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Rapid Isolation and Identification of Active Antioxidant Ingredients from Gongju Using HPLC-DAD-ESI-MS^n and Postcolumn Derivatization

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ABSTRACT: *Flos Chrysanthemi* (Gongju, GJ) is used to prepare a herbal tea that is commonly consumed as a health beverage in Asia and is believed to contain abundant beneficial antioxidants. To rapidly identify the chemical constituents and to obtain the profile related to antioxidant activity, an online analytical method combining high-performance liquid chromatography−diode-array detector−electrospray ionization−ion-trap time-of-flight mass spectrometry (HPLC-DAD-ESI-IT-TOF-MS^n) and postcolumn derivatization (PCD) has been applied for a precise and thorough identification of the chemical constituents. Meanwhile, the antioxidant profile has also been characterized by directly measuring the scavenging activity of each compound for the free radical produced by DPPH. As a result, 13 compounds have been identified in GJ, 7 of which account for its antioxidant activity. The established LC-MS^n-PCD system has proved to offer a useful strategy for correlating the chemical profile with the bioactivities of the components without their isolation and purification, and may be used for multicomponent analysis of active substances in other foods and herbs.

KEYWORDS: *Flos Chrysanthemi (Gongju), HPLC-DAD-ESI-IT-TOF-MS^n coupled with postcolumn derivatization (PCD), online identification, antioxidant constituents*

INTRODUCTION

Flos Chrysanthemi is the dried anthodium of *Chrysanthemum morifolium* Ramat.,[1] which grows in China, and has been commonly used for the preparation of health beverages for more than 2000 years.[2] In China, thousands of medicinal and edible chrysanthemum cultivar groups are available. According to the different origins and processing methods, chrysanthemums can be categorized into Hangju, Gongju, Chuju, Boju, etc. Gongju (GJ) was originally produced mainly in Shexian, Anhui Province. In 1896, it was first introduced to Deqing, Zhejiang Province,[3] which has since been known for the quality of its edible and medicinal chrysanthemum varieties. The harvest time of GJ starts in early November. Bloom time comes when the petals are flat and stamens are 60−70% spread. After being picked, the flowers are baked twice using charcoal as fuel. GJ possesses various bioactivities, including hydroxyl radical scavenging,[4] blood pressure reducing effects, and regulation of blood lipid levels.[5] According to previous studies, caffeoylquinic acids (CQAs) and flavonoids are regarded as major constituents of GJ.[6] Several flavonoids, such as acacetin-7-*O*-*β*-D-glucoside, apigenin-7-*O*-*β*-D-glucoside, and luteolin-7-*O*-*β*-D-glucoside, have been isolated from GJ.[6] Nevertheless, to the best of our knowledge, the chemical constituents of GJ and their antioxidative activities have yet to be investigated systematically, efficiently, and rapidly.

High-performance liquid chromatography coupled with diode-array detector sequential mass spectrometry (HPLC-DAD-MS^n) and ion-trap time-of-flight mass spectrometry (IT-TOF-MS) have various advantages, such as high resolution, accurate mass measurement, and high sensitivity,[7,8] and so have been widely used for structure identification of components in complex matrices. However, mass spectrometry is unable to provide some precise additional structural information, such as the positions of free phenolic or methoxyl groups or the linkage sites of sugar moieties. Postcolumn derivatization (PCD)[9−11] combined with HPLC-DAD-MS^n offers an online complementary analytical technique that is widely used to identify phenolic compounds by inducing shifts in their ultraviolet (UV) absorption maxima. In some recent studies,[9,12,13] PCD coupled with LC-MS/MS has been successfully applied to identify the structures of phenolic compounds in *Blumea gariepina*, sugar cane, and *Trifolium*.

In the present study, after identifying the compounds in GJ by means of HPLC-ESI-MS coupled with postcolumn derivatization (PCD), an online HPLC-DAD-DPPH assay was developed and applied to evaluate the radical-scavenging activities of the main components. LC-ESI-MS^n-PCD analysis gave the accurate molecular weights, and the fragmentation patterns acquired from multistage mass fragmentation gave some additional structural information, such as the position of free phenolic and methoxyl groups, and the linkage sites of sugar moieties, thus enabling a comprehensive understanding of the chemical structures in GJ. Meanwhile, to explore the active

