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Chemical fingerprint and quantitative analysis of *Salvia plebeia* R.Br. by high-performance liquid chromatography

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ABSTRACT

To control the quality of *Salvia plebeia* R.Br., a simple and reliable method of high-performance liquid chromatography coupled with photodiode array detector (HPLC-DAD) was developed both for fingerprint analysis and quantitative determination of seven bioactive compounds, namely caffeic acid, luteolin-7-glucoside, nepetin-7-glucoside, homoplantaginin, luteolin, nepetin and hispidulin. In fingerprint analysis, twelve peaks were selected as characteristic peaks. In quantitative analysis, seven compounds showed good regression ($R^2 > 0.9995$) within test ranges and the recovery of the method was in the range of 91.7–103.2%. The content ranges (mg/g) of seven compounds in the collected samples of *S. plebeia* were 0.80–1.67 (hispidulin), 2.18–5.75 (homoplantaginin), 0.52–1.22 (nepetin), 1.56–3.48 (nepetin-7-glucoside), 0.12–0.24 (luteolin), 0.97–2.22 (luteolin-7-glucoside) and 0.21–0.44 (caffeic acid), respectively. From the results obtained, the content of homoplantaginin was the highest. In addition, luteolin and luteolin-7-glucoside were isolated for the first time from *S. plebeia*.

1. Introduction

Salvia plebeia R.Br. is an annual or biennial grass, widely distributed in many countries, especially in China and India. It has been used as a folk medicine for the treatment of hepatitis, cough, inflammation and haemorrhoids. Phytochemical studies on *S. plebeia* revealed that it contained flavonoids [1–3], lignans [4,5], diterpenoids [6], aliphatic compounds [7] and caffeic acid [8]. Due to different cultivation areas and climatic conditions, its chemical constituents may vary significantly. Modern pharmacological and clinical studies have shown that *S. plebeia* possesses different biological activities, such as antimicrobial [8], hepatoprotective [9,10], antioxidative [3,11] and antitumor activity [12].

However, no HPLC method was established for analysis of this herbal medicine. Since application of *S. plebeia* is growing steadily, development of a suitable quality control method for it is urgently required. Chemical quality control of herbal medicine should consist of two aspects. One is the qualitative and quantitative analysis of one or several high-content components. The other is analysis of chemical fingerprint [13], which has been introduced and accepted by the WHO [14], FDA (2000) [15] and other authorities as a strategy for quality assessment of herbal medicines. In the past decade, the chromatographic fingerprints established by HPLC, HPTLC, GC, and CE have been recognized as rapid and reliable means for the identification and qualification of herbal medicines [16,17]. Among all methods mentioned above, HPLC is the most popular method and is widely used in fingerprint analysis.

In our present study, a HPLC-DAD method was built for fingerprint analysis of *S. plebeia*. At equal pace, seven phenolic components were simultaneously determined for the first time. So we can control the quality of the plant in different angles.

2. Experimental

2.1. Plant materials and reagents

Ten batches of the raw material samples of *S. plebeia* were collected from different provinces in China. All the voucher specimens, identified by Professor Yan-hua Lu, were deposited at Institute of Biochemistry, East China University of Science and Technology, Shanghai 200237, China.

HPLC-grade methanol was purchased from Caledon Laboratories Ltd., Ontario, Canada. Redistilled water was prepared in our own lab. Glacial acetic acid was of analytical grade from Shanghai Chemical Co., Shanghai, China. All the solutions were filtered through 0.45 μm membranes (Schleicher & Schuell, Dassel, Germany) and degassed by ultrasonic bath before use.

