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Full Length Research Paper

Simultaneous analysis of eight phenolic compounds in *Phyllanthus simplex* Retz by HPLC-DAD-ESI/MS

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A new, simple and sensitive high-performance liquid chromatography-diode array detector (HPLC-DAD) method was developed for the simultaneous determination of eight phenolic components in *Phyllanthus simplex* Retz. The chemical profile of the eight components, including gallic acid, caffeic acid, quercetin, ferulic acid, luteolin-7-*O*-glucoside, 8,9-epoxy brevifolin, brevifolin and globulariacitrin were acquired by using high-performance liquid chromatography-diode array detector coupled to an electrospray ionization mass spectrometer (HPLC-DAD-ESI/MS). The optimal condition of separation was achieved on a ZORBAX Extend C_{18} column (5 µm, 250×4.6 mm i.d.) with a gradient elution of methanol and 1% acetic acid at a flow rate of 1.0 ml/min. Under ultra violent (UV) detection at 275 nm, the recoveries of the analytes were in the range of 99.2 to 99.8% with a RSD of 0.41 to 1.50%. The limits of detection and quantification for the analytes were ranged from 55 to 140 ng/ml and from 155 to 320 ng/ml, respectively. The intra-day RSD% ranged from 0.32 to 0.82% and the inter-day RSD% were not higher than 5.0%. The proposed method is suitable for quantitative and qualitative determination of the eight bioactive phenolic components in *P. simplex* Retz.

Key words: *Phyllanthus simplex* Retz, phenolic components, high-performance liquid chromatography-diode array detector coupled to an electrospray ionization mass spectrometer (HPLC-DAD-ESI/MS).

INTRODUCTION

The genus *Phyllanthus* (Euphorbiaceae) consists of about 600 species mainly distributed in tropical and subtropical areas of the world, there are about 33 species grown in China. Many species of *Phyllanthus* are widely been used as traditional Chinese medicine (TCM) owing to their therapeutic effects on dysentery, diarrhea, enteritis, nephritis edema, hepatitis and urinary tract infection (Niu et al., 2006). Because of their bioactivities, some species of *Phyllanthus* had been studied, and the presences of various phenolic compounds are well documented (Zhou et al., 2007; Fan et al., 2006; Niu et al., 2008; Zhang et al., 2000). In our laboratory, we have firstly carried out phytochemical studies on *Phyllanthus simplex* Retz, a traditional Chinese herbal plant widespread in China. Finally, eight phenolic compounds, that is, gallic acid, caffeic acid, quercetin, ferulic acid, luteolin-7-*O*-glucoside, 8,9-epoxy brevifolin, brevifolin and globulariacitrin have been isolated from the ethanol extract of the whole plant of *P. simplex* Retz., and their molecular structures (Figure 1) have also been elucidated by means of various spectroscopic methods.

Plant phenolics are defined as compounds possessing one or more aromatic ring bearing a hydroxyl substituent (s), and are widely distributed in the plant kingdom. The importance of antioxidant activities of phenolic compounds and their possible usage in processed foods as a natural antioxidant have reached a new high in recent years (Rocha-Guzmán et al., 2009; Altunkaya and Gökmen, 2009; Mousavinejad et al., 2009). Antioxidants are considered key-compounds in the fight against of various diseases (e.g. cancer, chronic inflammation, atherosclerosis and cardiovascular disorder) and ageing processes. Moreover, the relevance of using antioxidants from natural sources has been considerably enhanced by consumer's preference for natural products and concerns about the toxic effects by synthetic antioxidants (Zubia et al., 2009; Ito et al., 1986; Kohen and Nyska, 2002). Due to the foregoing reasons, eight phenolic compounds isolated from the plant, maybe accounted for the biological activity