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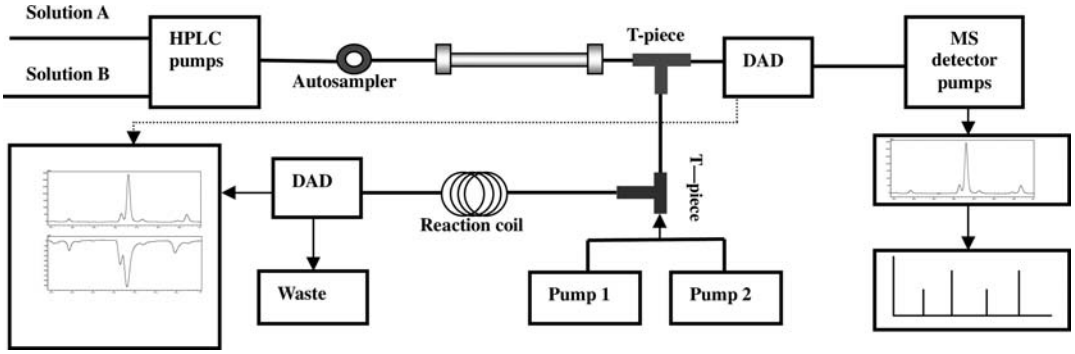

Figure 1. Schematic of online HPLC-DAD-ESI-MS^n-PCD system for identification and screening of antioxidants. An *n*-BuOH extract of GJ was the sample solution used; only one pump (pump 1) was used for the HPLC-DAD-DPPH assay; both pumps 1 and 2 were used for HPLC-DAD-UV-PCD analysis, and pump 2 was used to pump NaOH to adjust the pH; the split-flow ratio of the 3-way T-piece was 1:1; the reaction coil was PEEK tubing (10 m, 0.25 mm i.d.).

compounds responsible for the antioxidant activity of GJ, the antioxidant profile was investigated by HPLC-DAD-PCD, which involved direct detection of the scavenging activities of the free radical produced by DPPH of each peak in the fingerprint. HPLC-DAD-ESI-IT-TOF-MS^n-PCD (Figure 1) is an efficient method for the isolation and identification of active compounds, and might prove to be a powerful technique for rapid online evaluation of antioxidant activities in other food and herb samples.

MATERIALS AND METHODS

Chemicals and Materials. Petroleum ether (60−90 °C), *n*-butanol, and ethanol were of analytical grade and purchased from Beijing Chemical Plant (Beijing, China). Methanol, formic acid, and acetonitrile were of HPLC grade and purchased from Fisher (Fair Lawn, NJ, USA). The standard compounds, including luteolin, apigenin, diosmetin, and acacetin, were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (approximately 90%) was purchased from Sigma (Sigma-Aldrich, Shanghai, China). Boric acid, hydrochloric acid, sodium acetate, and aluminum chloride for the PCD system were purchased from Suzhou Yacoo Chemical Reagent Corporation (Jiangsu, China). Ultrapure water for chromatography was obtained from an Arium reversed osmosis system (Sartorius Stedim Biotech GmbH, Goettingen, Germany).

GJ, which originated from Shexian, Anhui Province, was purchased from an urban market in Anguo, Hebei Province, China. It was identified as *Chrysanthemum morifolium* Ramat. by Professor Hong Wang, associate professor of the School of Pharmaceutical Sciences, Peking University. Voucher specimens (*Flos Chrysanthemi*, No. GJS0102) were deposited in the Herbarium of the School of Pharmaceutical Sciences, Peking University.

DPPH Reagent and PCD System Solutions. DPPH solutions (0.12×10^{-3} M and 0.06×10^{-3} M) were prepared in methanol for off-line and online assays and were protected from light during the detection process.

For the PCD system, 0.01 mol·L^{-1} (M) NaOH (pH 12), 0.3 M $AlCl_3$ (pH 5.0), and 0.5 M NaOAc (pH 8.0) were prepared by dissolving the reagents in ultrapure water. A mixed solution (pH 6.0) of 0.1 M NaOAc and 0.7 M H_3BO_3 (1:1, v/v), and 0.3 M $AlCl_3$/HCl (adjusted to pH 3.5 by HCl) were also used as UV-shift reagents.

Sample Preparation. GJ was finely pulverized and then sieved through a 20 mesh sieve. A total of 20 g of GJ was extracted with ethanol/water (3 × 100 mL, 95%, v/v) with the assistance of ultrasonic oscillation (40 kHz, 250 W). The combined extracts were then filtered, and the filtrate was concentrated in vacuo to remove the ethanol. The remaining solid was freeze-dried to provide 2.03 g of extract, which was suspended in water (60 mL). The aqueous suspension was extracted with petroleum ether (3 × 20 mL) and with *n*-butanol (20 mL). Each solvent extract was concentrated in vacuo, and the remaining solids were freeze-dried to provide 0.71 g from the petroleum ether extract, 0.36 g from the *n*-butanol extract, and 0.67 g from the aqueous phase.

Instrumentation. HPLC-DAD analysis of GJ extract was performed on a Shimadzu (Kyoto, Japan) series 2020 liquid chromatography system. The components were separated on a YMC C_{18} column (150 mm ×4.6 mm i.d.; 5 μm particle size) connected to a Diamonsil C_{18} column (250 mm ×4.6 mm i.d.; 5 μm particle size). HPLC-DAD-ESI-MS^n was performed on a Shimadzu (Kyoto, Japan) series directly after the DAD measurements. Spectrophotometric determination was performed on Shimadzu 1800 (Kyoto, Japan). Ultrasonic extraction of GJ was performed with a KQ250DE ultrasonic cleaner (Kunshan, China).