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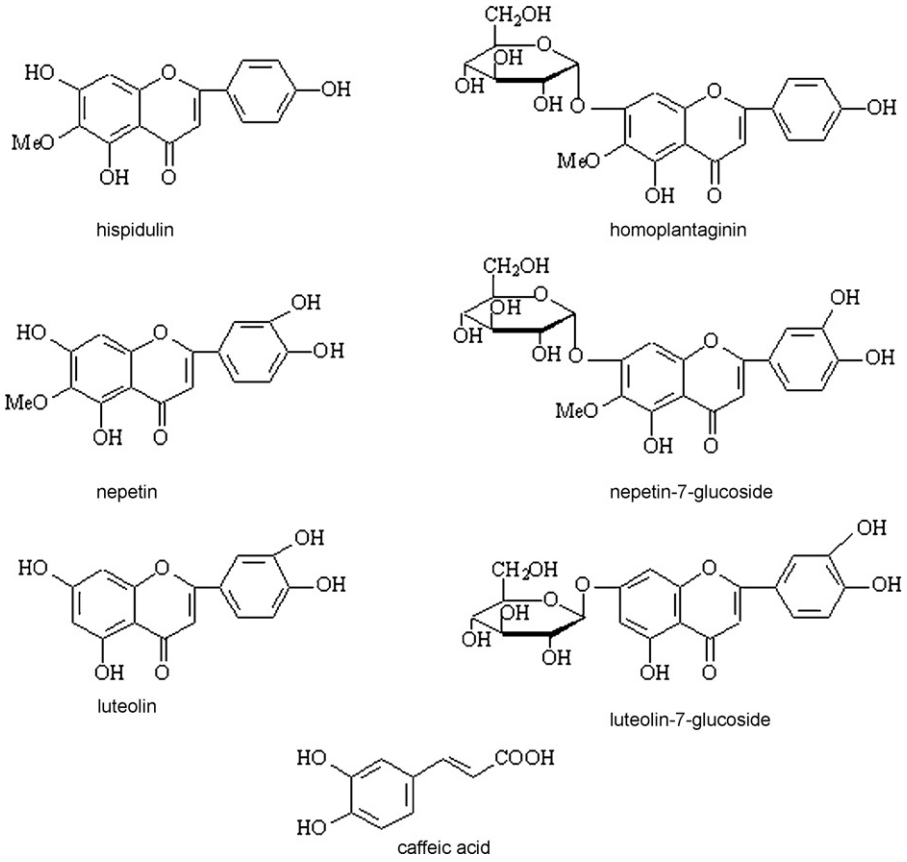

Fig. 1. Chemical structures of hispidulin, homoplantaginin, nepetin, caffeic acid, luteolin, luteolin-7-glucoside and nepetin-7-glucoside.

2.2. Apparatus and chromatographic conditions

Chromatographic analysis was performed on an Agilent 1100 liquid chromatography system, equipped with a diode array detector working in the range of 190–400 nm, a quaternary solvent delivery system, a column temperature controller and an autosampler. The chromatographic data was recorded and processed with Agilent Chromatographic Work Station software. Analysis was carried out at 30 °C on a Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm). A linear gradient elution of eluents A (0.5%, v/v aqueous glacial acetic acid) and B (methanol) was used for the separation. The elution programme was optimized and conducted as follows: a linear gradient of 38–42% B with the range of 0.0–14.0 min, a linear gradient of 42–45% B with the range of 14.0–17.0 min, a linear gradient of 45–48% B with the range of 17.0–17.1 min, a linear gradient of 48–50% B with the range of 17.1–32.0 min and a linear gradient of 50–85% B with the range of 32.0–40.0 min. This was followed by a 10 min equilibration period prior to the injection of each sample. The peaks were recorded using DAD

