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Chemical Fingerprint Analysis of *Gardenia jasminoides* Fruit by High-Performance Liquid Chromatography

YUE DING[1], LE-YUAN XIANG[1], TONG ZHANG[1]*, SHEN JI[2] AND LIAN-YING XU[1]

[1.] Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China
[2.] Shanghai Institute for Food and Drug Control, Shanghai 201203, China

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ABSTRACT

A high performance liquid chromatography-ultraviolet detector-electrospray ionization tandem mass spectrometry (HPLC/UV/ESI/MS^n) method was used for the chromatographic fingerprint analysis of *Gardenia jasminoides* fruit, Fructus Gardeniae (zhi-zi in Chinese). Thirteen batches of samples collected from 8 provinces were analyzed to standardize the fingerprint of zhi-zi. The thirteen peaks in the fingerprints of all the 13 batches of samples with reasonable height and good resolution were assigned as "characteristic peaks". Seven "characteristic peaks" were identified by comparing their retention time and MS (mass spectrum) with those of the reference substances for the first time. The Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine software was used to evaluate the similarities among the 13 batches of zhi-zi samples. The results indicated that samples from different origins had similar HPLC fingerprint and the method could be applied for the quality control of zhi-zi. In addition, the fingerprint can be used to analyze the differences of zhi-zi samples with different harvest time and different parts of *Gardenia jasminoides* including calyx, root, fruit (zhi-zi), leaf and stem. The results revealed that the chemical compounds in some samples picked in November varied significantly from samples in September and October. The content of geniposide in zhi-zi picked in September and October was much higher than that picked in November. Calyx, leaf, stem and root of *Gardenia jasminoides* had similar chemical components that were significantly different from those of zhi-zi.

Key words: Fructus Gardeniae, zhi-zi, chromatographic fingerprint, HPLC/UV/ESI/MS^n

INTRODUCTION

Gardenia jasminoides Ellis of the Rubiaceae family is a widely used traditional Chinese medicine (TCM). Its dried ripe fruit, Fructus Gardeniae (zhi-zi in Chinese), has been frequently used in many diseases, such as hyperpyrexia, jaundice with urine abnormality, conjunctiva congestion, contused wound, *etc*. It has effects of purging pathogenic fire, reducing fever to induce urination and cooling blood to remove pathogenic heat[(1)]. Phytochemical studies on zhi-zi have revealed that it contains geniposide, gardenoside, genipin-1-β-gentiobioside, crocin-1, chlorogenic acid, geniposidic acid[(2)], *etc*. Modern pharmacological studies have shown that geniposide is a major active component in zhi-zi that has similar curative effects of zhi-zi, which can be used for liver protection and facilitation of biliation[(3-7)], and has been designated as the marker compound for quality evaluation of zhi-zi in Chinese Pharmacopoeia. However, studies have shown other chemical components such as chlorogenic acid, geniposidic acid and crocin-1 also have similar curative effects as zhi-zi[(8-10)]. Therapeutic efficacy of herbal drug is always attributed to its bioactive components but not to any single ingredient according to traditional Chinese medicine's principle. Therefore, quality evaluation method by one component is insufficient due to variances of chemical composition and clinical efficacy of herbs. It is necessary to establish a comprehensive method to control the quality of zhi-zi for its medical applications.

Many analytical methods have been used to analyze the fingerprint of zhi-zi including high performance liquid chromatography with ultraviolet detector (HPLC/UV)[(11-16)], high performance capillary electrophoresis (HPCE)[(17,18)] and gas chromatography with mass spectrometry

* Author for correspondence. Tel: +86-21-51322332;
Fax: +86-21-51322337; E-mail: zhangtdmj@hotmail.com,
dingyue-2001@hotmail.com

(GC/MS)[19]. However, fingerprints in all the previous reports provided only the chromatographic information, without adequate chemical information about zhi-zi. Therefore, these methods could not truly reflect the pharmaceutical activity of the corresponding products.

Thus, in the present study, we described a method to establish the fingerprint of zhi-zi by HPLC/UV/ESI/MS^n. Firstly, the chromatographic fingerprints of zhi-zi were established by the high performance liquid chromatography-ultraviolet detector (HPLC/UV) method. The method validation result indicated that the method for the fingerprint analysis of zhi-zi was adequate and accurate. Then 13 batches of samples of zhi-zi were collected and their chromatographic fingerprints were analyzed by HPLC/UV to establish a representative standard chromatographic fingerprint. And 7 characteristic peaks in the fingerprint were identified by HPLC/ESI/MS^n with the same column and elution program of HPLC/UV to acquire more chemically related information. The representative standard fingerprint could show the types and relative contents of the components in zhi-zi systematically. Then the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine software was applied to the similarity analysis of chromatographic patterns. The similarity analysis result of the 13 batches of zhi-zi samples indicated that samples from different origins had similar HPLC fingerprints. At last, the fingerprint had been used to analyze the differences of zhi-zi samples with different harvest time and different parts of *Gardenia jasminoides* including calyx, root, fruit (zhi-zi), leaf and stem for the first time. The result showed that zhi-zi samples picked in September and October had similar chemical components, but were significantly different from samples picked in November. Furthermore, analysis of the different parts of *Gardenia jasminoides* indicated the chemical components of calyx, leaf, stem and root of *Gardenia jasminoides* were similar to each other, but significantly different from zhi-zi.