Off-Line DPPH Assay. Using an improved method based on that of Larrauri and Yokozawa,[14,15] a series of samples were prepared in methanol as follows: 0.15 mL of sample solution was diluted with 2.85 mL of methanolic DPPH solution (0.12 mmol·L^{-1}), and the mixture was incubated at 37 °C for 30 min. The absorbance at 515 nm was measured using a spectrophotometer. Each sample was measured three times, and the average absorbance was used to determine the DPPH radical-scavenging rate, which is indicative of antioxidant activity strength.

HPLC-DAD-ESI-IT-TOF-MS^n-PCD System and Analysis Conditions. HPLC-DAD analysis was performed on a Shimadzu (Kyoto, Japan) series 2020 liquid chromatography system. The active extract (50 mg) was diluted to 25 mL with methanol, and the solution was filtered through a 0.22 μm membrane (Millipore). The components were then separated on a YMC C_{18} column (150 mm ×4.6 mm i.d.; 5 μm) coupled with a Diamonsil C_{18} column (250 mm ×4.6 mm i.d.; 5 μm) at a flow rate of 1.0 mL·min^{-1} using water/formic acid (100:0.08, v/v) (solvent A) and acetonitrile/formic acid (100:0.08, v/v) (solvent B) as mobile phases (0→15 min, 12% B→20% B; 15→55 min, 20% B→21% B; 55→60 min, 21% B→25% B; 60→80 min, 25% B→34% B; 80→100 min, 34% B→40% B; 100→110 min, 40% B→60% B; and 110→120 min, 60% B→100% B). The DAD was set to monitor absorbance at 335 nm.

A Shimadzu HPLC-IT-TOF (Kyoto, Japan) spectrometer with an electrospray ionization (ESI) interface was used under the following conditions. The interface voltage and current were set at 4.50 kV and 1.6 μA, respectively, in the positive-ion mode. The flow rate of the nebulizing gas was 1.5 L/min. The curved desolvation line (CDL) and heating block temperatures were both 200 °C. The end-cap acceleration voltage in the positive-ion mode was −4.0 V. The relative energy in all collisions was 50%. The detector voltage of the TOF analyzer was 1.65 kV. Argon of ultrahigh purity was used as the collision gas in the CID experiments. Masses from 50 to 1000 Da were calibrated using sodium trifluoroacetate solution (2.5 mmol·L^{-1}). For data acquisition and processing, the LCMS Solution 3.3 software supplied with the instrument was used.

HPLC-DAD-DPPH/UV postcolumn derivatization was performed according to the method previously described in the literature.[9,11,16] Diagnostic reagents were added to accurately determine the type of flavonoid and the numbers and locations of substituents. The DPPH solution was added to detect fingerprints of the antioxidant activity of the chemicals online. The instrumentation is shown schematically in Figure 1. The postcolumn derivatization conditions are presented in Table 1.

Table 1. Experimental Conditions for Postcolumn Addition of UV-Shift Reagents and Methanolic DPPH Solution

pump 1	flow 1 ($mL \cdot min^{-1}$)	pump 2	flow 2 ($mL \cdot min^{-1}$)	temperature (°C)
NaOH	1.0	$AlCl_3$	0.3	90
NaOH	0	$AlCl_3$/HCl	0.3	90
NaOH	0.9	NaOAc	0.5	90
NaOH	0.5	NaOAc/H_3BO_3	0.6	90
DPPH	0.2			55

RESULTS AND DISCUSSION

Antioxidant Capacities of the Extracts from GJ by the off-Line DPPH Assay. Each extract was measured three times by the off-line DPPH assay, and the average absorbance was used to determine the DPPH radical-scavenging rate, which indicates antioxidant activity strength. The DPPH scavenging rate was calculated according to the following equation:

$$[(A_{control} - A_{sample})/A_{control}] \times 100\%$$

where $A_{control}$ and A_{sample} are the absorbances of the control and the sample solution after the addition of DPPH solution, respectively. The parameter IC_{50} indicates the concentration of extract required ($mg \cdot L^{-1}$) to clear 50% of the DPPH free radicals. The DPPH free-radical scavenging capacities of different solvent extracts of GJ are shown in Figure 2.