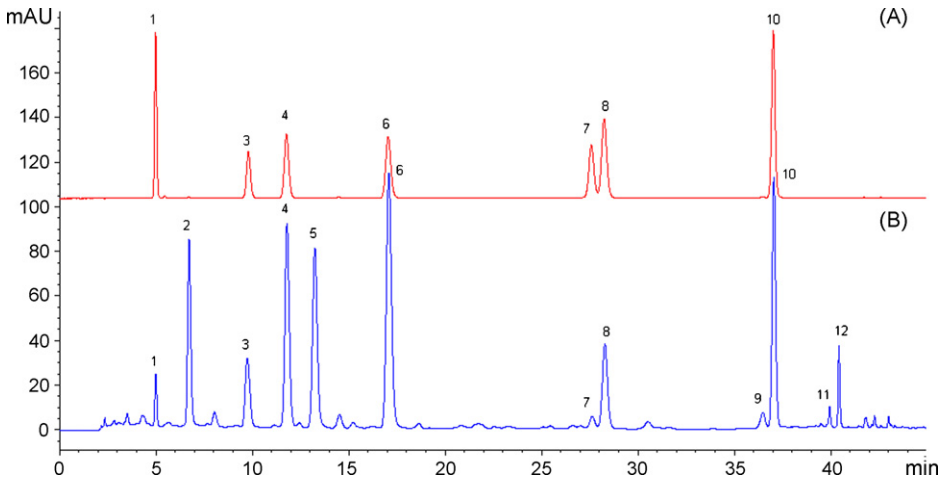

Fig. 2. (A) Chromatogram of mixture standard compounds: (1) caffeic acid; (3) luteolin-7-glucoside; (4) nepetin-7-glucoside; (6) homoplantaginin; (7) luteolin; (8) nepetin; (10) hispidulin. (B) Standardized chromatographic fingerprint by HPLC-DAD at 342 nm.

absorbance at 342 nm and the solvent flow rate was kept at 1.0 ml/min.

2.3. Reference compounds prepared form S. plebeia

An amount of dried and minced herb of *S. plebeia* (Anhui, 0609) was exhaustively extracted with 95% ethanol for 1 week at room temperature. The extract was concentrated with a rotary evaporator under reduced pressure to give a residue, and then was suspended in deionized water and extracted with petroleum, chloroform, ethyl acetate and *n*-butanol, respectively [18].

The ethyl acetate extract was applied to a silica gel column (200 meshes) and eluted with chloroform/methanol to obtain four fractions. Fraction 3 was repeatedly chromatographed on Sephadex LH-20 to obtain hispidulin, nepetin and luteolin.

The *n*-butanol extract was applied to a silica gel column (200 meshes) and eluted with chloroform/methanol to obtain five fractions. Fractions 3 and 5 were repeatedly chromatographed on Sephadex LH-20. Nepetin-7-glucoside, homoplantaginin and luteolin-7-glucoside were obtained from Fraction 3, and caffeic acid was obtained from Fraction 5.

The chemical structures of seven compounds (hispidulin, homoplantaginin, nepetin, nepetin-7-glucoside, luteolin, luteolin-7-glucoside and caffeic acid; Fig. 1) were confirmed by UV, IR, ESI-MS, ^{1}H NMR and ^{13}C NMR, and the chromatogram of mixture standard compounds was shown in Fig. 2A. Luteolin and luteolin-7-glucoside were isolated for the first time from *S. plebeia*. The purity of each compound was determined to be higher than 98% by normalization of the peak area detected by HPLC. These reference compounds were accurately weighed and dissolved in methanol and then diluted to appropriate concentration ranges for the establishment of calibration curves. All stock and working standard solutions were stored at 4 °C until used for analysis.

2.4. Sample preparation

All the samples were milled into powder and oven-dried at 50 °C until constant weight was reached. 1.0 g powder of each dried sample was extracted with 20 ml 70% (v/v) aqueous methanol in an ultrasonic bath for 2 h and then cooled at room temperature. The extract was filtered through glass wool for sample clean up and diluted to 25 ml with 70% methanol. The sample solution was filtered through a 0.45 μm membrane filter prior to HPLC analysis and the injection volume was 5 μl.

2.5. Data analysis

Data analysis was performed by professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A), which was recommended by State Food and Drug Administration. The correlation coefficients of entire chromatographic profiles of samples were calculated, while the simulative mean chromatogram was calculated and generated. The similarities of different chromatographic patterns were compared with mean chromatogram among tested samples.

3. Results and discussion

3.1. Optimization of HPLC condition

For giving the most chemical information and better separation in the chromatograms, the column, mobile phase, detection wavelength and conditions for gradient elution were investigated in this study.