MATERIALS AND METHODS

I. *Materials and Reagents*

(I) *Plant Materials*

Thirteen batches of zhi-zi from 8 provinces of China were collected (Table 1). These herbal samples were authenticated by Professor Tong Zhang from the Shanghai University of Traditional Chinese Medicine, Shanghai, China. The voucher specimens were stored in the Technology Experiment Center, Shanghai University of Traditional Chinese Medicine.

(II) *Reference Compounds and Reagents*

Gardenoside, scandoside methyl ester, genipin-1-β-gentiobioside and crocin-1 were isolated from zhi-zi by preparative liquid chromatography in our own laboratory. Their chemical structures were unambiguously identified by UV, IR, ESI/MS, ^{1}H-NMR and ^{13}C-NMR with recorded literatures[20, 21]. Purity of each compound was determined to be higher than 98% by normalization method of the peak area with HPLC/UV. Geniposide and chloroenic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Shanghai, China). Geniposidic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC-grade methanol was purchased from Shanghai Ambrosia Pharmaceutical, Ltd. (Shanghai, China), and ultra-pure water was used for all analyses. Phosphoric acid of analytical-reagent grade was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

II. *Instrumentation and Chromatographic Condition*

(I) *HPLC/UV Instrumentation and Chromatographic Condition*

The analyses were performed on an Agilent series 1100 HPLC system equipped with a quaternary pump, a variable wavelength detector (VWD), a manual injector, and a column compartment. Chromatographic separation was carried out using an Akzo Nobel Kromasil 100-5C_{18} column (250 mm × 4.6 mm i.d., 5 μm), operated at 25°C. The mobile phase consisted of methanol (A) and 0.4% (v/v) acetate acid in water (B) with a linear gradient elution at a flow rate of 1.0 mL/min. The elution program was as follows: 10-60% A (0-75 min); 60% A (75-80 min). The detection wavelength was 238 nm.

Table 1. Collection location of zhi-zi samples

Sample number	Collection location
1	Nanyang, Henan, China
2	Huangcheng, Jiangxi, China
3	Xingan, Jiangxi, China
4	Xinyu, Jiangxi, China
5	Pujiang, Zhejiang, China
6	Songyang, Zhejiang, China
7	Jiangjin, Chongqing, China
8	Yibin, Sichuang, China
9	Guangyuan, Sichuang, China
10	Qichun, Hubei, China
11	Nanjing, Jiangsu, China
12	Nantong, Jiangsu, China
13	Lianqiao, Hunan, China

(II) *HPLC/ESI/MS Instrumentation and Chromatographic Condition*

The MS analysis was performed on an Agilent 1100 Series LC/MSD Trap system, equipped with an ion trap mass spectrometer with electrospray ionization interface and a diode array detector (DAD). The mobile phase consisted of methanol (A) and 0.4% (v/v) acetate acid in water (B) with a linear gradient elution at a flow rate of 1.0 mL/min. The same column and elution program were used for HPLC/ESI/MS analysis. The condition of ESI source was as follows: source voltage, 4500V; drying gas (N_2) flow rate, 8.0 L/min; drying gas temperature, 350°C; pressure of nebulizer, 30 psi. The ESI/MS/MS was set with fragment amplification 2.0V. Scan range of both MS and MS/MS was set between *m/z* 50 and 1000. The two most abundant ions in each scan were selected and subjected to MS and MS^n analyses.

(III) *Software*

The Similarity Evaluation System for Chromatographic Fingerprint of TCM software was published by the Chinese Pharmacopoeia Commission (Version 2004AB) and mainly applied to the similarity analysis of chromatographic patterns[22,23]. The mathematical theories of the software were based on principal component analysis (PCA) and fuzzy information analysis, which were suitable for complicated system.

(IV) *Sample Preparation*

All samples were dried at 60°C for 4h before use. Each dried sample was grounded to fine powder (40 mesh, 450 μm i.d.) using a grinder. An aliquot of 0.5 g of each sample powder was accurately weighed and extracted with 25 mL of methanol by ultrasonication for 30 min and then filtered through a 0.45 μm filter membrane prior to use. An aliquot of 10 μL of each sample solution was injected into the HPLC system for analysis.

(V) *Data Analysis of Fingerprint Chromatogram*

(1) *The Analysis of RRT and RPA in Fingerprint*

To standardize the fingerprint of zhi-zi, multi-batches of samples from different origins were analyzed. Some peaks that existed in all multi-batches of zhi-zi samples with reasonable heights and good resolution were assigned as "characteristic peaks" for identification of the plant. Among them, the tallest peak or a peak in the middle of the chromatogram was called peak S and selected as reference to calculate the relative retention time (RRT) and relative peak area (RPA). The formulas of RRT and RPA were RRT = $RT_{peak}/Rt_{peak\ S}$ and RPA = $PA_{peak}/Pa_{peak\ S}$, respectively. The RT_{peak} and PA_{peak} represented absolute retention time and absolute peak area of the peak whose RRT need to be calculated, and the $Rt_{peak\ S}$ and $Pa_{peak\ S}$ represented absolute retention time and absolute peak area of the peak S, respectively. RRT and RPA made the various absolute values stable, which could semi-quantitatively reflect the constituents displayed in the chromatographic profile of Fructus Gardeniae.