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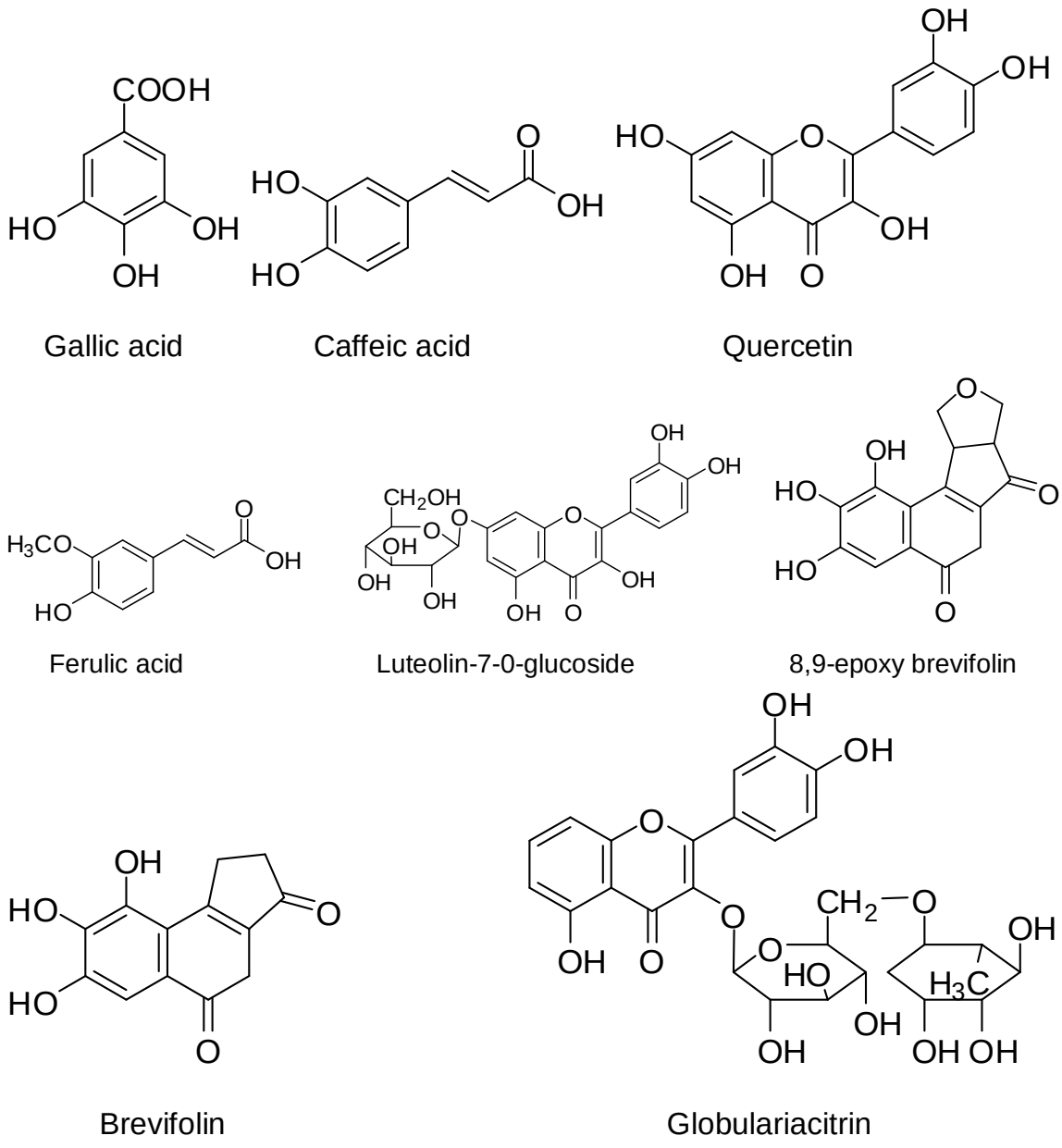

Figure 1. The structures of the eight phenolic compounds from *P. simplex* Retz.

of their plants to some extent, and the levels of the contents can be an important norm in the evaluation of these plants. Therefore, quantitative analysis of these constituents is of great significance for the crude drug's quality control.

Efficient detection and rapid characterization of natural products play an important role as an analytical support in the work of natural products chemists. Existing methods for the analysis of phenolic compounds involved high-performance liquid chromatography (HPLC) (Weisz et al., 2009; Proestos et al., 2005), thin layer chromatography (TLC) (Malbaša et al., 2004), capillary electrophoresis (CE) (Hinneburg et al., 2004; Peng et al., 2005), and gas chromatography (GC) (Du et al., 2009). Among several chromatographic methods, HPLC is the most widely used technique for both qualitative and quantitative analysis of phytochemical substance. During the past decade, HPLC and coupled techniques, especially DAD and MS, have been proved to be a powerful approach for the rapid identification of the constituents in natural materials because DAD and MS as a sensitive detector could provide abundant structural

information and thus facilitate the structural identification of unknown compounds. Until now, to the best of ourknowledge, no data have been reported on the simultaneous determination of these eight phenolic compounds in *P. simplex*.