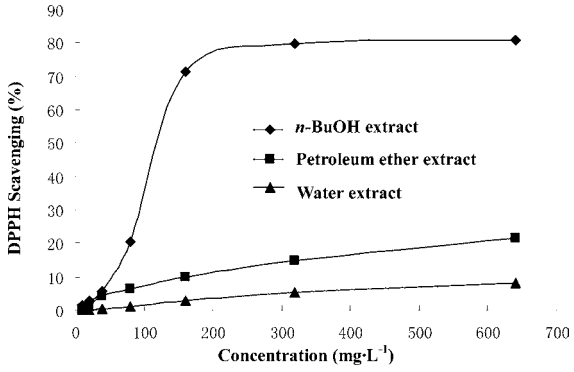

Figure 2. DPPH free-radical scavenging capacities of GJ fractions extracted by different solvents.

The IC_{50} of the *n*-butanol extract was estimated with a nonlinear regression algorithm (SPSS 18.0 software), while the values for the petroleum ether and aqueous extracts were predicted on the basis of a linear regression algorithm. While both the petroleum ether and *n*-butanol extracts of GJ possessed DPPH radical-scavenging activity, the *n*-butanol extract was much more potent than the petroleum ether extract (IC_{50} = 169 vs 795 $mg \cdot L^{-1}$). The aqueous extract possessed minimal radical-scavenging activity (IC_{50} = 3826 $mg \cdot L^{-1}$). The *n*-butanol extract possessing the highest antioxidant activity was passed through a 0.22 μm pore size filter and further analyzed by the online HPLC-DAD-ESI-IT-TOF-MS^n-PCD assay to quickly identify its chemical constituents and to obtain the profile related to antioxidant activity.

Identification of the Major Chemical Constituents in the *n*-Butanol Extract of GJ. The *n*-butanol extract of GJ showed a relatively high antioxidant activity. Therefore, it was necessary to determine the active components present in this extract and their composition. With the optimized parameters, the HPLC-DAD chromatogram of the *n*-butanol extract was recorded at 335 nm (Figure 3). The samples were analyzed by

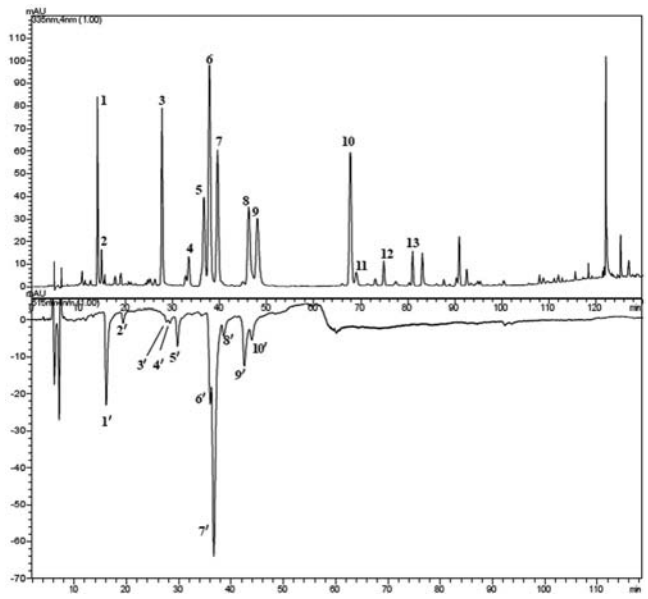

Figure 3. Chromatograms and activity profiles from online HPLC-DAD-MS^n-DPPH experiments.

mass spectrometry in both positive- and negative-ion modes for precise elucidation of the structures of the compounds in GJ. The total ion current (TIC) chromatogram of the sample in positive-ion mode is shown in Figure 4. It can be seen that most of the constituents were well separated under the applied conditions. Detailed MS data of the identified compounds and their fragments in the MS^n spectra are listed in Table 2. $[M + H]^+$ and $[M + H]^-$ ions of these compounds were used to calculate their molecular weights. Due to the characteristic ultraviolet (UV) absorption patterns of flavonoid molecules, the structures of the detected flavonoids and their modified analogues could be rapidly confirmed from their typical UV spectra. Diagnostic reagents were added to accurately determine the types of flavonoid and the numbers and locations of their substituents. This was done to supplement structure identification by mass spectrometry. The HPLC-UV data after the postcolumn addition of UV-shift reagents are given in Table 3. A total of 13 compounds were identified in the *n*-butanol fraction by the experimental procedures described above, including five chlorogenic acids and eight flavonoids, as shown in Table 4.