(2) *The Similarity Analysis of the Fingerprint of Zhi-zi*

The similarity analysis of the zhi-zi fingerprint was finished with the Similarity Evaluation System for Chromatographic Fingerprint of TCM software. The software used the correlation coefficient for evaluating the similarities of different chromatograms. And the calculation of correlation coefficient was mainly based on the peak area and retention time. Firstly, the chromatograms of multi-batches of samples should be introduced in the form of AIA (*.cdf), which included the information of peak area and retention time. Then peak S was chosen by the user and all other characteristic peaks would be matched. Subsequently, the standard chromatogram was produced, the mean value of correlation coefficient was set to 1, and the correlation coefficient of all introduced chromatograms relative to that of standard chromatogram were calculated. If the correlation coefficient was higher than 0.950, it would suggest that the sample quality was relatively similar to the standard group. If the correlation coefficient was lower than 0.950, the sample quality was dissimilar to the standard group.

RESULTS AND DISCUSSION

I. *Optimization of HPLC Condition*

It was very difficult to separate chemical constituents of zhi-zi because of their similar physicochemical properties, so chromatographic parameters were optimized to achieve a higher separation quality of the fingerprint and more chemical information in shorterned analysis time if possible.

Four types of reversed-phase columns, Waters Xterra TM RP18 column (250 mm × 4.6 mm i.d., 5 μm), Akzonobel Kromasil 100-5C_{18} column (250 mm × 4.6 mm i.d., 5 μm), Elite Hypersil C_{18} column (250 mm × 4.6 mm i.d., 5 μm), Shimpack CLC-ODS column (250 mm × 4.6 mm i.d., 5 μm) were investigated. The results showed that the Akzonobel Kromasil 100-5C_{18} column was suitable and provided good peak separation and sharp peaks.

The effect of mobile phase composition on chromatographic separation was investigated and an obvious distinction between methanol-water and acetonitrile-water was found. Considering the good peak separation and sharp peaks, the binary mixture of methanol-water was chosen and 0.4% (v/v) acetate acid was added to improve the peak shape. The compounds in the sample

were so complicated and different that the gradient elution was employed in HPLC analysis. Satisfactory separation was achieved in 80 min by gradient elution using the HPLC conditions as described in section HPLC/UV Instrumentation and Chromatographic Condition.

The wavelength for the detection of the constituents in the plant was selected according to the analyzed result of 3D-plots by the DAD. Since the maximum absorption wavelengths (λ_{max}) of the characteristic components in zhi-zi were different, the wavelengths of 238 nm (λ_{max} of geniposide), 327nm (λ_{max} of chlorogenic acid) and 440nm (λ_{max} of crocin-1) were selected to compare the number and separation of peaks. The result showed the chromatograms monitored at 238 nm adsorption revealed more peaks than 327 and 440 nm. Therefore, 238 nm was selected as ultraviolet detection wavelength.

II. *Optimization of Extraction Methods*

Three related extraction conditions were designed and evaluated, which involved the following factors and corresponding levels: extraction method (ultra-sonication, reflux, decoction), methanol concentration(0, 50 ,75 and 100%, v/v), solvent volume (10, 25 and 50 mL) and ultra-sonication time (0.25, 0.5 and 1 h). By comparing the total number and characteristic peak area in each chromatogram of different factors, the optimal condition for extraction of zhi-zi was selected as 0.5 g of each dried sample extracted with 25 mL methanol in an ultrasonic bath for 0.5 h.

III. *Validation of Methodology*

The criterion for method validation is the Fingerprint Technical Requirements (provisional) of Traditional Chinese Medicine Injection published in 2000 by the State Food and Drug Administration of China. The method was validated in terms of precision, repeatability and stability test. The relative standard deviations (RSD) of RRT and RPA must be lower than 3.0%.

The injection precision was determined by replicated injection of the same sample six times in a day. The RSD