In this paper, we described a HPLC method on identifying eight phenolic compounds in *P. simplex*. Using a high-performance liquid chromatography-diode array detector coupled to an electrospray ionization mass spectrometer (HPLC-DAD-ESI/MS) technique, the eight phenolic compounds were identified and characterized. The MS spectra and ultra violent (UV) data obtained were applied to produce a library that allowed the complete identification of characteristic peaks in chromatographic spectrum of *P. simplex*. Their contents in *P. simplex* also were determined by authentic standards and could be used to evaluate the crude drug's quality. In addition, a simple, rapid and accurate analysis method was presented.

MATERIALS AND METHODS

Chemicals and reagents

Methanol was purchased from Merck (Darmstadt, Germany) and of HPLC grade. Ultrapure water was prepared by a Milli-Q50 SP reagent water system (Millipore Corporation, MA, USA). Acetic acid was purchased from Tedia (Ohio, USA) and of analytical grade. All other organic solvents used in this study were of analytical grade from Tianjing Chemical Reagent Corporation (Tianjing, China).

The reference phenolic compounds, gallic acid, caffeic acid, quercetin, ferulic acid, luteolin-7-*O*-glucoside, brevifolin and globulariacitrin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and 8,9-epoxy brevifolin was isolated from *P. simplex* Retz by our laboratory, purity analysis suggested that its purity was above 98%.

Ten batches of *P. simplex* Retz were collected in five consecutive years (2005 to 2009), two batches per year, one was wild (w) grown in Shaanxi province while another was cultured (c) in Xi'an Jiaotong University. A voucher specimen of these collections was identified by Prof. Yingli Li and deposited at Herbarium of Faculty of Pharmacy, Medical School of Xi'an Jiaotong University, Xi'an, P. R. China.

Instrumentation and analytical conditions

The HPLC system used for the phenolic compounds separation was an Agilent-1100 HPLC system with diode array detector coupled with a LC/MSD Trap XCT electrospray ionization mass spectrometer in series both from Agilent (Agilent 1100), equipped with quaternary pump, vacuum degasser, autosampler and column heater-cooler. The chromatographic separation was performed on a ZORBAX Extend C_{18} column (5 µm, 250×4.6 mm i.d.) (Agilent Corporation, MA, USA) with the column temperature set at 25℃. Lin ear gradient elution was used with methanol from pump A and 1% acetic acid from pump B at a flow rate of 1.0 ml/min, with the procedure as followed (v/v): 5 to 25% A (0 to 8 min), 25 to 55% A (8 to 20 min) and 55 to 100% A (20 to 30 min). The column was re-equilibrated with the initial condition for 15 min before the next injection. Analytes were injected via an autosampler and eluate was monitored by a DAD detector, set to scan between 200 and 600 nm. The effluent from the column passed through the DAD detector was delivered into the MS detector by solvent splitting with 0.2 ml/min portion of the column effluent was delivered into the ion source of mass spectrometry for analysis. MS spectra were acquired in positive and negative ion mode and parameters for acquisition of mass spectral data were as follows, drying gas (N_2, 8 ml/min), capillary temperature (350℃), pressure of nebulizer (30 psi), ESI n eedle voltage (3.5 KV), scan range (100 to 800 m/z). Data acquisition was performed on a Chemstation software (Agilent Corporation, MA, USA). The temperature of the column during analysis was maintained at 25℃. The injection volume was 20 µL each time.

Preparation of standard solutions

Reference standards, gallic acid, caffeic acid, quercetin, ferulic acid, luteolin-7-O-glucoside, 8,9-epoxy brevifolin, brevifolin and globulariacitrin, were accurately weighted, then dissolved in methanol and diluted to appropriate concentration, respectively. The stock solutions were stored at 4℃ and brought to room temperature before their use. Calibration standard working solutions were freshly prepared by appropriate dilution of the stock solutions giving final concentration at the range of 2.2 to 220.0 µg/ml for gallic acid, 2.0 to 200.0 µg/ml for caffeic acid, 3.5 to 350.0 µg/ml for quercetin, 3.0 to 450.0 µg/ml for ferulic acid, 2.5 to 250.0 µg/ml for luteolin-7-O-glucoside, 1.5 to 150.0 µg/ml for 8,9-epoxy brevifolin, 2.0 to 200.0 µg/ml for brevifolin, and 7.5 to 750.0 µg/ml for globulariacitrin.