Two kinds of reversed-phase columns, Zorbax Eclipse SB-C18 column (250 mm × 4.6 mm, 5 μm) and Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm) were investigated, the Zorbax Eclipse XDB-C18 column was found to be more suitable and gave good peak separation and sharp peaks.

The effect of mobile phase composition on chromatographic separation was investigated and found there was no obviously distinguish between methanol–water and acetonitrile–water. Considering the high-toxicity and price of acetonitrile, the binary mixture of methanol–water was chosen, and 0.5% (v/v) acetic acid was added to improve the peak shape. Because of the long retention time of some of the late-eluting peaks in isocratic runs, gradient elution was employed in HPLC analysis. Satisfactory separation was achieved in 45.0 min by gradient elution using the HPLC conditions as described earlier in Section 2.2.

The wavelength for the detection of the constituents in the plant was selected by the DAD. Due to a full-scan experiment of the seven active components, the wavelengths of 254, 280 and 342 nm were selected to compare the number and separation of peaks. The chromatograms monitored at 254 and 280 nm adsorption revealed more peaks than 342 nm in the first 5 min. However, due to the serious baseline noise, some peaks obtained under 254 and 280 nm were not well separated. Therefore, 342 nm was selected as detection wavelength.

3.2. Optimization of extraction methods

Four related extraction conditions were designed and evaluated, which involved the following factors and corresponding levels: extraction method (ultrasonication, reflux), methanol concentration (50, 70 and 100%, v/v), solvent volume (10, 20 and 30 ml) and extraction time (1, 2 and 2.5 h). By comparing the sum numbers and areas of characteristic peaks in each chromatogram of different factors, the optimal condition for extraction of *S. plebeia* was selected as 1.0 g powder of each dried sample was extracted with 20 ml 70% (v/v) aqueous methanol in an ultrasonic bath for 2 h.

3.3. Method validation of quantitative analysis

The method was validated in terms of linearity, limits of detection and quantification (LODs and LOQs), precision, repeatability and recovery test.

Linearity was examined with standard solutions. A mixed stock solution consisted of hispidulin (0.216 mg/ml), homoplantaginin (0.224 mg/ml), nepetin (0.224 mg/ml), nepetin-7-glucoside (0.200 mg/ml), luteolin (0.200 mg/ml), luteolin-7-glucoside (0.220 mg/ml) and caffeic acid (0.190 mg/ml) was prepared. 0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 ml of the stock solution each was put into a 10 ml volumetric flask and adjusted with methanol for the standard curves. Each calibration curve contained six different concentrations and was performed in triplicate. An aliquot (10 μl) of each standard working solution was subjected to HPLC-DAD analysis. The linearity for each compound was established by plotting the peak area (y) versus concentration (x) of each analyte which was expressed by the equation given in Table 1. All calibration curves showed good linear regression ($R^2 > 0.9995$) within test ranges.

The LODs and LOQs under the present HPLC-DAD method were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. Standard solution containing seven reference compounds was diluted to a series of appropriate concentrations with methanol and an aliquot (10 μl) of the diluted solutions was injected into HPLC for analysis. The LODs and the LOQs for the analytes were less than 1.42 and 4.73 ng.

Intra- and inter-day variations were utilized to determine the precision. The intra-day variation was determined by analyzing the

Table 1
Calibration curves, detection limits and quantification limits of the analytes ($n = 6$) by HPLC-DAD

Analyte	Calibration curve	Linear range (μg/ml)	R^2 ($n = 6$)	LOD (ng)	LOQ (ng)
Caffeic acid	$y = 14.436x + 6.2160$	1.90–38.0	0.9999	0.41	1.36
Luteolin-7-glucoside	$y = 6.9116x + 1.9549$	2.20–44.0	0.9999	1.42	4.73
Nepetin-7-glucoside	$y = 10.881x + 15.882$	2.00–40.0	0.9997	1.00	3.33
Homoplantaginin	$y = 11.746x + 0.7418$	2.24–44.8	0.9999	1.22	4.07
Luteolin	$y = 11.252x + 3.7138$	2.00–40.0	0.9999	0.14	0.48
Nepetin	$y = 14.759x + 10.272$	2.24–44.8	0.9998	0.21	0.69
Hispidulin	$y = 22.063x + 2.6187$	2.16–43.2	0.9999	0.24	0.80

y, peak area; x, the concentration of each reference compound (μg/ml); R^2, correlation coefficient of regression equations; LOD, limit of detection (S/N = 3); LOQ, limit of quantification (S/N = 10).