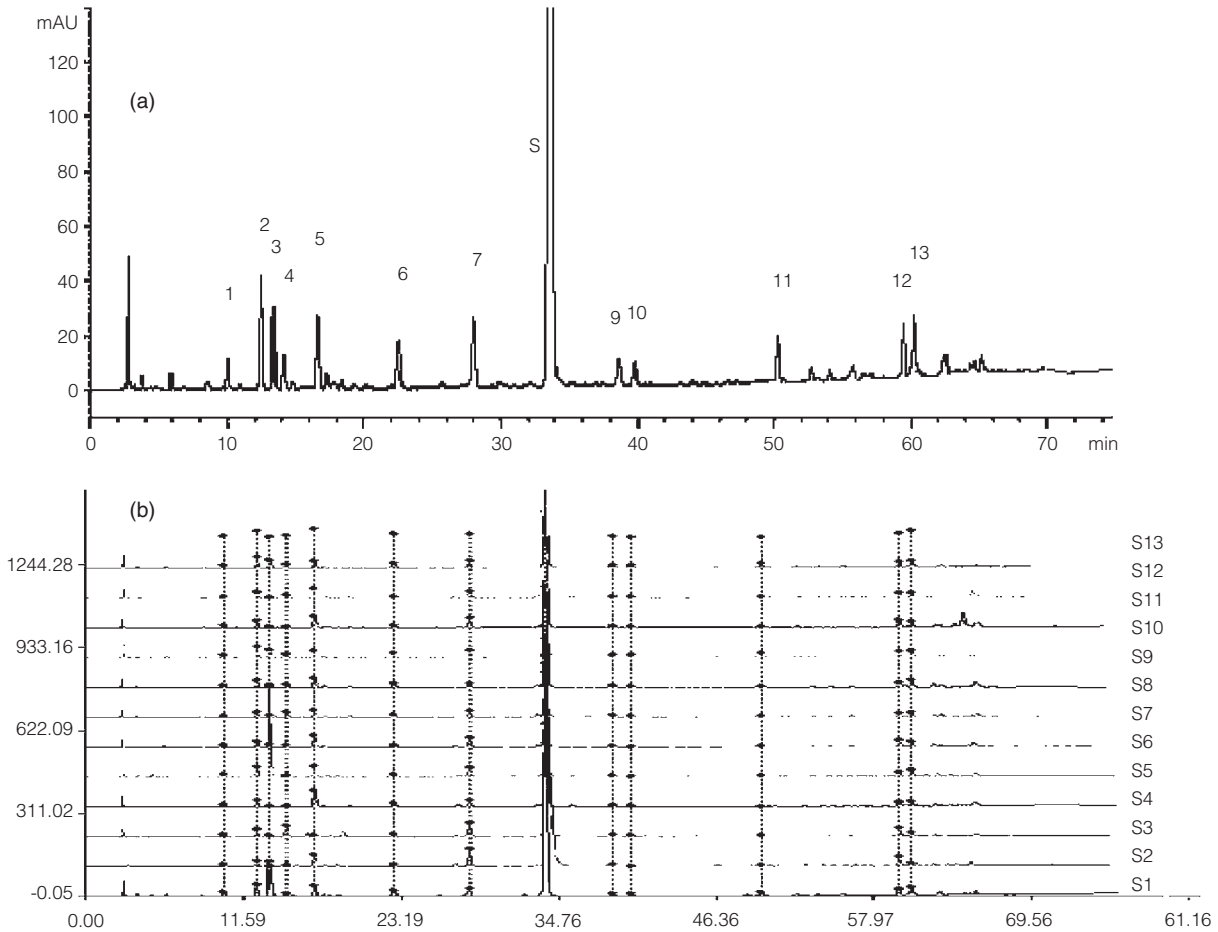

Figure 1. (a) The representative standard fingerprint chromatogram of zhi-zi based on 13 batches of samples analyzed by high performance liquid chromatography-variable wavelength detector (HPLC-VWD) at 238nm. (b) Similarity evaluation of the HPLC fingerprint chromatogram of 13 batches of zhi-zi samples from different sources as S1, Nanyang, Henan; S2, Huangcheng, Jiangxi; S3, Xingan, Jiangxi; S4, Xinyu, Jiangxi; S5, Pujiang, Zhejiang; S6, Songyang, Zhejiang; S7, Jiangjin, Chongqing; S8, Yibin, Sichuang; S9, Guangyuan, Sichuang; S10, Qichun, Hubei; S11, Nanjing, Jiangsu; S12, Nantong, Jiangsu; S13, Lianqiao, Hunan.

of RRT and RPA of 13 characteristic peaks were lower than 0.29% and 1.42%, respectively. The correlation coefficients of chromatograms were not lower than 0.999.

The repeatability was evaluated by analyzing 6 independently prepared samples of zhi-zi. The RSD of RRT and RPA of 13 characteristic peaks were lower than 0.21% and 2.93%, respectively. The correlation coefficients of chromatograms were not lower than 0.999.

The stability test was assessed by successive injection of the same sample in 0 h, 3 h, 6 h, 12 h, 24 h and 48 h. The RSD of RRT and RPA of thirteen "characteristic peaks" were lower than 0.38% and 2.82%, respectively. And the correlation coefficients of chromatograms were not lower than 0.999. All the results indicated that the method for the fingerprint analysis of zhi-zi was adequate and applicable.

IV. *HPLC Fingerprints of Zhi-zi*

To standardize the fingerprint of zhi-zi, 13 batches of samples from different origins were analyzed. Characteristic peaks that existed in all the 13 batches of samples with reasonable heights and good resolution were assigned as characteristic peaks for identification of the plant. There were 13 characteristic peaks (from peak 1 to peak 13) in the fingerprint chromatogram (Figure 1a). The representative standard fingerprint of zhi-zi was generated from multi-samples by the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine software. The software was also used to evaluate the similarity of these chromatograms (Figure 1b). Peak 8 (geniposide) which was one of the most important active constituents in zhi-zi and whose position was in the middle of the chromatogram, called peak S, was chosen to calculate the relative retention time (RRT) and relative peak area (RPA). RRT and RPA of characteristic peaks in 13 batches of samples are shown in Table 2 and 3. The correlation coefficients of chromatograms of 13 batches of samples comparing with the representative standard fingerprint, which were developed with the median of all chromatograms, are shown in Table 4.

The correlation coefficients of chromatograms of 13 batches of zhi-zi samples comparing with that of the representative standard fingerprint were very close to 1 (Table 4). The data showed that the chromatograms of 13 batches of zhi-zi samples were consistent, and the whole chromatograms provided applicable means of assessing the quality of zhi-zi.

