of 82:18 (0 m), 70:30 (30 m), and 60:40 (50 m). The column was C18 YMC (ODS-A 5 μm, 4.6 × 250 mm), with the column temperature at 40°C, and the flow rate of the mobile phase was 0.5 mL/min. The detection wavelength was 260 nm and the injection volume was 5 μL.

2.7 Validation of the method

As a process of verifying the validity of the analysis method, various parameters were determined. In this study, the validity of the analysis method was verified as specificity, linearity, accuracy, precision, and limit of detection (LOD) and limit of quantitation (LOQ) were determined in accordance with the validation guidelines for pharmaceuticals announced by the MFDS (Korean Food and Drug Administration, 2015).

2.7.1 Specificity

Specificity refers to the ability to accurately analyze an analyte in a sample in which various substances are mixed. By analyzing a standard mixture solution of a sample and each indicator component, the chromatogram was obtained and visually evaluated. Then, UV spectrum was evaluated by comparing UV spectrum maximum absorption wavelength of the index component of the sample extract and purchased standard product mixture solution by HPLC.

2.7.2 Linearity

Linearity refers to the ability to obtain a linear measurement value within a certain range in proportion to the amount or concentration of the analyte in the sample. After diluting the index components to six concentrations, a calibration curve was prepared based on the results obtained after performing analysis three times, and linearity was evaluated by obtaining a regression equation from the calibration curve.

2.7.3 Limit of detection

The LOD refers to the minimum amount of substance to be analyzed that can be detected in the sample, and it is not necessary to be quantifiable. Based on the prepared calibration curve, calculation was made using Equation 1:

$$\text{Limit Of Detection (LOD)} = 3.3 \times \frac{\text{Standard deviation}(\sigma)}{\text{Gradient(S)}} \quad (1)$$

2.7.4 Limit of quantification

Quantification limit refers to the minimum amount of a sample that can be quantified. Similar to LOD, calculation was made using Equation 2:

$$\text{Limit of quantification (LOQ)} = 10 \times \frac{\text{Standard deviation }(\sigma)}{\text{Gradient(S)}} \quad (2)$$

2.7.5 Precision and accuracy

Precision refers to the dispersion degree of measured values when a single sample is analyzed several times. Relative standard deviation (RSD%) values were calculated to evaluate precision, using the Inter-day method, which measures the concentration of the standard 10, 40, and 100 μg/mL three times within a day, and the Inter-day method, which measures the concentration three times. Accuracy refers to the degree to which the measured value is close to the known true value, and the recovery rate (%)

was calculated after repeating the three-section concentrations of 10, 40, and 100 μg/mL of the standard sample three times.

2.7.6 Content evaluation

Using the identical analysis method, the average and standard deviation of peak area values were calculated from the *Puerariae Flos* extract by ethanol concentration, and then substituted into the regression equation obtained by linearity evaluation to calculate the content.

2.7.7 Statistical processing

All experimental results presented in this experiment are expressed as mean ± standard deviation (mean ± SD) repeatedly measured three times. Statistical processing was performed using the Statistical Package for the Social Sciences (SPSS), and one-way analysis of variance (ANOVA) was performed for statistical significance tests between each experimental group. In case of significance, Tukey's test was conducted, and it was determined that there was significance when $P < 0.05$ and the significance in correlation was marked using Pearson's correlation coefficient.

3 RESULTS AND DISCUSSION

3.1 Identification of indicator components

Under HPLC-DAD analysis conditions, six major peaks of *Puerariae Flos* extract were identified in the absorption wavelength range of 190–400 nm. After six single components were separated using preparative HPLC, the isoflavone compound was determined when molecular weight of each separated compound 1–6 was identified by LC/MS ESI spectrum analysis method (Figure 1 and Table 1). In general, the maximum absorption of isoflavone UV wavelength is 259–265 nm, and flavone is 263–293 nm (Lu et al., 2013). It was confirmed that the separated compound 1–6 has the same maximum absorption wavelength as isoflavones. The molecular weight of compound 1 was measured as $[M+H]^+$ 447 m/z, and it was confirmed as a 285 m/z fragment ion from which the glucose residue was removed. Also, on HPLC, the retention time (RT) and mass fragmentation pattern of the glycitin standard were compared and identified as $C_{22}H_{22}O_{10}$ 446.2 m/z of glycitin (Chen et al., 2005; Lee et al., 2015). Compound 2 confirmed the molecular weight of $[M+H]^+$ 433 m/z, and the glucose residue of 271 m/z fragment ion and 225 m/z fragment ion in which CHO + H_2O was lost. By analyzing the fragmentation pattern with RT and ESI mass spectrum of the genistin standard on HPLC, it was confirmed as $C_{21}H_{20}O_{10}$ 432.3 m/z genistin (Chen et al., 2005; Lee et al., 2015; Liang et al., 2018). Compound 3 confirmed a molecular weight of $[M+H]^+$ 463 m/z, a 301 m/z fragment ion with glucose residue removed, and a 285 m/z fragment ion with a loss of methane (CH_4). Also, by comparing RT and mass spectrum fragmentation pattern on HPLC of tectoridin standard, it was confirmed as $C_{22}H_{22}O_{11}$ 462.3 m/z tectoridin (Shu et al., 2010). Compound 4 confirmed the molecular weight $[M+H]^+$ 285 m/z and $[M-H]^-$ 283 m/z fragment ions. By comparing the RT and fragment pattern of the glycitein standard on HPLC, it was identified as $C_{16}H_{12}O_5$ 284.1 m/z glycitein (Lee et al., 2015; Raju et al., 2015). Compound 5 confirmed the molecular weight $[M+H]^+$ 271 m/z and $[M-H]^-$ 269 m/z fragment ions. By comparing the RT and fragmentation pattern on HPLC of the genistein standard, it was identified as $C_{15}H_{10}O_5$ 270.2 m/z genistein compound (Lee et al., 2015; Raju et al., 2015). Compound 6 confirmed molecular weight $[M+H]^+$ 301 m/z and

