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Development of high-performance liquid chromatographic fingerprints for distinguishing Chinese Angelica from related umbelliferae herbs

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Abstract

A high-performance liquid chromatographic (HPLC) fingerprint of Chinese Angelica (CA) was developed basing on the consistent chromatograms of 40 CA samples (*Angelica sinensis* (Oliv.) Diels). The unique properties of this HPLC fingerprints were validated by analyzing 13 related herbs including 4 Japanese Angelicae Root samples (JA, *A. acutiloba* Kitagawa and *A. acutiloba* Kitagawa var. *sugiyame* Hikino), 6 Szechwan Lovage Rhizome samples (SL, *Ligusticum chuanxiong* Hort.) and 3 Cnidium Rhizome samples (CR, *Cnidium officinale* Makino). Both correlation coefficients of similarity in chromatograms and relative peak areas of characteristic compounds were calculated for quantitative expression of the HPLC fingerprints. The amount of senkyunolide A in CA was less than 30-fold of that in SL and CR samples, which was used as a chemical marker to distinguish them. JA was easily distinguished from CA, SL and CR based on either chromatographic patterns or the amount of coniferyl ferulate. No obvious difference between SL and CR chromatograms except the relative amount of some compounds, suggesting that SL and CR might have very close relationship in terms of chemotaxonomy. Ferulic acid and *Z*-ligustilide were unequivocally determined whilst senkyunolide I, senkyunolide H, coniferyl ferulate, senkyunolide A, butylphthalide, *E*-ligustilide, *E*-butylidenephthalide, *Z*-butylidenephthalide and levistolide A were tentatively identified in chromatograms based on their atmospheric pressure chemical ionization (APCI) MS data and the comparison of their UV spectra with those published in literatures.
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Keywords: *Angelica* spp.; *Ligusticum chuanxiong*; *Cnidium officinale*; Umbelliferae herbs; Fingerprint

1. Introduction

Chinese Angelica (*Radix Angelicae Sinensis*, CA) is the root of *Angelica sinensis* (Oliv.) Diels, which has been used as one of the traditional Chinese medicines (TCM) for more than 2000 years. It has been mostly used as one of the herbal ingredients in prescriptions of TCM to treat gynecological diseases. As an estimate, more than 80 composite formulae of TCM contain CA in China. Moreover, CA also has been used as a health supplement, ingredient included in cosmetic, etc. [1–3].

Apart from macroscopic and microscopic authentication, chemical identification of TCM materials is an important and useful means as it directly associates with the medicinal functions of TCM materials. Ferulic acid and ligustilide were usually chosen as marker compounds to assess the quality of CA and their products in literatures [4–9]. Pharmacological and clinical studies indicated that they were both bioactive compounds with reported activities to inhibit platelet aggregation, relax uterus, tracheal muscle, smooth muscle, prevent gynecological disease, treat menstrual disorders, urgent premature birth, hypertension, etc. [10–15]. However, ferulic acid and ligustilide are also found in other plants such as the roots of *A. acutiloba* Kitagawa and *A. acutiloba* Kitagawa var. *sugiyamae* Hikino, the rhizomes of *Ligusticum chuanxiong* Hort, *Cnidium officinale* Makino, etc. All these are the crude sources of official medicinal materials of Japanese Angelicae Root (*Angelica Radix*, JA), Szechwan Lovage Rhizome (*Rhizoma Chuanxiong*, SL) and Cnidium Rhizome (*Cnidii Rhizoma*, CR), respectively [16–23]. Therefore, chemical identification of CA by using ferulic

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acid and ligustilide as marker compounds seems to be insufficient. However, a unique chemical compound for CA identification is not yet available for qualitatively distinguishing CA from its related umbelliferae herbs. In light of this, characteristic fingerprint/chromatogram is developed for this purpose.

Although there were some publications associated with CA fingerprints or multi-component chromatograms using HPLC, none of these involved multi-samples analysis, comparison between herbal species or identification using characteristic chemical components [4,24–27]. In other literature reports, volatile compounds were qualitatively or quantitatively analyzed by GC-flame ionization detection (FID) or GC–MS in CA samples, however, the stability as well as reproducibility of these methods or relevant compounds were not mentioned [6,28,29]. Therefore, there still exists a void of satisfactory chemical means for CA identification.

The present study has been focused on developing a chemical method to identify CA samples and its related umbelliferae herbs, including JA, SL and CR. Multi-sample batches of these species were collected in order to generate a representative picture. HPLC-diode array detection (DAD) technique, in particular the analysis of three-dimensional plots of retention time-absorbance-wavelength (3D-plots), was used to identify characteristic peaks among the various samples and determine the optimal detection wavelength for the characteristic chromatograms of each herb. The chemical constituents were identified in the fingerprints based on the on-line HPLC-atmospheric pressure chemical ionization (APCI)–MS and UV techniques in order to retrieve more chemically related information. A combination of mathematics and computer approaches was also employed to study the relationship of chromatographic patterns in this study.