V. *Identification of Characteristic Peaks in Fingerprint Chromatograms of Zhi-zi*

In order to qualitatively express the chemical constituents in the fingerprints of zhi-zi plants, the online ESI/MS techniques were used to identify their structures. Both positive and negative ion modes were attempted to ionize the standards.

Table 2. The RRT of thirteen characteristic peaks in zhi-zi samples

Sample number	The RRT of characteristic peaks												
	1	2	3	4	5	6	7	S	9	10	11	12	13
1	0.305	0.376	0.402	0.444	0.499	0.672	0.836	1.000	1.156	1.188	1.491	1.762	1.791
2	0.299	0.371	0.396	0.441	0.496	0.67	0.834	1.000	1.161	1.193	1.452	1.775	1.805
3	0.285	0.356	0.382	0.429	0.485	0.663	0.797	1.000	1.167	1.2	1.603	1.814	1.843
4	0.299	0.371	0.399	0.44	0.494	0.67	0.834	1.000	1.152	1.184	1.498	1.772	1.793
5	0.3	0.372	0.399	0.44	0.494	0.67	0.833	1.000	1.153	1.185	1.499	1.774	1.796
6	0.302	0.374	0.4	0.444	0.499	0.672	0.835	1.000	1.159	1.191	1.526	1.77	1.800
7	0.294	0.365	0.39	0.436	0.492	0.667	0.833	1.000	1.162	1.195	1.516	1.78	1.812
8	0.303	0.377	0.402	0.445	0.500	0.673	0.837	1.000	1.155	1.187	1.49	1.749	1.778
9	0.302	0.375	0.399	0.443	0.499	0.673	0.838	1.000	1.158	1.19	1.528	1.749	1.780
10	0.302	0.376	0.4	0.444	0.499	0.674	0.838	1.000	1.157	1.189	1.539	1.755	1.785
11	0.301	0.374	0.399	0.443	0.499	0.672	0.837	1.000	1.157	1.189	1.558	1.757	1.787
12	0.302	0.376	0.399	0.444	0.499	0.673	0.838	1.000	1.158	1.194	1.551	1.748	1.780
13	0.305	0.376	0.399	0.444	0.499	0.673	0.838	1.000	1.16	1.19	1.553	1.746	1.796
Mean	0.3	0.372	0.397	0.441	0.496	0.671	0.833	1.000	1.158	1.19	1.523	1.765	1.796
R.S.D.(%)	1.77	1.57	1.39	1.01	0.86	0.45	1.32	0.0	0.34	0.36	2.54	1.06	0.97

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Table 3. The RPA of thirteen characteristic peaks in zhi-zi samples

Sample number	The RPA of characteristic peaks												
	1	2	3	4	5	6	7	S	9	10	11	12	13
1	0.018	0.050	0.047	0.012	0.042	0.024	0.085	1.000	0.021	0.018	0.026	0.066	0.046
2	0.010	0.034	0.043	0.029	0.049	0.016	0.087	1.000	0.012	0.011	0.015	0.055	0.024
3	0.008	0.020	0.045	0.028	0.040	0.010	0.078	1.000	0.004	0.006	0.026	0.042	0.010
4	0.012	0.044	0.044	0.014	0.044	0.029	0.040	1.000	0.018	0.015	0.024	0.039	0.043
5	0.018	0.052	0.058	0.019	0.048	0.020	0.046	1.000	0.016	0.020	0.002	0.054	0.048
6	0.016	0.055	0.050	0.018	0.041	0.025	0.073	1.000	0.014	0.020	0.019	0.076	0.054
7	0.018	0.057	0.048	0.017	0.043	0.020	0.077	1.000	0.011	0.013	0.012	0.066	0.051
8	0.018	0.050	0.047	0.037	0.047	0.022	0.069	1.000	0.024	0.012	0.028	0.048	0.040
9	0.020	0.054	0.044	0.015	0.048	0.018	0.116	1.000	0.014	0.011	0.027	0.164	0.078
10	0.014	0.049	0.041	0.031	0.045	0.025	0.077	1.000	0.012	0.009	0.025	0.050	0.018
11	0.014	0.053	0.049	0.014	0.040	0.021	0.064	1.000	0.011	0.014	0.019	0.068	0.053
12	0.012	0.045	0.043	0.024	0.077	0.025	0.074	1.000	0.024	0.013	0.026	0.070	0.051
13	0.013	0.046	0.047	0.025	0.036	0.021	0.071	1.000	0.012	0.015	0.026	0.057	0.045
Mean	0.015	0.047	0.047	0.022	0.046	0.021	0.074	1.000	0.015	0.014	0.021	0.066	0.043
R.S.D.(%)	24.8	21.4	9.2	35.8	21.7	22.7	25.3	0.0	38.4	30.1	36.0	48.0	40.7

In the positive ESI/MS of zhi-zi (Table 5), the quasi-molecular ion peaks always appeared as $[M+Na]^+$ ions. A series of fragment ions arising from the loss of glycoside residues and aglycone ($[M+Na-Glc]^+$) was the major fragmentation pathways in MS or MS/MS. For example, the positive spectrum of compound 8 (geniposide) shows the quasi-molecular ion peak appeared at *m/z* 411 $(M+Na)^+$, and another ion peak arising from the loss of glucosyl appeared at *m/z* 249 $(M+Na-Glc)^+$. In the negative ESI/MS of zhi-zi (Table 5), the base peaks always appeared as $[M-H]^-$ ions. The negative spectrum of compound 8 (geniposide) showed a base peak at *m/z* 387 $(M-H)^-$. Further comparison of the retention time and MS of ions of the compound with corresponding reference compound confirmed identification.