Table 2
Analytical results of intra- and inter-day variability and repeatability for the analytes in *S. plebeia* (Zhejiang (0709))

Analyte	Intra-day ($n = 6$)		Inter-day ($n = 6$)		Repeatability ($n = 5$)	
	Content (mg/g)	R.S.D. (%)	Content (mg/g)	R.S.D. (%)	Content (mg/g)	R.S.D. (%)
Caffeic acid	0.39	1.7	0.38	2.8	0.41	1.6
Luteolin-7-glucoside	1.80	1.5	1.78	3.2	1.81	2.0
Nepetin-7-glucoside	3.17	1.4	3.21	2.7	3.19	1.3
Homoplantaginin	4.02	1.1	3.98	2.6	4.01	1.4
Luteolin	0.14	1.6	0.15	2.5	0.14	1.5
Nepetin	0.67	1.8	0.68	2.8	0.65	1.8
Hispidulin	0.78	1.5	0.76	2.9	0.79	1.7

Table 3
Recovery of each analyte determined by standard addition method ($n = 3$)

Analytes	Original (mg)	Spiked (mg)	Found (mg)	Recovery (%)	R.S.D. (%)
Caffeic acid	0.40	0.42	0.80	95.2	3.1
Luteolin-7-glucoside	1.81	1.80	3.56	97.2	2.8
Nepetin-7-glucoside	3.18	3.21	6.48	102.8	2.6
Homoplantaginin	4.02	4.03	8.18	103.2	3.3
Luteolin	0.12	0.12	0.23	91.7	3.1
Nepetin	0.64	0.64	1.24	93.8	2.4
Hispidulin	0.80	0.82	1.58	95.1	2.9

The data was present as average of three determinations. The sample type used to carry out the recovery study was Zhejiang 0709. Recovery (%) = 100 × (amount found − original amount)/amount spiked.

six replicate samples (1.0 g, Zhejiang, 0709) within 1 day and inter-day variation was determined on three consecutive days. To confirm the repeatability, five different working solutions prepared from the same sample (1.0 g, Zhejiang, 0709) were analyzed. Variations were expressed as relative standard deviations (R.S.D.). Table 2 showed the results of the tests of precision and repeatability. It indicated that the R.S.D. values of the overall intra- and inter-day variations were less than 3.2% and the repeatability was less than 2.0% for the analytes.

The recovery test was determined by standard addition method. Seven phenolic components were spiked into the samples (1.0 g, Zhejiang, 0709), and then extracted, processed and quantified in accordance with the established procedures (Section 2.4). The results of recovery test were summarized in Table 3. The average recoveries of the seven phenolic compounds were 91.7–103.2% and their R.S.D. values were less than 3.3%. Therefore, the HPLC-DAD method was precise, accurate and sensitive enough for simultaneously quantitative evaluation of seven phenolic compounds in *S. plebeia*.

3.4. HPLC fingerprint and quantitative analysis

To standardize the fingerprint, 10 samples were analyzed. Peaks that existed in all 10 samples with reasonable heights and good resolution were assigned as "characteristic peaks" for identification of the plant. There were twelve characteristic peaks (from peak 1 to peak 12) in the fingerprint chromatogram (Fig. 2B). Peaks purity was identified by comparison of retention time and DAD spectra. The software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine was used to evaluate these chromatograms. Peak 6 (homoplantaginin) which is one of the most important active constituents in *S. plebeia* was chosen to calculate the relative retention time (RRT) and relative peak area (RPA). RRT and RPA of characteristic peaks in 10 samples were shown in Table 4. The similarities of chromatograms of 10 samples comparing with the reference fingerprint, which was developed with the median of all chromatograms, were shown in Table 5.