The *Computer Aided Similarity Evaluation System* was a recently developed computer software based on chemometrics developed by the Research Center of Modernization of Traditional Chinese Medicines (Central South University, Changsha, China) and mainly applied in the similarity study of chromatographic and spectral patterns [30–33]. In this study, this software was employed to synchronize the chromatographic peaks, to calculate the correlation coefficients between entire chromatographic profiles and to do quantitative comparison among different samples, as well as to compute and generate the mean chromatogram as a representative standard fingerprint/chromatogram for a group of chromatograms. Furthermore, principal component analysis (PCA) was also utilized to generate a visual 3D-projection plot for qualitative evaluation on the resemblance and difference of tested samples [34]. Besides, the relative retention time (RRT) and relative peak area (RPA) of each characteristic peak related to the reference peak were calculated for quantitative expression of the chemical properties in the chromatographic pattern of herbs. The generated data provided valuable insights about the application of fingerprint in the analysis/quality control of herbs. In this study, 40 CA samples with 13 related umbelliferae herb samples, i.e. 4 JA samples, 6 SL samples and 3 CR samples, were analyzed (Table 1). Eleven common compounds were identified in the HPLC fingerprints (Table 2 and Fig. 1). A reproducible HPLC fingerprinting technique was developed for identification and differentiation of the CA samples with other related herbs.

2. Experimental

2.1. Instrumentation

An Agilent/HP 1100 series HPLC–DAD system consisting of a vacuum degasser, binary pump, autosampler, thermostated column compartment and DAD (Agilent, Palo Alto, CA, USA) was used for acquiring chromatograms, UV spectra and 3D-plots. An Applied Biosystems/PE-SCIEX API 365 LC–MS–MS system with atmospheric pressure chemical ionization source (Applied Biosystems, Foster City, CA, USA) was used for mass spectrometric measurements. Branson 5210E-MTH ultrasonic bath (Branso Ultrasonics Corporation, CT, USA) was used for sample extraction. For chromatographic analysis, an Alltima C_{18} column (5 μm, 250 mm × 4.6 mm) with a suitable guard column (C_{18}, 5 μm, 7.5 mm × 4.6 mm) was used. The mobile phase consisted of 1.0% acetic acid in water (A) and acetonitrile (B) using a gradient program of 19% (B) in 0–18 min, 19–100% (B) in 18–60 min and 100% (B) in 60–75 min. The flow rate was 1.0 mL/min and column temperature was maintained at 30 °C. DAD detector was set at 280 nm for acquiring chromatograms. UV spectra were acquired from 200 to 400 nm. The APCI–MS spectra were acquired in both of the positive and negative ion modes.

The software of *Computer Aided Similarity Evaluation System*, which was coded in MATLAB 5.3 for windows and run on a Pentium III 850 (Intel) personal computer, was employed to calculated correlation coefficients, to generate mean chromatograms and to carry out PCA of three principal components.

2.2. Solvents and chemicals

Analytical grade methanol (Labscan, Bangkok, Thailand) and formic acid (Unichem, Warsaw, Poland) were used for sample preparation. HPLC grade acetonitrile (Labscan, Bangkok, Thailand), deionized water obtained from a Milli-Q water system (Millipore, Bedford, MA, USA) and analytical grade glacial acetic acid (Unichem, Warsaw, Poland) were used for preparation of mobile phase.

2.3. Reference compounds

Ferulic acid was purchased from the Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). *Z*-ligustilide was extracted, isolated and purified

Table 1
A summary of the tested samples

No.	Sample code	Source	Sampling part	Year of collection
1	CA-1	Minxian, Gansu, China	Whole root	2001
2	CA-2	Minxian, Gansu, China	Whole root	2001
3	CA-3	Minxian, Gansu, China	Whole root	2001
4	CA-4	Minxian, Gansu, China	Whole root	2002
5	CA-5	Minxian, Gansu, China	Whole root	2001
6	CA-6	Minxian, Gansu, China	Whole root	2002
7	CA-7	Minxian, Gansu, China	Whole root	2002
8	CA-8	Minxian, Gansu, China	Whole root	2003
9	CA-9	Minxian, Gansu, China	Whole root	2003
10	CA-10	Minxian, Gansu, China	Whole root	2003
11	CA-11	Minxian, Gansu, China	Whole root	2003
12	CA-12	Minxian, Gansu, China	Root head	2001
13	CA-13	Minxian, Gansu, China	Root head	2002
14	CA-14	Minxian, Gansu, China	Rootlets	2002
15	CA-15	Weiyuan, Gansu, China	Whole root	2002
16	CA-16	Weiyuan, Gansu, China	Whole root	2001
17	CA-17	Weiyuan, Gansu, China	Root head	2001
18	CA-18	Weiyuan, Gansu, China	Root head	2001
19	CA-19	Weiyuan, Gansu, China	Rootlets	2002
20	CA-20	Weiyuan, Gansu, China	Rootlets	2002
21	CA-21	Zhangxian, Gansu, China	Whole root	2002
22	CA-22	Zhangxian, Gansu, China	Root head	2002
23	CA-23	Dangchang, Gansu, China	Whole root	2002
24	CA-24	Dangchang, Gansu, China	Whole root	2002
25	CA-25	Dangchang, Gansu, China	Rootlets	2002
26	CA-26	Pingwu, Sichuan, China	Whole root	2003
27	CA-27	Pingwu, Sichuan, China	Whole root	2003
28	CA-28	Pingwu, Sichuan, China	Whole root	2003
29	CA-29	Pingwu, Sichuan, China	Whole root	2003
30	CA-30	Pingwu, Sichuan, China	Whole root	2003
31	CA-31	Jiuzaigou, Sichuan, China	Whole root	2003
32	CA-32	Diqing, Yunnan, China	Whole root	2002
33	CA-33	Heqing, Yunnan, China	Whole root	2003
34	CA-34	Hong Kong, China	Whole root	2003
35	CA-35	Hong Kong, China	Whole root	2003
36	CA-36	Hong Kong, China	Whole root	2003
37	CA-37	Hong Kong, China	Whole root	2003
38	CA-38	Hong Kong, China	Root head	2002
39	CA-39	Hong Kong, China	Root head	2002
40	CA-40	Hong Kong, China	Root head	2002
41	JA-1	Japan	Whole root	2004
42	JA-2	Japan	Coarse granule of root	2003
43	JA-3	Japan	Whole root	2004
44	JA-4	Japan	Coarse granule of root	2003
45	SL-1	Pengzhou, Sichuan, China	Rhizome	2003
46	SL-2	Dujiangyan, Sichuan, China	Rhizome	2004
47	SL-3	Chongzhou, Sichuan, China	Rhizome	2004
48	SL-4	Changqi, Sichuan, China	Rhizome	2004
49	SL-5	Nanzheng, Shanxi, China	Rhizome	2004
50	SL-6	TCM Market of Chengdu, China	Rhizome	2003
51	CR-1	Japan	Rhizome	2004
52	CR-2	Japan	Rhizome	2004
53	CR-3	Maoxian, Sichuan, China	Rhizome	2003