Lastly, structures of thirteen characteristic peaks were analyzed by comparing the mass spectrum and retention time of the components of zhi-zi with those of the corresponding standard compounds. A total of 7 compounds were unambiguously identified (Table 5). Peaks 2, 3, 4, 6, 7, S and 12 could be unambiguously assigned as gardenoside (2), geniposidic acid (3), scandoside methyl ester (4), chlorogenic acid (6), genipin-1-β-gentiobioside (7), geniposide (S), crocin-1 (12), respectively. Peaks 9 and 13 were speculated as croceic acid and crocin-2 by comparing the mass spectrum with references[19,24,25]. The molecular formulas of peaks 1, 5 and 10 were speculated by their molecular weight.

Table 4. The similarity (correlation coefficient) of zhi-zi samples comparing with the reference fingerprint

Sample number	similarity
1	0.99
2	0.994
3	0.996
4	0.995
5	0.984
6	0.993
7	0.994
8	0.98
9	0.996
10	0.994
11	0.982
12	0.996
13	0.998
Mean	1

Table 5. MS and MS^2 of ions of the peaks in Figure.1 and proposed identification

Peak no.	HPLC/ESI/MS fragment ions (*m/z*)	HPLC/ESI/MS^2 fragment ions (*m/z*)	M (molecular weight)	Compound identity
1	$345[M-H]^-/369[M+Na]^+$		346	$C_{17}H_{14}O_8$
2	$403[M-H]^-/427[M+Na]^+$	$MS^2(+)$:409.1[M+Na-H_2O],265[M+Na-Glc],233[M+Na-Glc-H_2O-CH_2] $MS^2(-)$:240.9[M-H-Glc]	404	Gardenoside[a]
3	$373[M-H]^-/397[M+Na]^+$	$MS^2(+)$:379[M+Na-H_2O],235[M+Na-Glc],217[M+Na-H_2O-Glc] $MS^2(-)$:210.9[M-H-Glc], 166.9, 122.9	374	Geniposidic acid[a]
4	$403[M-H]^-/427[M+Na]^+$	$MS^2(+)$:395[M+Na-H_2O-CH_2],265[M+Na-Glc] $MS^2(-)$:240.9[M-H-Glc]	404	Scandoside methyl ester[a]
5	$345[M-H]^-/369[M+Na]^+$		346	$C_{17}H_{14}O_8$
6	$353[M-H]^-/377[M+Na]^+$	$MS^2(+)$:355[M+H],163 $MS^2(-)$:190.9[M-H-Glc]	354	Chlorogenic acid[a]
7	$549[M-H]^-/573[M+Na]^+$	$MS^2(+)$:365[M+ Na-gentiobiose] $MS^2(-)$:517.1[M-H- H_2O -CH_2],323[M-H-gentiobiose-H_2O]	550	Genipin-1-β-genitiobioside[a]
s	$387[M-H]^-/411[M+Na]^+$	$MS^2(+)$:379[M+Na- H_2O - CH_2],249[M+Na-Glc],231[M+Na-Glc-H_2O] $MS^2(-)$:224.9[M-H-Glc]	388	Geniposide[a]
9	$353[M+Na]^+$		330	Croceic acid[b]
10	$453[M+Na]^+$		430	$C_{19}H_{27}O_{11}$
11	$513[M+Na]^+$		490	Unknown
12	$975[M-H]^-/999[M+Na]^+$		976	Crocin-1[a]
13	$813[M-H]^-/837[M+Na]^+$		814	Crocin-2[b]

Glc is the abbreviation of glucose in Table 5.
[a]Compounds identified by comparing the mass spectrum and retention time with corresponding standard compound.
[b]Compounds identified by comparing the mass spectrum with reference.

VI. *The HPLC Fingerprint Analysis of Different Samples by the Standard Fingerprint of Zhi-zi*

In this paper, the zhi-zi samples with different harvest time and the different parts of *Gardenia Jasminoides* were collected for difference analysis using HPLC fingerprints.

(I) *The Differences between Zhi-zi Samples with Different Harvest Time*

Nine batches of zhi-zi planted in Xingan of Jiangxi province were picked at different times for this investigation (Table 6). The HPLC fingerprint chromatograms of the nine samples were analyzed. The correlation coefficients are shown in Table 6. The correlation coefficients of the samples picked in September and October were higher than 0.99, whereas the correlation coefficients of the

Table 6. The similarity (correlation coefficient) of 9 batches of zhi-zi samples

Sample number	The harvest time	Similarity
1	3/9/03	0.994
2	3/9/03	0.994
3	3/9/03	0.994
4	15/10/03	0.994
5	15/10/03	0.994
6	15/10/03	0.994
7	2/11/03	0.952
8	2/11/03	0.913
9	2/11/03	0.845
standard		1.000