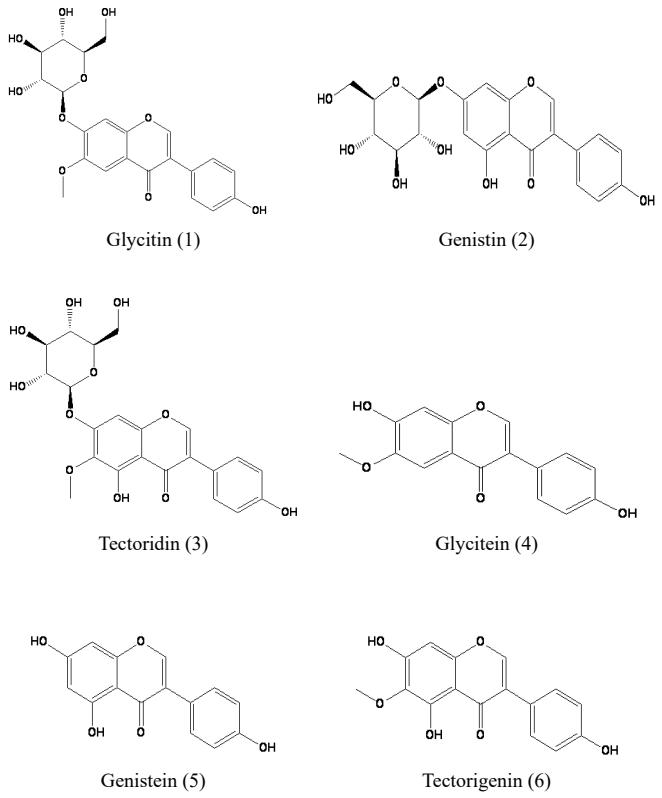

Figure 1. Structures of isoflavone compounds 1–6 from *Puerariae Flos* extract.

Table 1. LC/MS ESI analysis of compounds 1–6 identified from *Puerariae Flos* extract.

Peak No.	tR (min)	UV (λmax)	$[M+H]^+$ (m/z)	Fragment ions (+)	$[M-H]^-$ (m/z)	Fragment ions (-)	Identification	References
1	14.7	258, 322	447	252, 285, 371	445	206, 283, 431	Glycitin	18, 19
2	20.6	261	433	271, 301	431	269	Genistin	18, 19, 20
3	21.5	265	463	285, 301	461	299	Tectoridin	21
4	36.6	257, 321	285	188, 205, 285	283	-	Glycitein	19, 22
5	46.4	260	271	-	269	-	Genistein	19, 22
6	47.6	264	301	105, 252, 271	299	269	Tectorigenin	17, 21, 23

$[M-H]^-$ 299 m/z. By comparing the RT value and fragmentation pattern of the tectorigenin standard in HPLC, it was identified as $C_{16}H_{12}O_6$ 300.0 m/z tectorigenin (Bhat et al., 2014; Lu et al., 2013; Shu et al., 2010).

3.2 Specificity

The same RT value was measured as a result of HPLC-DAD analysis of the mixed solution of the sample and the index component. As a result of comparing the UV-spectrum of the index component peak, it was confirmed that the pattern was consistent (Figure 2).

3.3 Linearity, limit of detection and limit of quantitation

A total of six index components were diluted to a concentration of 10–100 μg/mL and repeatedly analyzed three times by HPLC. The measured values were used as a regression equation to obtain a calibration curve with an R^2 value of 0.9997 or higher, and the LOD and LOQ were calculated from the calibration curve (Table 2).