CA-1 to CA-40: Chinese Angelica (*Angelica sinensis* (Oliv.) Diels); JA-1 to JA-4: Japanese Angelicae Root (*A. acutiloba* Kitagawa for JA-1 and JA-2; *A. acutiloba* Kitagawa var. *sugiyamae* Hikino for JA-3 and JA-4); SL-1 to SL-6: Szechwan Lovage Rhizome (*Ligusticum chuanxiong* Hort); CR-1 to CR-3: Cnidium Rhizome (*Cnidium qfficinale* Makino).

Table 2
The on-line detected chromatographic and spectrometric data of these identified compounds extracted with methanol–formic acid (95:5) in the HPLC fingerprints

Peak no.	t_R (min)	$[M+H]^+$ (*m*/*z*)	$[M-H]^-$ (*m*/*z*)	Other positive ions (*m*/*z*)	Other negative ions (*m*/*z*)	λ_{max} (nm)	Identification
1	14.1	–	193	–	179	298sh, 323	Ferulic acid
2	25.9	225	–	207, 189	–	277	Senkyunolide I
3	27.6	225	–	207	–	278	Senkyunolide H
4	40.3	–	355	163, 131, 103	193	270, 299sh, 318	Coniferyl ferulate
5	43.3	193	–	175, 165, 147, 137	–	280	Senkyunolide A
6	44.1	191	–	173, 145	–	228, 274, 281	Butylphthalide
7	45.6	191	–	173, 145, 83	–	290sh, 328	*E*-Ligustilide
8	46.4	189	–	171, 153, 83	–	239, 261, 312	*E*-Butylidenephthalide
9	46.9	191	–	173, 145, 83	–	282, 327	*Z*-Ligustilide
10	47.3	189	–	171, 153, 83	–	236, 260, 312	*Z*-Butylidenephthalide
11	53.9	381	–	191	–	232, 278	Levistolide A

from fresh roots of *A. sinensis* (Oliv.) Diels in our laboratory. Purified *Z*-ligustilide was identified by electron impact ionization (EI) MS, ^{1}H-NMR and ^{13}C-NMR spectrometric techniques. The purity was found to be > 98% based on the percentage of total peak area by HPLC analysis. The detailed procedures for isolation and spectrometric identification will be reported in another paper.

2.4. Plant materials

The identity, sampling part and sample source of the 53 tested samples are summarized in Table 1. These herbal samples were authenticated by Dr. Zhong-Zhen Zhao (School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China). Voucher specimens are stored at the Herbarium Center of this institution.

Representative samples were cut into smaller pieces and further ground into powder, passed through a 20-mesh (0.9 mm) sieve. The ground powders were stored at about 4 °C before use.

2.5. Sample preparation

An accurately weighed sample powder of 0.5 g was introduced into 60 mL amber vial and 25 mL of methanol–formic acid (95:5) was added. The weight of vial was record and the vial was sealed and sonicated for 100 min. The original solvent weight was restored. The extract was filtered through a 0.2 μm membrane filter. An aliquot of 10 μL solution was injected for HPLC analysis.

2.6. Data analysis of chromatogram

The correlation coefficients of entire chromatographic patterns among samples were calculated, and the simulative mean chromatogram was calculated and generated using the