Preparation of sample solution

After being air-dried and crushed into 50 mesh powder, ten batches of *P. simplex* Retz were accurately weighed (each 5.0 g) and then ultrasonically extracted with 80 ml methanol for 45 min. The solution was cooled down to the ambient temperature, and diluted with methanol to volume. The obtained solution was filtrated through a membrane filter (0.45 µm pore size) prior to injection.

Method validation

Precision tests were carried out by replicated injections, and the results showed that relative standard deviation (RSD) of the peak area of the eight phenolic compounds were 0.93% for gallic acid, 1.02% for caffeic acid, 0.55% for quercetin, 0.75% for ferulic acid, 0.80% for luteolin-7-O-glucoside, 0.91% for 8,9-epoxy brevifolin, 0.67% for brevifolin, and 0.49% for globulariacitrin respectively (n = 5).

The stability of the assay was evaluated by inter-day variability. The standard solution was analyzed on five consecutive days, and the RSDs of the phenolic compounds were not higher than 5% (n = 5).In order to verify the accuracy and precision of the analytical procedure, the recoveries of the eight compounds were carried out by the method of standards addition. Suitable amounts (about 50% of the content) of the eight standards were spiked into a sample of *P. simplex* Retz (lot No. 200905c), which had been determined previously. The mixture was extracted and analyzed using the proposed procedure. For comparison, an unspiked sample was concurrently prepared and analyzed simultaneously. As shown in Table 1, the mean recoveries of the compounds were in the range of 99.2 to 99.8%, with RSD values ranged from 0.41 to 1.50% (n = 5).

RESULTS

Optimization of chromatographic conditions

The optimization of chromatographic conditions was

Table 1. Statistical results of recovery of the eight phenolic compounds (n = 5).

Compounds	Added amount (mg)	Recorded amount (mg)	Recovery (%)	R.S.D. (%)
Gallic acid	2.3	2.289 ± 0.014	99.5 ± 0.6	0.43
Caffeic acid	1.0	0.992 ± 0.020	99.2 ± 2.0	1.50
Quercetin	1.4	1.396 ± 0.018	99.7 ± 1.2	0.94
Ferulic acid	1.2	1.195 ± 0.008	99.5 ± 0.8	0.58
Luteolin-7-O-glucoside	1.5	1.494 ± 0.009	99.6 ± 0.6	0.41
8, 9-epoxy brevifolin	1.0	0.998 ± 0.014	99.8 ± 1.4	0.93
Brevifolin	1.0	0.996 ± 0.006	99.6 ± 0.6	0.42
Globulariacitrin	3.0	2.994 ± 0.028	99.8 ± 0.9	0.68

guided by the need of obtaining chromatograms with better resolution of adjacent peaks, especially when numerous similar components were to be analyzed. According to reference (Weisz et al., 2009), owing to the phenolic feature of the analytic compounds, an acidified elution was used, which allowed for a satisfactory separation. To improve the peak shape (restrain the peak tailing), acetic acid was added as a mobile phase modifier to inhibit the dissociation of the phenolic hydroxy group of the analytes. The preliminary studies indicated that acetic acid provided efficient separation. Since the polarity of the eight analytes differ greatly from each other and the slight diversities of some structures, which present special problems for their simultaneous separation and determination. When methanol-1% acetic acid or acetonitrile-1% acetic acid with isocratic was used as the mobile phase, it is difficult to separate them and a bad resolution or long run time occurred. In an effort to achieve more rapid and efficient separation of the eight phenolic compounds, much better resolution was achieved by gradient elution. After trying several types of gradients and varying duration, an optimum solvent system was found to be, methanol from pump A and 1% acetic acid from pump B at a flow rate of 1.0 ml/min, with the procedure as followed: (v/v), 5 to 25% A (0 to 8 min), 25 to 55% A (8 to 20 min), 55 to 100% A (20 to 30 min), which led to good resolution and satisfactory peak shape. In our study, it was observed that separation could not be affected obviously by column temperature, so the column temperature was set at 25℃ during analysis.