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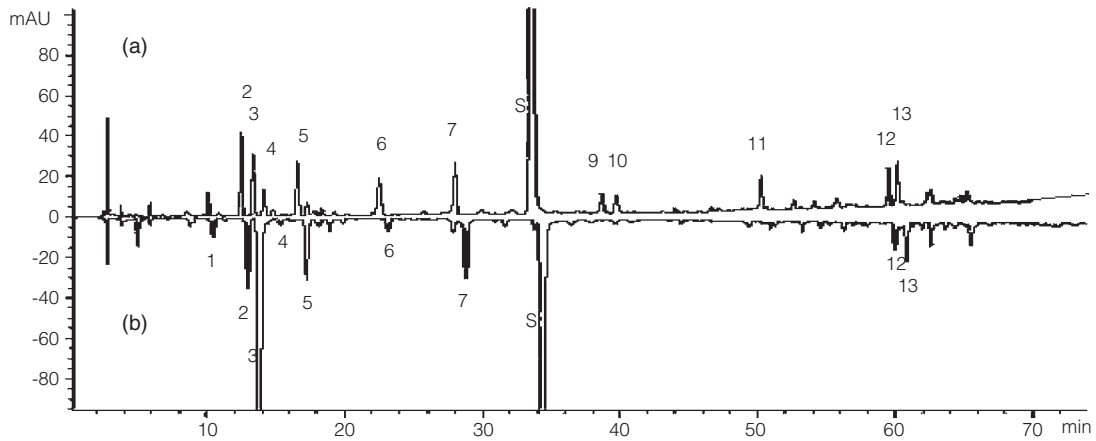

Figure 2. Comparison of HPLC fingerprint chromatograms of zhi-zi harvested in September and November. (a) fingerprint chromatogram of zhi-zi harvested in September and (b) fingerprint chromatogram of zhi-zi harvested in November. The chromatograms were all analyzed by high performance liquid chromatography- variable wavelength detecto.

samples picked in November were the lowest at 0.845. The result indicated the chemical components of zhi-zi samples picked in September and October were similar to each other, but significantly different from some samples picked in November, such as sample 8 and 9, with the correlation coefficients lower than 0.950. And from the Table 6, the correlation coefficients of the samples picked in November were different from each other. We could conclude that the quality of some samples picked in November was dissimilar to the standard group and not stable.

By visually comparing the HPLC fingerprints of different samples, RPA of peak 3 (geniposidic acid) varied remarkably from September to November, while the area of peak 3 (geniposidic acid) increased and the area of peak 8 (geniposide) decreased. By calculation, in sample 8 and 9 picked in November, the RPA of peak 3 was 30.2 and 25.4%, much higher than 4.7% of the samples picked in September and October. Its relative content might be high enough to affect the result of similarity. In addition, peaks 6 (chlorogenic acid), 9, 10 and 11 could not be detected in sample 8 and 9 picked in November (Figure 2). The results showed that the chemical components in sample 8 and 9 picked in November were dissimilar with the sample picked in September and October. So the comparison of RPA was consistent with similarity analysis. More importantly, the analysis of the RPA of characteristic peaks provided more relevant chemical information about the differences between zhi-zi samples with different harvest times.

The harvest time of zhi-zi recorded in Chinese Pharmacopoeia was from September to November. And our research showed the chemical compounds in zhi-zi varied significantly in some samples picked after October, especially the contents of geniposide in zhi-zi picked in September and October were much higher. Therefore, the harvest time of zhi-zi requires further investigation for better clinical use.

(II) *The Difference Analysis of the Different Parts of Gardenia jasminoides*

Different parts of *Gardenia jasminoides* including calyx, root and stem were collected for difference analysis by the HPLC fingerprint of zhi-zi. The correlation coefficients of different parts of *Gardenia jasminoides* are shown in Table 7. The correlation coefficients of different parts of *Gardenia jasminoides* including calyx, leaf, root and stem to its fruit were lower than 0.120, indicating that the chemical components of calyx, leaf, stem and root of *Gardenia jasminoides* were similar to each other, but significantly different from zhi-zi.

By visually comparing the HPLC fingerprints of different parts of *Gardenia jasminoides* to its fruits, the area of peak 3 (geniposidic acid) was increasing and the area of peak S (geniposide) was decreasing in the fingerprint chromatograms of *Gardenia jasminoides*' calyx, leaf, root and stem. This observation indicated that the chemical components varied in different parts (Figure 3). The small peaks such as peaks 4, 5, 6, 9, 10, 11, 12 and 13 were also decreasing. By calculation, the RPA of peak 3 in *Gardenia jasminoides*' calyx, leaf, root and stem varied between 20.5 and 51730.0%, much higher than 4.7% of zhi-zi. From the analysis of the RPA of peaks in these fingerprints, the main compound in *Gardenia jasminoides*' fruit was peak 8, identified as geniposide. While in other parts of *Gardenia jasminoides*, the main compound might be peak 3, identified as geniposidic acid. The RPA of peaks varied significantly in the fingerprint chromatograms of *Gardenia jasminoides*' calyx, leaf, root and stem from zhi-zi.