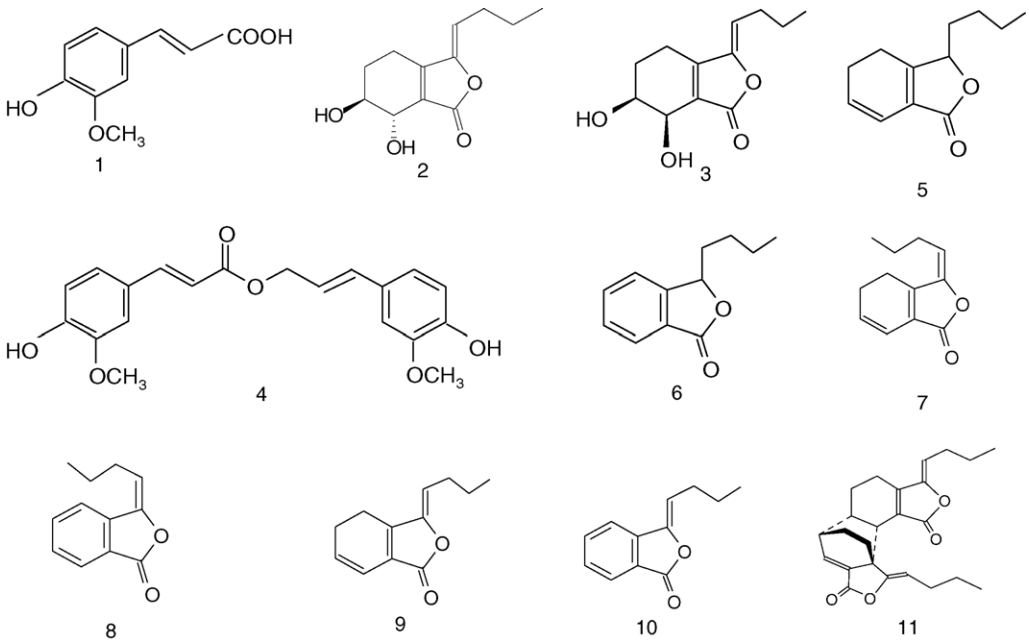

Fig. 1. Chemical structures of the identified compounds in the HPLC fingerprints: (1) ferulic acid; (2) senkyunolide I; (3) senkyunolide H; (4) coniferyl ferulate; (5) senkyunolide A; (6) butylphthalide; (7) *E*-ligustilide; (8) *E*-butylidenephthalide; (9) *Z*-ligustilide; (10) *Z*-butylidenephthalide; (11) levistolide A.

Computer Aided Similarity Evaluation System. The similarities of the entire chromatographic profiles were analyzed among tested samples. Three principal components obtained by PCA were used to evaluate the resemblance and differences of tested samples. The RRT and RPA of each characteristic peak to reference peak were also calculated in the chromatograms.

3. Results and discussion

3.1. Selection of chromatographic conditions

Both ferulic acid (**1**) and *Z*-ligustilide (**9**) are commonly found in CA and other related umbelliferae herbs. Compound **9** was often used as marker compound owing to its bioactivity and present in relatively higher content [4,13–16]. Although the content of compound **1** was comparatively lower in CA, it was demonstrated previously as a bioactive compound and also chosen as an additional marker for assessing the quality of CA and its products [5,7–9,12,35]. Therefore, both compounds **1** and **9**, which belong to different chemical classes of compounds, were first chosen as characteristic compounds for detection in this study. It is note worthy that compound **1** was not chosen as marker in some reported HPLC fingerprint of CA sample in literatures [3,25,26].

Regarding the choice of solvent for optimal extraction, organic solvents including hexane, aqueous methanol and ethyl acetate–methanol (70:30) were used for developing CA fingerprints in previous studies [3,4,24–26]. Methanol was the preferred choice of extraction solvent in the present study as a variety of compounds with different polarity can be co-extracted effectively. These included compounds **1**, coniferyl ferulate (**4**), senkyunolide A (**5**), ligustilide and so on. Besides, the interference from sugars in the raw herbs could also be minimized by extraction using methanol. However, it was observed that compounds **1** and **4** were found to be unstable by extraction using methanol. The peak height of compound **4** was found decreasing whilst that of compound **1** was increasing during the storage period of sample solution. It was proposed that compound **4** was hydrolyzed into compound **1** and coniferyl alcohol probably accounted for the change in HPLC chromatographic pattern. Extraction efficiency of methanol–formic acid at ratios of 99:1, 97:3, 95:5, 93:7 and 90:10 were examined, respectively by ultrasonic extraction. It was observed that the chromatograms of different extracts with methanol–formic acid by sonication were similar. The stability of compounds **1** and **4** in the sample solution extracted with methanol–formic acid (95:5) was further evaluated by determining their peak areas after storage for 0–40 h, respectively. By comparing the chromatographic peak areas, the recovery of compounds **1** and **4** were found having relative standard deviation (R.S.D.) of 1.16 and 2.1% ($n = 8$), respectively. This indicated that both compounds **1** and **4** were relatively stable in methanol–formic acid (95:5). Therefore, extraction using methanol–formic acid (95:5) was chosen in this study.

Selection of detection wavelength was one of the key factors contributing to a reliable and reproducible HPLC fingerprint of CA, JA, SL and CR. The wavelengths of 210, 320, 270 or 284 nm were used for detection in published CA HPLC fingerprint and multi-components analyses [3,4,24–27]. In the present study, analyzing the 3D-plots of chromatograms acquired from HPLC–DAD system of the four umbelliferae species revealed that the 3D-plot of CA was obviously different from that of JA but similar to those of SL and CR with the exception of compound **5**. Therefore, compound **5** was chosen as characteristic marker to distinguish CA from SL and CR. It was also observed that the UV absorption maximum for compound **5** was located at 280 nm in which most of other compounds in the chromatogram possessed strong UV absorbance at this wavelength (Table 2). Hence, characteristic chromatographic patterns were obtained to distinguish CA from JA, SL and CR by using 280 nm detection.