Diode array detector (DAD) was employed at wavelength range of 200 to 600 nm to investigate the UV spectra of the eight phenolic compounds and the methanol extract from *P. simplex* Retz. The wavelength was adjusted to 275 nm for the measurement of the eight phenolic compounds and the methanol extract from *P. simplex* Retz, for the eight analytes showed strong absorbance near 275 nm, and at this detection wavelength, it could properly represent the profile of the constituents of the methanol extract with good separation and high sensitivity. Under the proposed conditions, the ten batches of samples were analyzed and their chromatograms are shown in Figure 2.

Identification of eight phenolic compounds in *P. simplex* Retz

In the present study, the effluent from the column passed through the DAD detector was delivered into the MS detector by solvent splitting; with 0.2 ml/min portion of the column effluent was delivered into the ion source of mass spectrometry for analysis. The MS spectra of major components from *P. simplex* Retz were acquired in positive and negative ion mode and their total ion chromatograms (TIC) were obtained under the conditions mentioned previously.

Table 2 represents specific fragmentation behavior of the eight phenolic compounds, their fragmentation patterns were well matched with their chemical structures. For the eight phenolic compounds, clearly discernible quasi-molecular ions $[M+H]^+$, $[M+Na]^+$ or $[M-H]^-$ could be observed, which were the base peaks. Other fragmentations, for example, losing of $-CH_3$, -CO and –OH, etc., could also be observed in their MS^2 spectra.

On the basis of the m/z value, UV spectra, retention feature and by comparison with authentic standards, the eight phenolic compounds were identified from methanol extract in ten batches of *P. simplex* Retz.

Linear ranges and detection limits of the eight phenolic compounds

The calibration was based on the three duplicate analysis of working solutions at six concentration levels. The regression equation was calculated in the form of $y = ax + b$, where y and x were the values of peak area and concentration of each reference compound, respectively.

Table 3 lists the regression equations, correlation coefficients, linear ranges for calibration graphs and limits of detection (LODs) and quantification (LOQs) for the eight analytes in the optimum conditions. All the phenolic compounds showed good linearity ($r^2 \geq 0.9990$) in a relatively wide concentration range. The limits of detection (LOD) were also determined with a signal-to-noise ratio of 3 and ranged from 55 to 140 ng at 275 nm, which showed a high sensitivity under these chromatographic conditions.