3.4 Precision and accuracy

Concentrations in three sections of 10, 40, and 100 μg/mL of the six standards, which have linearity confirmed, were repeated three times for analysis, and the RSD value was calculated to determine the precision. As a result, both intra-day and inter-day were found to be in the range of 0.05–0.61% and the value was confirmed to be below 2.0%, which is the standard guideline of the MFDS. To check the accuracy, the concentrations of 10, 40, and 100 μg/mL of the six standards were measured three times and analyzed. Ranges in intra-day showed as glycitin 99.08–101.22%, genistin 99.34–100.71%, tectoridin 98.98–102.96%, glycitein 99.38–100.51%, genistein 99.46–100.41%, and tectorigenin 99.23–100.82%. Ranges in inter-day showed as glycitin 99.87–100.41%, genistin 99.97–100.10%, tectoridin 99.69–100.82%, glycitein 99.87–100.41%, genistein 99.92–100.20%, and tectorigenin 99.97–100.10% (Table 3).

3.5 Stability

To check whether the chemical stability of the six indicator components of *Puerariae Flos* was maintained or not, the final concentration of 100 μg/mL was stored at room temperature for 0–24 h and the changes in content were measured (Table 4).

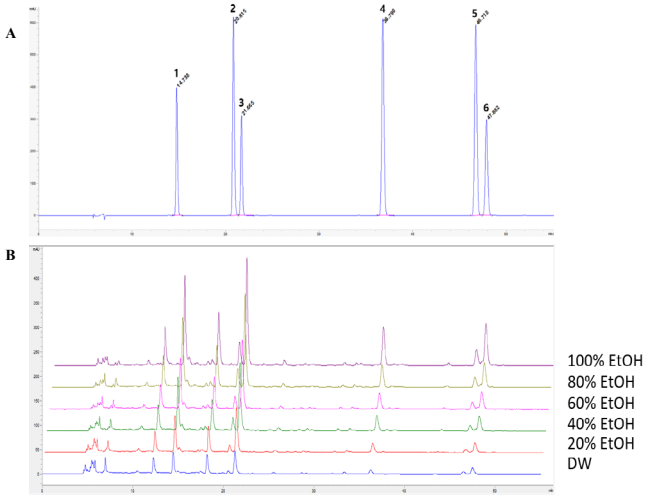

Figure 2. HPLC analytical chromatogram of marker compounds and *Puerariae Flos* extract. (A) HPLC traces of simultaneous analysis of isoflavone marker compounds 1–6 and (B) HPLC analysis chromatogram of *Puerariae Flos* extract.

Table 2. Linearity, LOD, and LOQ for six marker compounds.

Compound	Concentration (μg/mL)	Regression equation	R^2	σ	LOD (μg/mL)	LOQ (μg/mL)
Glycitin	9.8–98.0	y = 46.682x-46.898 y = 46.264x-35.943 y = 45.991x-24.770	0.9999 0.9999 0.9997	11.06	0.7884	2.3890
Genistin	9.9–99.0	y = 79.321x-69.426 y = 78.695x-50.769 y = 78.324x-36.188	0.9999 0.9998 0.9999	16.66	0.6979	2.1148
Tectoridin	9.8–98.0	y = 40.403x-44.801 y = 40.100x-36.925 y = 39.854x-27.219	0.9999 0.9998 0.9999	8.81	0.7244	2.1952
Glycitein	9.8–98.0	y = 100.28x-84.835 y = 99.657x-35.595 y = 99.072x-44.806	0.9999 0.9998 0.9999	20.06	0.6640	2.0122
Genistein	9.8–98.0	y = 104.91x-72.954 y = 104.44x-64.236 y = 104.29x-55.718	1.0000 0.9999 0.9998	8.62	0.2720	0.8243
Tectorigenin	9.8–98.0	y = 52.123x-41.354 y = 51.953x-38.894 y = 51.651x-28.809	0.9999 0.9998 0.9999	6.65	0.4226	1.2806

As a result, the rate of change before and after 24 h was 0.65–1.42%, which was measured to be below 2% of the standard guideline of the MFDS. With the results provided, the chemical stability of 6 types of indicator components for 24 h was confirmed.

3.6 Analysis of the content of Puerariae Flos extract

Table 5 shows the results of analyzing the content of the indicator components by diluting the extract for each concentration of *Puerariae Flos* ethanol by 50 times in methanol and by applying the HPLC analysis method.