Method reproducibility and repeatability were evaluated by the analysis of seven injections of sample solution and six replicates of solid sample, respectively. Precision of retention times and peak areas of compounds **1**–**5**, **7** and **9** for replicated injection were found in the range of 0.01–0.08 and 0.22–2.89% of R.S.D. ($n = 7$), respectively. The R.S.D. of peak area of compounds **1**–**5**, **7** and **9** in sample replicates were estimated to be 1.1–2.9% ($n = 6$). All results indicated that the conditions for the fingerprint analysis were satisfactory.

3.2. HPLC fingerprints of Chinese Angelica

Altogether 40 CA samples were analyzed including 28 whole root samples, 8 root head samples and 4 rootlet samples (Table 1). These samples were collected from a variety of sources and conditions. These included different cultivation areas, various cultivating environments, different processing methods, different parts of roots or harvesting years, etc. The results indicated that their chromatographic patterns were generally consistent although the absorption intensity of some peaks was different (Fig. 2a and Table 3). The correlation coefficient of each chromatogram to their simulative mean chromatogram was 0.981 ± 0.017 (mean $\pm$ S.D., $n = 40$). The projected dots of the 40 CA chromatograms were localized in a confined cluster in the 3D-projection plot of PCA (Fig. 3). The observation indicates that these chromatograms are associated with similar chemical properties/components of CA.

The HPLC chromatograms of CA samples were further quantitatively expressed in terms of RRT and RPA. Peak 9 (*Z*-ligustilide) was assigned as the reference peak as it was the highest peak in the chromatogram and the major compound of the volatile components in CA. Besides, peaks 1–5 and 7 were also chosen as characteristic peaks as they are bioactive compounds, belonged to the same class and characteristic chemical markers in their chromatograms

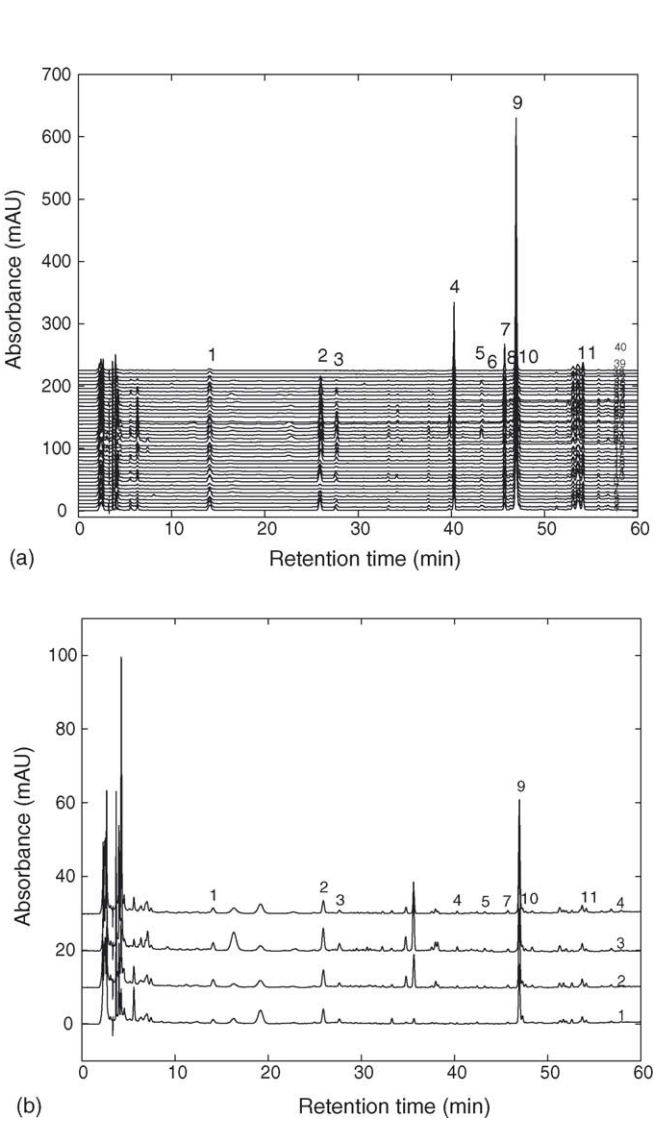

Fig. 2. HPLC chromatograms of (a) 40 Chinese Angelica samples; (b) 4 Japanese Angelicae Root samples; (c) 6 Szechwan Lovage Rhizome samples; and (d) 3 Cnidium Rhizome sample (analytical column: Alltima C_{18}, 5 μm, 250 mm × 4.6 mm; guard column: C_{18}, 5 μm, 7.5 mm × 4.6 mm; injected sample volume: 10 μL; mobile phase: 1.0% acetic acid in water (A) and acetonitrile (B) using a gradient program of 19% (B) in 0–18 min, 19–100% (B) in 18–60 min and 100% (B) in 60–75 min; flow rate: 1.0 mL/min; temperature: 30 °C; measured at UV 280 nm).