Previous studies[26] have shown that geniposidic acid was the active compound in Cortex Eucommiae (or du-zhong in Chinese) which had the effect including healing organs and strengthening bone and muscle. The geniposidic acid also played a role in an effective

anticancer product with the ability to decrease undesirable radiation damage to the hematologic tissue after high dose irradiation. If the effect of geniposidic acid had been proven, the different parts of *Gardenia jasminoides* could also be used for effective clinical use as new medicines with result of our research.

CONCLUSIONS

In this paper, the representative standard fingerprint of zhi-zi was generated from multi-samples by the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine software. Furthermore, 7 characteristic peaks in the common pattern were identified by comparing with the reference compounds based on their retention time and the data obtained by HPLC/UV/ESI/MS^n to further characterize the chromatographic fingerprint and contribute to the quality control of zhi-zi.

This standard fingerprint was used for the purpose of difference analysis of samples with different harvest time and quality evaluation of different parts. The fingerprints of zhi-zi were found to have great variation in different samples picked in different time. Our research also revealed significant differences among components in different parts of *Gardenia jasminoides*, *e.g.* geniposide was the main compound of zhi-zi and geniposidic acid was the main compound of other parts of *Gardenia jasminoides*. Further pharmacological investigation was necessary to determine whether the other parts of *Gardenia jasminoides* could be used for effective clinical use as new good medicines.

Finally, we have found the correlation coefficient and RPA were both important values for fingerprint analysis and were in line with each other in fingerprint analysis. The correlation coefficient indicated whether the fingerprint chromatogram of sample checked was relative to the standard chromatogram. If the correlation coefficients were higher than 0.950, the sample quality was relatively similar to the standard group. If the correlation coefficients were lower than 0.950, the sample quality were not similar to the standard chromatogram. Such as in section IV, the correlation coefficients of chromatograms of 13 batches of zhi-zi samples comparing with that of the representative standard fingerprint were very close to 1. The data showed that the chromatograms of 13 batches of zhi-zi samples were consistent. And in

Table 7. The similarity (correlation coefficient) of different parts of *Gardenia Jasminoides* Ellis

Sample number	Parts of *Gardenia Jasminoides* Ellis	Similarity
1	calyx	0.102
2	calyx	0.105
3	calyx	0.099
4	root	0.117
5	root	0.119
6	root	0.115
7	stem	0.106
8	stem	0.108
9	stem	0.109
10	leave	0.110
11	leave	0.108
12	leave	0.117
standard	fruit	1.000

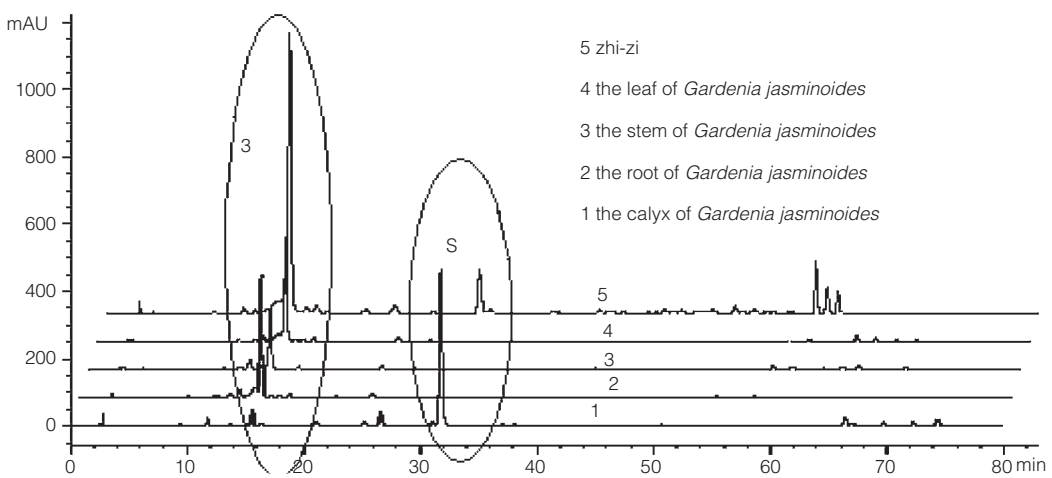

Figure 3. Comparison of HPLC fingerprint chromatograms of different parts of *Gardenia jasminoides* including calyx, root, leaf, fruit and stem. The chromatograms were all analyzed by high performance liquid chromatography-variable wavelength detector (HPLC-VWD) at 238nm.

section VI, the correlation coefficients of different parts of *Gardenia jasminoides* including calyx, leaf, root and stem to its fruits were lower than 0.120, indicating that the chemical components of calyx, leaf, stem and root of *Gardenia jasminoides* were similar to each other, but significantly different from zhi-zi. In addition, the analysis of the RPA of characteristic peaks provided more relevent chemical information about the differences of fingerprints. For example, in section VI, the analysis of the RPA of peaks indicated that the chemical components of calyx, leaf, stem and root of *Gardenia jasminoides* were similar to each other, but significantly different from zhi-zi. Note that peak 8, identified as geniposide, was the main compound in *Gardenia jasminoides*' fruit, while the peak 3, identified as geniposidic acid, was the main compound in other parts of *Gardenia jasminoides*.

Overall, this research sets a good example for systematical quality control of traditional Chinese medicines. Fingerprint is an effective method to comprehensively control the quality of complex herbs. Furthermore, 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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