4 CONCLUSION

Simultaneous analysis validation of the content analysis and analysis method of the indicator components was performed by the HPLC-DAD method for standardization of the components contained in *Puerariae Flos*. To verify the validity of the analysis method, the linearity showed that the regression equation and the calibration curve R^2 value were 0.9998 or higher. The detection and quantitation limits of each indicator component were measured in the range of 0.2720–0.7884 and 0.8243–2.3890 μg/mL. In the evaluation of precision and repeatability in the laboratory, the RSD value was measured to be less than 1%, and in accuracy, the recovery rate was 98.69–102.96%, which was confirmed to be less than 5% of the test method validation guideline for pharmaceuticals. The content change rate of the six indicator components before and after 24 h was 0.65–1.42%, which was below 2% or less according to the guidelines of the MFDS, confirming the stability for 24 h. Based on these research results, it is expected that basic data can be provided for the isoflavone content analysis of *Puerariae Flos*.

Table 4. Stability for six marker compounds.

Compounds	0 h	24 h	Differernce (%)
Glycitin	98.37 ± 0.57	99.58 ± 0.45	1.23
Genistin	99.30 ± 0.46	100.71 ± 0.36	1.42
Tectoridin	98.46 ± 0.53	99.94 ± 0.32	0.65
Glycitein	98.27 ± 0.41	99.50 ± 0.50	1.25
Genistein	98.28 ± 0.15	99.50 ± 0.23	1.24
Tectorigenin	98.28 ± 0.30	99.54 ± 0.32	1.28

Table 3. Precision and accuracy of six marker compounds.

Compounds	Concentration (μg/mL)	Intra-day			Inter-day		
		Mean ± SD (μg/mL)	RSD (%)	Accuracy (%)	Mean ± SD (μg/mL)	RSD (%)	Accuracy (%)
Glycitin	9.8	9.92 ± 0.04	0.40	101.22	9.84 ± 0.06	0.61	100.41
	39.2	38.84 ± 0.08	0.21	99.08	39.15 ± 0.04	0.10	99.87
	98.0	98.37 ± 0.57	0.58	100.38	98.02 ± 0.45	0.46	100.02
Genistin	9.9	9.97 ± 0.04	0.40	100.71	9.91 ± 0.06	0.61	100.10
	39.6	39.34 ± 0.02	0.05	99.34	39.59 ± 0.17	0.43	99.97
	99.0	99.30 ± 0.46	0.46	100.30	99.00 ± 0.36	0.30	100.00
Tectoridin	9.8	10.03 ± 0.03	0.30	102.96	9.88 ± 0.05	0.51	100.82
	39.2	38.80 ± 0.05	0.13	98.98	39.08 ± 0.16	0.41	99.69
	98.0	98.46 ± 0.53	0.54	100.47	98.04 ± 0.32	0.33	100.04
Glycitein	9.8	9.85 ± 0.05	0.51	100.51	9.84 ± 0.04	0.41	100.41
	39.2	38.96 ± 0.06	0.15	99.39	39.15 ± 0.10	0.26	99.87
	98.0	98.27 ± 0.41	0.42	100.28	98.02 ± 0.49	0.50	100.02
Genistein	9.8	9.84 ± 0.03	0.30	100.41	9.82 ± 0.06	0.61	100.20
	39.2	38.99 ± 0.07	0.18	99.46	39.17 ± 0.05	0.13	99.92
	98.0	98.28 ± 0.15	0.15	100.29	98.01 ± 0.23	0.23	100.01
Tectorigenin	9.8	9.88 ± 0.03	0.30	100.82	9.81 ± 0.05	0.51	100.10
	39.2	38.90 ± 0.10	0.26	99.23	39.19 ± 0.10	0.26	99.97
	98.0	98.28 ± 0.30	0.31	100.29	98.00 ± 0.31	0.32	100.00

Table 5. Major compound contents from *Puerariae Flos* extract

	Content (mg/g)					
Compounds	DW	20% EtOH	40% EtOH	60% EtOH	80% EtOH	100% EtOH
Glycitin	13.20 ± 0.04	22.34 ± 0.12	32.88 ± 0.09	31.39 ± 0.09	24.78 ± 0.68	55.34 ± 0.04
Genistin	1.75 ± 0.02	3.33 ± 0.05	5.69 ± 0.06	5.64 ± 0.03	7.59 ± 0.08	9.71 ± 0.03
Tectoridin	19.52 ± 0.15	35.83 ± 0.10	54.65 ± 0.22	56.06 ± 0.14	74.31 ± 1.11	86.72 ± 0.07
Glycitein	2.45 ± 0.11	4.22 ± 0.04	6.77 ± 0.14	7.19 ± 0.15	9.65 ± 0.18	16.27 ± 0.04
Genistein	1.63 ± 0.06	1.87 ± 0.01	2.81 ± 0.15	3.33 ± 0.22	4.12 ± 0.05	6.74 ± 0.50
Tectorigenin	6.18 ± 0.26	8.79 ± 0.15	13.09 ± 0.68	15.41 ± 0.98	20.05 ± 0.33	35.89 ± 2.38

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