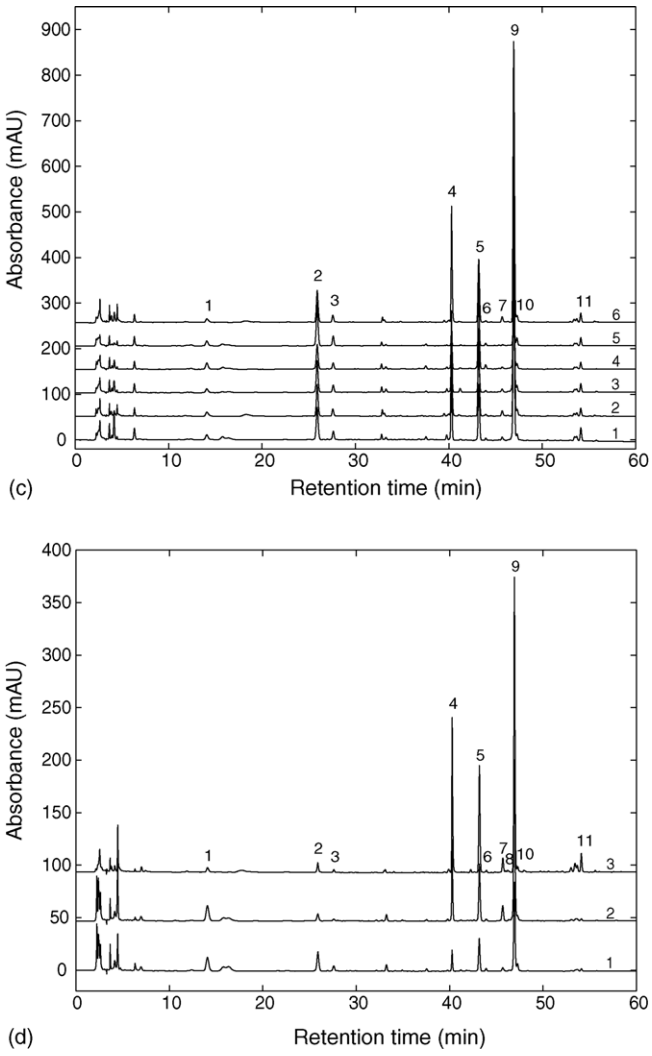

Fig. 2. (*Continued*).

[2,10,11,17,19,25,26,36]. The RRT and RPA of each characteristic peak with respect to the reference peak were calculated (Table 4). The data indicated that the relative amounts of these present compounds were similar in CA and different in other related umbelliferae species. Therefore, it may imply that the simulative mean chromatogram of the 40 tested CA samples can be applied as a standard HPLC fingerprint of CA

Table 3
Similarity comparison of the chromatographic pattern of these herbal samples

Sample	CA	JA	SL	CR
CA	0.981 ± 0.017[a] (*n* = 40)	0.502[b]	0.935[b]	0.910[b]
JA		0.922 ± 0.035[a] (*n* = 4)	0.444[b]	0.511[b]
SL			0.947 ± 0.087[a] (*n* = 6)	0.960[b]
CR				0.961 ± 0.038[a] (*n* = 3)

CA: Chinese Angelica, the root of *Angelica sinensis* (Oliv.) Diels; JA: Japanese Angelicae Root, the roots of *A. acutiloba* Kitagawa or *A. acutiloba* Kitagawa var. *sugiyamae* Hikino; SL: Szechwan Lovage Rhizome, the rhizome of *Ligusticum chuanxiong* Hort; CR: Cnidium Rhizome, the rhizome of *Cnidium officinale* Makino.

[a] The correlation coefficient of each chromatogram to themselves simulative mean chromatogram, mean ± S.D.

[b] The correlation coefficient between simulative mean chromatograms.

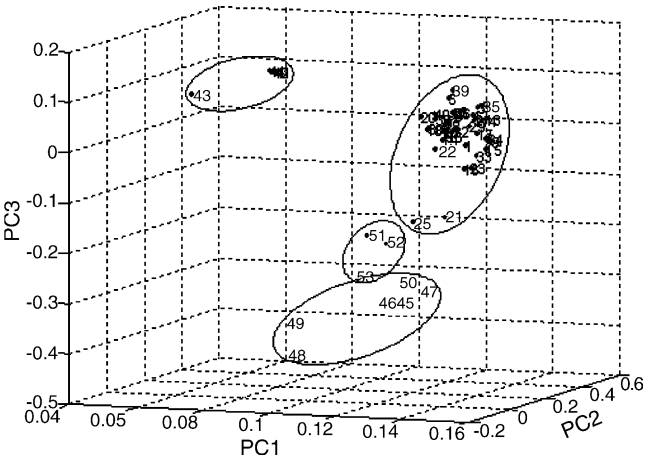

Fig. 3. 3D-projection plots of principal component analysis (PCA) of three principal components for the 53 samples. PC1, PC2 and PC3 are first three principal components using entire chromatographic profile as input data: 1–40, the samples of Chinese Angelica (CA); 41–44, the samples of Japanese Angelicae Root (JA); 45–50, the samples of Szechwan Lovage Rhizome (SL); and 51–53, the samples of Cnidium Rhizome (CR).

and can be used for differentiation of CA from other related species (Fig. 4a). The RRT and RPA data of the characteristic peaks may reflect the quantitative expression of the present HPLC fingerprint of CA.