Chlorogenic Acid Derivatives. UV absorptions at 242 and 327 nm of compounds 1, 2, 5, 6, and 8 were in agreement with those previously reported for caffeoylquinic acids.[17] The MS^n data for the isolated compounds are shown in Tables 2 and 3. In negative-ion mode, compounds 1 and 2 gave $[M - H]^-$ peaks at *m/z* 353.0878 and *m/z* 353.0908 (corrected value: *m/*

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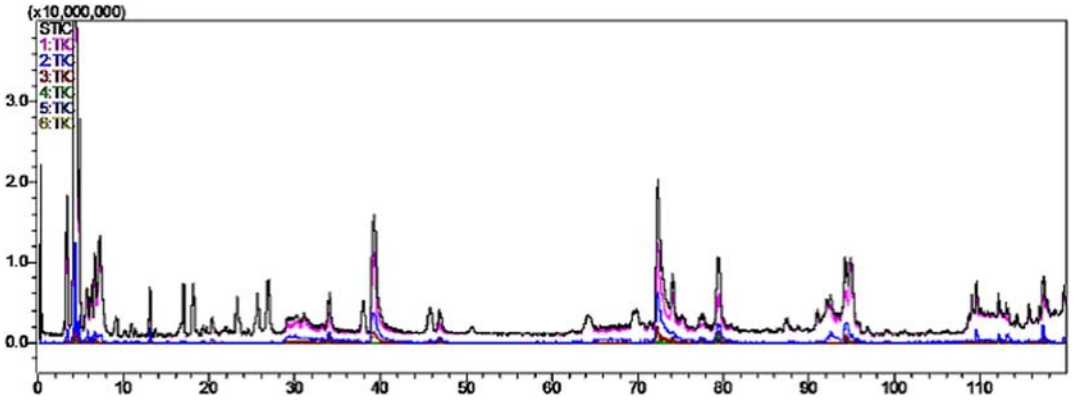

Figure 4. Total ion current (TIC) in positive-ion mode.

Table 2. Characterization of Constituents by LC-MS^n Analysis in Positive- and Negative-Ion Modes

no.	R_t (min)	experimental m/z (positive/ negative)	formula	(+)ESI-MS^n m/z (experimental)	(−)ESI-MS^n m/z (experimental)
			Chlorogenic Acids (CQA and DCQA)		
1	13.954	355.1029/353.0873	$C_{16}H_{18}O_9$	355.1057→163.0394→145.0296(117.0392)	353.0834→191.0572→85.0318
2	14.803	355.1029/353.0873	$C_{16}H_{18}O_9$	355.0998→163.0398→145.0285→117.0392	353.0859→173.0491→93.0334
5	36.613	517.1346/515.1190	$C_{25}H_{24}O_{12}$	517.1351→499.1197→319.0837→163.0356	515.1425→353.0883→ 191.0545(179.0485)→85.0318
6	37.789	517.1346/515.1190	$C_{25}H_{24}O_{12}$	517.1313→499.1260→319.0782→163.0380→ 145.0271→117.0339	515.1210→353.0879→ 191.0562(179.0404)→85.0408
8	46.151	517.1346/515.1190	$C_{25}H_{24}O_{12}$	517.1314→499.1197→319.0756→163.0356→ 135.0498	515.1191→353.0904→ 173.0449(179.0355)→93.0380
			Luteolin Type		
3	27.686	449.1084/447.0927	$C_{21}H_{20}O_{11}$	449.1082→287.0536→153.0204(135.0445)→ 97.0316	447.0887→285.0414→ 175.0379(199.0384)→147.0464
9	47.993	535.1088/533.0931	$C_{24}H_{22}O_{14}$	535.1049→449.1029→287.0535→15 3.0246(135.0498)	533.0891→489.1085→285.0441→175.0349
			Apigenin Type		
4	33.588	579.1714/577.1557	$C_{27}H_{30}O_{14}$	579.1669→433.1067→271.0577→15 3.0188(119.0485)	577.1568→269.0462→201.0557(183.0486)
7	39.930	433.1135/431.0978	$C_{21}H_{20}O_{10}$	433.1187→271.0619→153.0172(119.0414)→ 67.0192	431.0957→269.0488→149.0240
10	67.788	519.1139/517.0982	$C_{24}H_{22}O_{13}$	519.1108→433.1090→271.0630→15 3.0202(119.0573)	517.0816→269.0354→117.0355
11	69.054	475.1240/473.1084	$C_{23}H_{22}O_{11}$	475.1277→271.0601→153.0186(119.0472)→ 67.0152	473.1056→269.0489→117.0355
			Acacetin Type		
12	74.909	593.1870/591.1714	$C_{28}H_{32}O_{14}$	593.1869→447.1282→285.0775→270.0500→ 242.0577→153.0179	591.1857→283.0623→268.0402
13	81.047	447.1291/—	$C_{22}H_{22}O_{10}$	447.1345→285.0784→270.0517→242.0586→ 153.0194	—