Table 4
RRT and RPA of characteristic peaks in 10 samples

Peak no.	RRT	R.S.D. (%)	RPA	R.S.D. (%)
1	0.29	0.10	0.09	21.8
2	0.39	0.17	0.35	34.5
3	0.57	0.27	0.22	27.1
4	0.69	0.17	0.65	23.0
5	0.77	0.19	0.77	38.4
6	1.00	0.17	1.00	23.1
7	1.62	0.16	0.04	30.3
8	1.66	0.15	0.25	32.3
9	2.14	0.09	0.05	23.5
10	2.17	0.07	0.50	31.6
11	2.34	0.02	0.03	13.5
12	2.37	0.02	0.11	14.0

Table 5
The similarities of chromatograms of 10 samples

No.	Similarities
S1	0.868
S2	0.979
S3	0.993
S4	0.992
S5	0.994
S6	0.984
S7	0.989
S8	0.997
S9	0.990
S10	0.998

S1, Guangxi (0708); S2, Anhui (0609); S3, Bozou, Anhui (0701); S4, Anhui (0710); S5, Luan, Anhui (0710); S6, Hebei (0708); S7, Hebei (0709); S8, Hubei (0710); S9, Jinmen, Hubei (0708); S10, Zhejiang (0709).

Table 6
Content (mg/g) of seven analytes in different samples ($n = 3$)

Samples	1	3	4	6	7	8	10
Bozou, Anhui (0701)	0.36	1.44	2.65	4.32	0.13	0.61	0.90
Guangxi (0708)	0.21	0.97	1.56	2.18	0.20	1.15	1.67
Jinmen, Hubei (0708)	0.30	1.28	2.30	3.65	0.13	0.65	1.03
Hubei (0710)	0.31	1.38	2.52	3.44	0.14	0.69	0.85
Hebei (0708)	0.33	2.22	3.40	4.44	0.23	1.19	1.45
Zhejiang (0709)	0.40	1.81	3.18	4.02	0.12	0.64	0.80
Hebei (0709)	0.36	2.18	3.36	4.74	0.24	1.22	1.66
Anhui (0609)	0.44	2.21	3.37	5.75	0.12	0.52	0.87
Anhui (0710)	0.42	1.92	3.36	4.60	0.13	0.70	0.91
Luan, Anhui (0710)	0.41	2.03	3.48	4.68	0.14	0.76	0.96

(1) caffeic acid; (3) luteolin-7-glucoside; (4) nepetin-7-glucoside; (6) homoplantaginin; (7) luteolin; (8) nepetin; (10) hispidulin.

The developed analytical method was successfully applied to the simultaneous determination of hispidulin, homoplantaginin, nepetin, nepetin-7-glucoside, luteolin, luteolin-7-glucoside and caffeic acid, which were selected as the major pharmacological active constituents in *S. plebeia*. All samples were analyzed using the optimized extraction method under optimized HPLC conditions. Each sample was analyzed in triplicate to determine the mean content (mg/g) and the results were tabulated in Table 6.

The quantitative analysis results showed that the crude drugs generally contained the seven selected constituents. The content ranges (mg/g) were 0.80–1.67 (hispidulin), 2.18–5.75 (homoplantaginin), 0.52–1.22 (nepetin), 1.56–3.48 (nepetin-7-glucoside), 0.12–0.24 (luteolin), 0.97–2.22 (luteolin-7-glucoside) and 0.21–0.44 (caffeic acid), respectively. From the results obtained, the content of homoplantaginin was the highest among the selected constituents in the collected samples.

4. Conclusions

A HPLC-DAD chromatographic method was established for the quality control of *S. plebeia*. The method was well validated by systematically comparing chromatograms of all samples from different region, and certified helpfully to improve the quality control. Further, the method developed in this study will provide an important reference to establish the quality control method for other related traditional Chinese medicinal preparations.

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