3.3. Distinguishing Chinese Angelica from Japanese Angelicae Root, Szechwan Lovage Rhizome and Cnidium Rhizome

The validation of HPLC fingerprints for chemical identification of CA was further studied by comparing with related umbelliferae herbs. Several batches of JA, SL and CR samples were analyzed. The differentiation of CA, JA, SL and CR samples were described as follows:

3.3.1. Distinguishing of Chinese Angelica from Japanese Angelicae Root

JA is officially used as medicinal material in Japan and it is the roots of *A. acutiloba* Kitagawa or *A. acutiloba* Kitagawa var. *sugiyamae* Hikino [21]. Two samples of each *A. acutiloba* (JA-1 and JA-2) and *A. acutiloba* var. *sugiyamae* (JA-3 and JA-4) were analyzed. The chromatograms of the four JA samples were found resembling to each other (Fig. 2b). The correlation coefficient of each chromatogram to their simulative mean chromatogram was 0.922 ± 0.035 (mean $\pm$ S.D., $n = 4$) (Table 3). However, the chromatogram of JA showed drastic differences from of that of CA (Figs. 2a, b and 4a, b). The correlation coefficient of each the four JA chromatograms to the CA simulative mean chromatogram was only 0.465 ± 0.072 (mean $\pm$ S.D., $n = 4$), and the correlation coefficient of the simulative mean chromatogram of CA to that of JA was 0.502. The 3D-projection plot of PCA in three principal components also shows that samples CA and JA are significantly

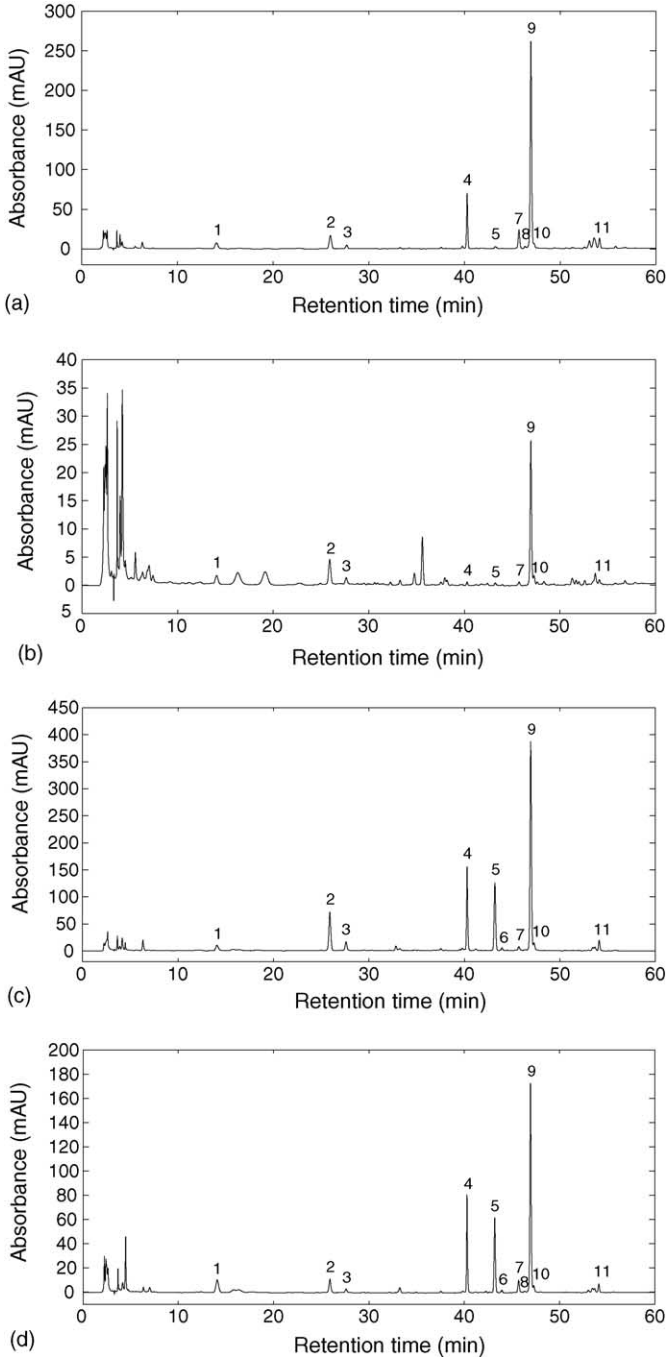

Fig. 4. Simulative mean chromatograms of (a) Chinese Angelica; (b) Japanese Angelicae Root; (c) Szechwan Lovage Rhizome; and (d) Cnidium Rhizome: (1) ferulic acid; (2) senkyunolide I; (3) senkyunolide H; (4) coniferyl ferulate; (5) senkyunolide A; (6) butylphthalide; (7) *E*-ligustilide; (8) *E*-butylidenphthalide; (9) *Z*-ligustilide; (10) *Z*-butylidenephthalide; (11) levistolide A.

different (Fig. 3). Comparing the chemical components in the CA and JA chromatograms, compound **9** was the highest peak in the CA chromatogram whilst the peak with retention time at about 4.2 min was the highest in the JA chromatogram. Besides, the RPA of compound **4** in CA sample was 11 times higher than that in the JA sample (Table 4).