Table 3. On-Line Ultraviolet Data of Compounds Determined in the Absence and Presence of Shift Reagents[a]

			shifted UV spectrum (nm)							
	UV spectrum (nm)		NaOAc		NaOAc/H_3BO_3		$AlCl_3$		$AlCl_3$/HCl	
compd	I	II	I	II	I	II	I	II	I	II
3	348	254 266 (sh)	345	254 266 (sh)	362	258	406	270	383	270
4	336	267	340	266	338	266	337 373	273 294	337 374	273 295
7	336	267	339	266	339	266	331 371	273 294	337 374	273 295
9	347	253 266(sh)	343	250 265(sh)	364	258	411	270	382	268
10	336	267	339	266	339	266	331 374	273 294	337 374	273 295
11	336	267	338	266	337	266	337 373	273 294	337 373	273 295
12	332	267	335	267	336	266	336 376	274 296	337 375	273 296
13	332	267	335	266	335	266	336 374	274 295	337 374	274 296

[a]sh, shoulder peak.

z 353.0873), respectively. The formula was calculated as $C_{16}H_{18}O_9$, which was consistent with the molecular weight of a caffeoylquinic acid. The MS^2 base peak of compound 1 was at m/z 191.0576 (corrected value: m/z 191.0556, $C_7H_{11}O_6^-$), and

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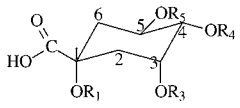
Table 4. Chemical Structures of the Identified Compounds[a]



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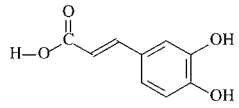

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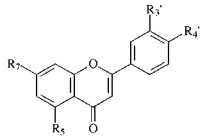
No.	Name	R_1	R_3	R_4	R_5
1	5-CQA	H	H	H	*C*
2	4-CQA	H	H	*C*	H
5	1,5-DCQA	*C*	H	H	*C*
6	3,5-DCQA	H	*C*	H	*C*
8	4,5-DCQA	H	H	*C*	*C*



No.	Name	R_5	R_7	$R_{3'}$	$R_{4'}$
3	luteolin-7-*O*-glucoside	OH	*O*-glucose	OH	OH
4	apigenin-7-*O*-rutinoside[a]	OH	*O*-rutinose	H	OH
7	apigenin-7-*O*-glucoside	OH	*O*-glucose	H	OH
9	luteolin-7-O-(6"-malonyl)glucoside[a]	OH	*O*-(6"-malonyl)glucose	OH	OH
10	apigenin -7-*O*-(6"-malonyl)glucoside[a]	OH	*O*-(6"-malonyl)glucose	H	OH
11	apigenin-7-O-(6"-acetyl)glucoside[a]	OH	*O*-(6"-acetyl)glucose	H	OH
12	linarin	OH	*O*-rutinose	H	OCH_3
13	acacetin-7-*O*-glucoside	OH	*O*-glucose	H	OCH_3

[a]Compounds identified in GJ for the first time.

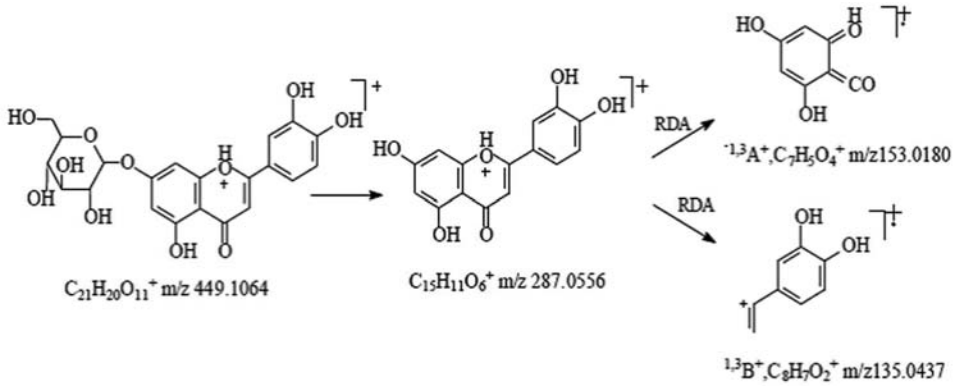


Figure 5. Fragmentation scheme for flavone in positive-ion mode (taking luteolin-7-*O*-glucoside as an example).