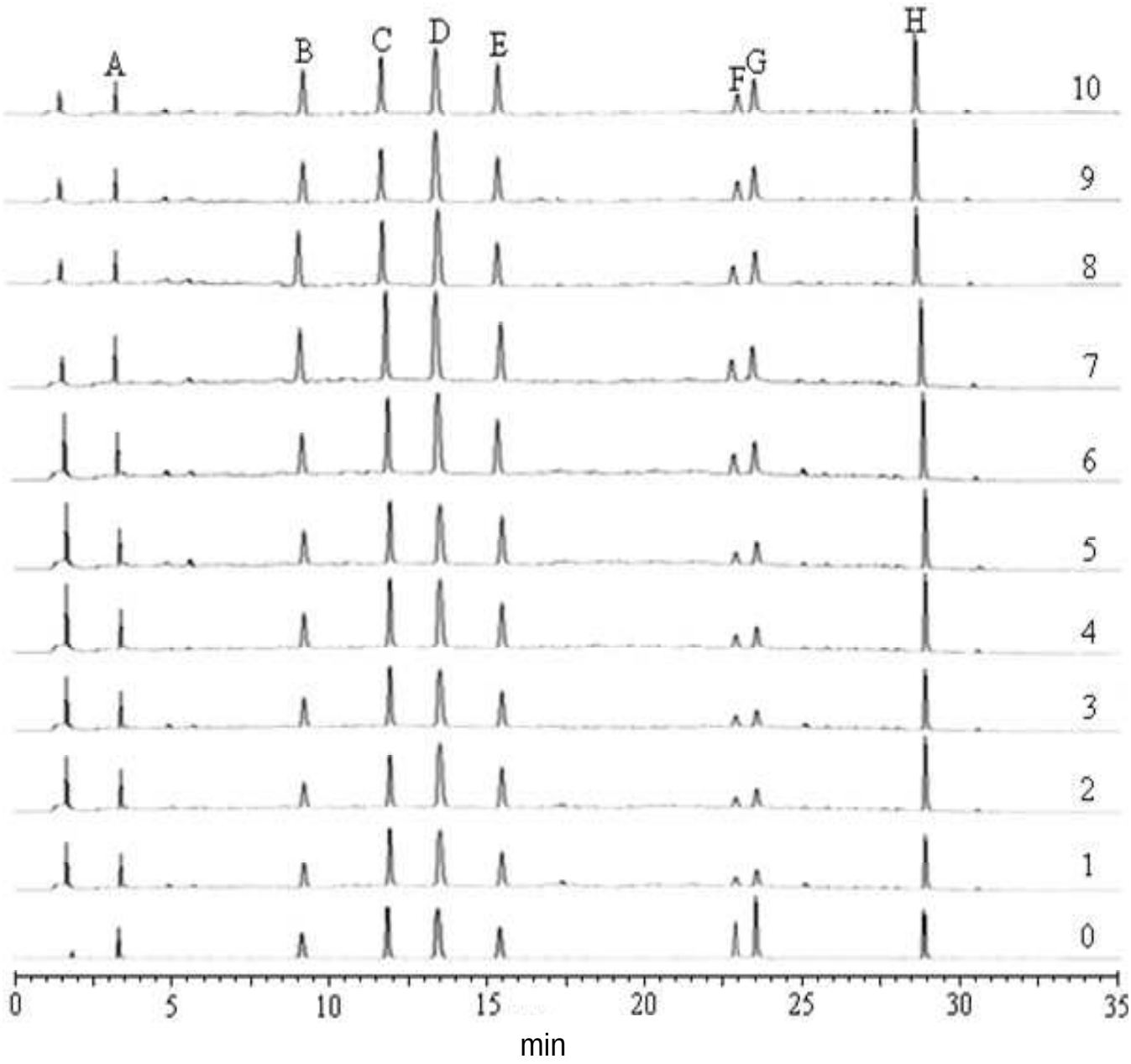

Figure 2. HPLC chromatograms of standard mixture solution (0) and the methanol extract of ten batches of *P. simplex* Retz, lot No.200505w (1), lot No.200505c (2), lot No.200606w (3), lot No.200605c (4), lot No.200705w (5), lot No.200705c (6), lot No.200806w (7), lot No.200805c (8), lot No.200905w (9), lot No.200905c (10). The peaks marked were A = gallic acid, B = caffeic acid, C = quercetin, D = ferulic acid, E = luteolin-7-O-glucoside, F = 8,9-epoxy brevifolin, G = brevifolin, and H = globulariacitrin, respectively.

Quantification of eight phenolic compounds in *P. simplex* Retz

The eight phenolic compounds in *P. simplex* Retz were simultaneously determined by the proposed HPLC-DAD method under the conditions mentioned in the foregoing. The quantitative analyses were performed by means of the external standard methods. Typical chromatograms of the eight phenolic compounds and the methanol extract of *P. simplex* Retz were shown in Figure 2. Data of the quantitative analyses were expressed as mean ± standard deviation (Table 4). Compared with those of the wild and the cultured batches of *P. simplex* Retz, the contents of almost all the components were a little higher

Table 2. HPLC-ESI/MS data of the methanol extract of *P. simplex* Retz.

Peak	Positive ions (m/z)		Negative ions (m/z)	
	MS	MS^2	MS	MS^2
A	170.1 $[M+H]^+$	153.0 $[M+H-OH]^+$ 126.1 $[M+H-COOH]^+$	169.2 $[M-H]^-$	—
B	180.0 $[M+H]^+$	163.0 $[M+H-OH]^+$ 136.1 $[M+H-COOH]^+$	179.0 $[M-H]^-$	—
C	303.2 $[M+H]^+$ 325.0 $[M+Na]^+$	275.0 $[M+H-CO]^+$ 258.0 $[M+H-CO-OH]^+$	301.1 $[M-H]^-$	—
D	195.3 $[M+H]^+$	180.1 $[M+H-CH_3]^+$ 178.0 $[M+H-OH]^+$	193.0 $[M-H]^-$	178.0 $[M-CH_3]^-$
E	449.4 $[M+H]^+$	287.3 $[M+H-C_6H_{11}O_5]^+$	285.2 $[M-H-Glu]^-$	—
F	289.1 $[M+H]^+$	—	287.2 $[M-H]^-$	—
G	247.1 $[M+H]^+$	230.2 $[M+H-OH]^+$	245.0 $[M-H]^-$	—
H	611.1 $[M+H]^+$	—	609.2 $[M-H]^-$ 463.0 $[M-H-Rha]^-$ 301.1 $[M-H-Rha-Glu]^-$	—

Table 3. Calibration graphs, LODs and LOQs for the eight analytes.