the MS^3 base peak was at *m/z* 85.0319. In addition, the relative abundance of the ion fragment at *m/z* 127.0582 was greater than 25%. According to the literature[11,17,18] and the absence of a fragment ion at *m/z* 179 in the MS^2, the compound was either 1-CQA or 5-CQA. Compound 1 was identified as 5-CQA by comparison of its UV spectrum, MS^n data, and relative retention time with those of a standard sample of 5-CQA. The identity of compound 2 was confirmed as 4-CQA based on the base peaks at *m/z* 173.0471 in the MS^2 and *m/z* 93.0380 in the MS^3, which were in accordance with literature values.[17−19] Compounds 5, 6, and 8 showed $[M - H]^-$ peaks at *m/z* 515.1645, 515.1199, and 515.1865 (corrected value *m/z* 515.1190), respectively. The formula was calculated as $C_{25}H_{24}O_{12}$, which has the appropriate molecular weight for a dicaffeoylquinic acid. Compound 5 was confirmed as 1,5-DCQA on the basis of the following base peak values: MS^2, *m/z* 353.0876 (corrected value *m/z* 353.0873, $C_{16}H_{17}O_9^-$); MS^3, *m/z* 191.0553 (corrected value *m/z* 191.0556); MS^4, *m/z* 85.0318 (corrected value *m/z* 85.0290), and the absence of an ion fragment peak at *m/z* 179 in the MS^3. These values were in agreement with the mass spectral data for 1,5-DCQA.[17−19] Compound 6 was confirmed as 3,5-DCQA by comparison with a standard 3,5-DCQA sample. In addition, its base peak values in MS^3, *m/z* 191.0560 (corrected value *m/z* 191.0556), with a significant ion fragment peak at *m/z* 179.0351, MS^4, *m/z* 85.0273 (corrected value of *m/z* 85.0290), were in accordance with the MS^n data of 3,5-DCQA.[17−19] The base peak values of compound 8 were found as MS^3, *m/z* 173.0468, MS^4, *m/z* 93.0380, which were consistent with 3,4-DCQA or 4,5-DCQA. Since compound 8 did not show a fragment at *m/z* 335 in MS^2, a possible structure was 4,5-DCQA,[19] and this was confirmed by comparison with literature data.[17]

Flavonoid Derivatives. All of the flavonoids identified from GJ had an aglycone structure with a 5,7-dihydroxy motif and formed glycosides with a sugar via the 7-OH group, as determined by flavonoid MS cracking patterns and postcolumn derivatization results. All of the flavonoids produced fragments $^{1,3}A^+$ and $^{1,3}B^+$ through an RDA reaction. Their common characteristic was that all $^{1,3}A^+$ fragments appeared at *m/z* 153.0180 (theoretical value), suggesting that the A ring in GJ has a dihydroxy motif. Different substituents on the B-ring

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could be determined according to the $^{1,3}B^+$ fragment mass/charge ratios, as shown in Figure 5.

Postcolumn derivatization techniques[9,11,16] were used to determine the A ring and B ring substituents of each flavonoid. This method improves the reliability of mass spectrometric information. The postcolumn derivatization of the flavonoids showed the following: compared with the original UV spectra, band I for each compound was red-shifted by 21−43 nm after the addition of $AlCl_3$/HCl as a diagnostic reagent, indicating a structure with a free 5-hydroxy group. When NaOAc was added as a diagnostic reagent, there was no red shift of band II, indicating no free 7-hydroxy group in the structure, as shown in Figure 6.

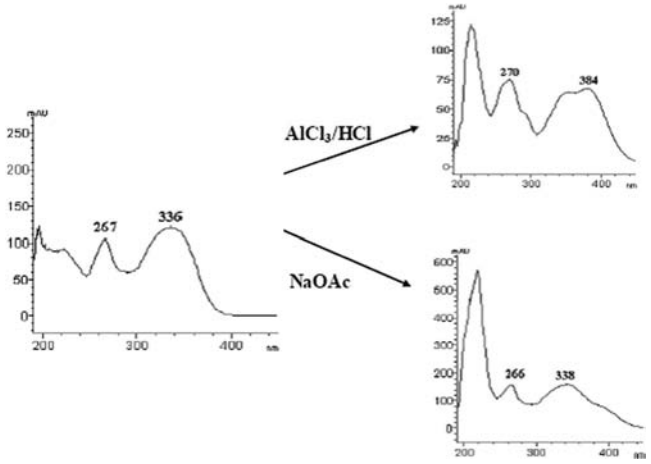

Figure 6. UV-derivatization spectra for flavonoids in Gongju.

As many as eight flavonoids (shown in Table 4) were identified based on mass spectrometric data coupled with UV-shift data from the PCD system. Among them, compounds 3, 7, 12, and 13 were unambiguously identified as luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, acacetin-7-*O*-glucoside, and linarin by comparing their experimental retention times, MS^n data, and UV-shift data with those of standards. Compounds 9 and 10 showed $[M + H-248]^+$ and $[M + H-86]^+$ fragments due to the loss of malonyl-glucose and malonyl ($-COCH_2COO^-$) from their molecular ions, suggesting that they were malonyl glucosides of luteolin and apigenin. The fragment ion $[M + H-204]^+$ at m/z 271.0606 ($[M + H-ace-glc]$) of compound 7 was indicative of the acetyl glucoside of apigenin. In the MS^n spectra of compound 4, $[M + H-146]^+$ at m/z 433.1096 (base peak) and $[M + H-146-162]^+$ at m/z 271.0582 were formed through the loss of rhamnose and glucose (relative abundance 31.65%). Therefore, compound 4 was identified as rutinoside. In the postcolumn derivatization of compounds 4, 9, 10, and 11, there were no red-shifts of band II after using NaOAc as a diagnostic reagent, indicating no free 7-hydroxy groups. Therefore, these compounds were identified as apigenin-7-*O*-rutinoside, apigenin-7-*O*-(6″-acetyl)glucoside, apigenin-7-*O*-(6″-malonyl)glucoside, and luteolin-7-*O*-(6″-malonyl)glucoside, respectively, and this is the first time that they have been identified in GJ. The compounds identified in GJ add to the chemical structure information for further study on the efficacious material basis of antioxidant activity.

Online Assay for Screening Compounds with Antioxidant Activity. A total of 13 compounds were separated and detected by the online HPLC-DAD-ESI-IT-TOF-MS^n-PCD method in the *n*-butanol extract of GJ. The chromatogram and online HPLC-DAD-DPPH profile are shown in Figure 3. Peaks in the HPLC fingerprint with antioxidant activity gave rise to counterparts in the corresponding inhibition profile, and the DPPH inhibition capability of a compound was proportional to the intensity of its negative peak. The strength of antioxidant activity of the compounds detected by online HPLC-DAD-DPPH was measured in terms of their contribution to the overall radical scavenging (RSP, radical scavenger profile), calculated according to the following equation:

$$RSP = [A_{(x)}/A_{(\text{total})}] \times 100\%$$

where $A_{(x)}$ is the area under the negative peak of a reactive compound at 515 nm, and $A_{(\text{total})}$ indicates the total peak area at 515 nm of all active compounds in the GJ extract.

For validation of the online method and further evaluation and comparison of the strength of antioxidant activity, seven reference standards were detected using off-line DPPH assay (taking rutin as a positive control). According to Figure 3 and Table 5, 5-CQA, 1,5-DCQA, 3,5-DCQA, and 4,5-DCQA showed high contributions to the total peak areas both in the HPLC fingerprint and the activity profile. Indeed, their total contributions amounted to more than 85%, suggesting that they were main ingredients and the principal compounds responsible for the antioxidant activity of GJ. Meanwhile, only relatively minor contributions to the total activity were observed for some flavonoids, including compounds 3′, 8′, and 10′ illustrated in Table 5. According to previous studies,[19−22] the antioxidant mechanism involves hydroxyl groups combined with oxygen-centered free radicals, which form semiquinoid free radicals and terminate chain reactions. The activities of the caffeoylquinic acids and flavonoids are different because of differences in the numbers and positions of the hydroxyl groups,[23−25] which was corroborated by the detection of standards using off-line DPPH assay. According to Table 5, all of the IC_{50} values of the caffeoylquinic acids were lower than those of flavonoid glycosides and rutin (positive control), indicating that they possessed stronger activity than flavonoids and were the main active antioxidant compounds.

Table 5. Peak Areas, RSP, and IC_{50} Values Obtained by Investigating Scavenging Activity on DPPH Radicals[a]

no.	compd	area	RSP (%)	IC_{50} ($mg \cdot L^{-1}$)
1′	5-CQA	825,056	22.28	123.13
2′		80,851	2.18	
3′	luteolin-7-*O*-glucoside	25,329	0.68	175.01
4′		14,303	0.39	
5′		223,288	6.03	
6′	1,5-DCQA	119,288	3.22	56.70
7′	3,5-DCQA	1,760,613	47.54	73.38
8′	apigenin-7-*O*-glucoside	100,613	2.72	169.81
9′	4,5-DCQA	447,287	12.08	53.86
10′	luteolin-7-*O*-(6″-malonyl) glucoside	106,438	2.87	171.04
	Rutin			200.71

[a]Rutin was used as an antioxidant standard (positive control); area, integral area of negative peak in the antioxidant profile; RSP, the relative contribution to the overall radical scavenging; IC_{50}, the concentration of standard required ($mg \cdot L^{-1}$) to clear 50% of the DPPH free radicals (using off-line DPPH assay).

Moreover, three unknown negative peaks (peaks 2′, 4′, and 5′) in the chromatograms exhibited favorable radical-scavenging activities, and these will be studied in due course.

In summary, an online HPLC-DAD-ESI-IT-TOF-MS-PCD system has been developed for structural identification and antioxidant activity detection of the constituents of GJ, among which four isolated compounds (4, 9, 10, and 11) have been identified in GJ for the first time. Seven of the identified compounds possess antioxidant activity; CQAs proved to be the main active compounds in GJ, contributing more than 85% to the total radical-scavenging rate. The rapid separation and identification of the main constituents of GJ has allowed elucidation of the efficacious material basis of its antioxidant activity. Moreover, the assay applied in this study, including both rapid structural identification and activity screening, offers a fast and effective method for the separation and identification of active ingredients and might be a powerful technique for the rapid online evaluation of the antioxidant activities of other complex food and herb samples.

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Notes

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