Compounds	Regression equation $y = ax + b$*	r^2	Linear range (μg/ml)	LOD† (ng/ml)	LOQ† (ng/ml)
Gallic acid	$y = 8795x + 1453$	0.9997	2.2-220.0	55	155
Caffeic acid	$y = 14523x + 2142$	0.9996	2.0-200.0	80	220
Quercetin	$y = 20341x - 4513$	0.9997	3.5-350.0	105	200
Ferulic acid	$y = 28957x + 5874$	0.9999	3.0-450.0	115	225
Luteolin-7-O-glucoside	$y = 15433x + 5000$	0.9996	2.5-500.0	85	245
8, 9-epoxy brevifolin	$y = 24578x + 5478$	0.9997	1.5-150.0	140	320
Brevifolin	$y = 17548x - 2124$	0.9996	2.0-200.0	120	280
Globulariacitrin	$y = 14563x + 2548$	0.9999	7.5-750.0	90	255

* *y*: Peak area; *x*: concentration of the analytes (μg/ml); *r*: correlation coefficient. † The LOD was defined as the concentration at the signal-to-noise ratio of 3 and the LOQ was defined as the concentration at the signal-to-noise ratio of 10.

in the wild batches than in the cultured batches, but the differences between the two kinds of batches were always slight, and sometimes the contents were the same, and during the long storage period (5 years), most content of the components was diminished slowly.

DISCUSSION

In conclusion, we developed a new high-performance liquid chromatography-diode array detector (HPLC-DAD) method for quantification of eight phenolic compounds in *P. simplex* Retz and validated it. This method was fully compatible with MS detection and allowed us to quantify these eight phenolic compounds in *P. simplex* Retz. Considering the bioactivities of these eight phenolic compounds, they can play an important role for the activity of *P. simplex* Retz, their contents in the plant may affect therapeutic effect extremely. The experiment results also indicate that the wild bathes have only lightly high quality, the cultured *P. simplex* Retz should produce almost the same therapeutic effects but at much lower

Table 4. Contents (mg/g) of the eight phenolic compounds in the ten batches of *P. simplex* Retz (mean ± deviation, *n* = 3).

Compounds	Content of each compound in ten batches of *P. simplex* Retz (mg/g)									
	200505c	200505w	200606c	200605w	200705c	200705w	200806c	200805w	200905c	200905w
Gallic acid	0.39±0.02	0.41±0.01	0.40±0.03	0.45±0.03	0.44±0.02	0.46±0.04	0.38±0.03	0.41±0.02	0.43±0.03	0.42±0.02
Caffeic acid	0.18±0.01	0.18±0.02	0.19±0.02	0.20±0.02	0.22±0.02	0.21±0.01	0.24±0.02	0.25±0.01	0.22±0.02	0.27±0.01
Quercetin	0.24±0.02	0.25±0.02	0.25±0.03	0.26±0.03	0.26±0.02	0.27±0.03	0.30±0.01	0.27±0.02	0.27±0.03	0.28±0.01
Ferulic acid	0.20±0.02	0.22±0.02	0.21±0.01	0.21±0.02	0.20±0.02	0.23±0.02	0.24±0.01	0.24±0.02	0.25±0.02	0.25±0.01
Luteolin-7-O-glucoside	0.25±0.03	0.27±0.02	0.26±0.02	0.26±0.03	0.25±0.01	0.29±0.02	0.30±0.02	0.28±0.01	0.29±0.01	0.31±0.02
8, 9-epoxy brevifolin	0.07±0.01	0.08±0.01	0.08±0.00	0.08±0.01	0.09±0.01	0.09±0.01	0.09±0.01	0.10±0.01	0.09±0.00	0.11±0.01
Brevifolin	0.12±0.02	0.12±0.01	0.13±0.01	0.13±0.02	0.13±0.01	0.14±0.02	0.14±0.01	0.14±0.00	0.15±0.01	0.15±0.01
Globulariacitrin	0.53±0.03	0.51±0.03	0.59±0.04	0.58±0.03	0.59±0.04	0.60±0.04	0.61±0.02	0.64±0.03	0.66±0.03	0.72±0.03

cost. The HPLC method developed here represent an excellent technique for quality control of herbal medicines.

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