Table 4
The relative retention time (RRT) and relative peak area (RPA) of characteristic peaks in Chinese Angelica, Japanese Angeliae Root, Szechwan Lovage Rhizome and Cnidium Rhizome samples

Peak no.	Compound	Chinese Angelica		Japanese Angelicae Root		Szechwan Lovage Rhizome		Cnidium Rhizome	
		RRT	RPA	RRT	RPA	RRT	RPA	RRT	RPA
1	Ferulic acid	0.30 ± 0.004	0.06 ± 0.03	0.30 ± 0.02	0.11 ± 0.03	0.31 ± 0.02	0.07 ± 0.06	0.31 ± 0.01	0.15 ± 0.12
2	Senkyunolide I	0.55 ± 0.003	0.10 ± 0.11	0.55 ± 0.001	0.25 ± 0.07	0.56 ± 0.01	0.41 ± 0.48	0.55 ± 0.005	0.14 ± 0.15
3	Senkyunolide H	0.59 ± 0.002	0.02 ± 0.02	0.59 ± 0.02	0.05 ± 0.01	0.59 ± 0.01	0.08 ± 0.08	0.59 ± 0.005	0.03 ± 0.03
4	Coniferyl ferulate	0.86 ± 0.001	0.22 ± 0.08	0.86 ± 0.001	0.02 ± 0.004	0.86 ± 0.01	0.32 ± 0.09	0.86 ± 0.01	0.36 ± 0.15
5	Senkyunolide A	0.92 ± 0.001	0.01 ± 0.01	0.92 ± 0.003	0.02 ± 0.003	0.92 ± 0.001	0.36 ± 0.22	0.92 ± 0.001	0.33 ± 0.01
7	*E*-Ligustilide	0.97 ± 0.001	0.08 ± 0.04	0.97 ± 0.001	0.02 ± 0.004	0.97 ± 0.001	0.02 ± 0.01	0.97 ± 0.001	0.05 ± 0.03
9	*Z*-Ligustilide	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

The data of RRT and RPA are the ratio of t_R and peak area of each characteristic peak to those of peak 9, respectively. The value is mean ± S.D. ($n = 40$ for Chinese Angelica, $n = 4$ for Japanese Angelicae Root, $n = 6$ for Szechwan Lovage Rhizome and $n = 3$ for Cnidium Rhizome).

Thus, CA and JA could be easily distinguished by either their chromatograms or the amount of compound **4** in their samples.

3.3.2. Distinguishing Chinese Angelica from Szechwan Lovage Rhizome and Cnidium Rhizome

SL and CR are related species of CA and belong to the Umbelliferae family. They are official medicinal materials in China and Japan, respectively [22,23]. Some components are commonly found in both species, such as compounds **1**, **4**, **7**, **9**, *Z*-butylidenephthalide and *E*-butylidenephthalide. Moreover, compound **9** is also the major compound in their volatile oils [4,17,18,25,29,36]. Therefore, it was not easy to identify them based on their chemical constituents. Six SL samples and three CR samples were then compared with general consistent chromatograms of each species (Fig. 2c and d). The correlation coefficients of each chromatogram to their simulative mean chromatograms were 0.947 ± 0.087 (mean ± S.D., $n = 6$) for SL samples and 0.961 ± 0.038 (mean ± S.D., $n = 3$) for CR samples. However, a unique characteristic peak in their chromatogram for qualitatively distinguishing CA from SL and CR is not available (Fig. 4a, c and d). Nevertheless, compound **5** was quantitatively different between CA from SL and CR samples. The RPA of compound **5** in SL and CR samples were 36 and 33 times of that in CA samples, respectively (Table 4). Using this information, it would be helpful to distinguish CA from SL and CR samples by comparing the RPA or amount of compound **5** in their chromatograms.

The correlation coefficient of chromatogram showed that CA sample was different from SL and CR. However, their difference was not much significant (Table 3). The correlation coefficient of each SL chromatogram to the CA simulative mean chromatogram (0.871 ± 0.123, mean ± S.D., $n = 6$) and that of each CR chromatograms to the CA simulative mean chromatogram (0.870 ± 0.044, $n = 3$) were not significantly smaller than the correlation coefficient of each CA chromatogram to CA simulative mean chromatogram (0.981 ± 0.017, $n = 40$). This indicated that there was resemblance in terms of chemical constituents of CA sample with SL and CR sample.

3.3.3. Distinguishing between Japanese Angelicae Root, Szechwan Lovage Rhizome and Cnidium Rhizome

JA sample can be easily distinguished from SL and CR by either their chromatographic patterns or characteristic compounds (Fig. 4b–d and Table 4). The correlation coefficients of JA simulative mean chromatogram to SL and CR simulative mean chromatograms were only 0.444 and 0.511, respectively. Meanwhile, the contents (peak area/sample weight, mAU s/g) of compounds **4** and **5** in JA sample were both less than those in SL and CR samples by two orders.

Although the contents (peak area/sample weight, mAU s/g) of compounds **2**–**5** and **9** in SL samples were more than two times of those in CR samples, which agreed with the reported data of Naito et al. [36], the chromatograms of the two species were quite similar (Figs. 2c, d and 4c, d). The correlation coefficient of the simulative mean chromatogram of SL to that of CR chromatogram was 0.960, which was close to the correlation coefficient of each SL chromatogram to their simulative mean chromatogram (0.947 ± 0.087, $n = 6$) and each CR chromatogram to their simulative mean chromatogram (0.961 ± 0.038, $n = 3$) (Table 3). It is worth noting that even the correlation coefficients between the samples of SL and CR are rather close, some overlapping in fingerprint patterns could be still observed for SL and CR samples in the 3D-projection plot of PCA in our present work (Fig. 3). More samples are needed to obtain a more representative population. Nevertheless, the results indicated that the main chemical components in SL and CR samples were similar, and demonstrated that the relationship of the two species was very close indeed. The analysis of two genes plastid *mat* K (maturase for lysine) and nuclear internal transcribed spacer (ITS) also revealed that the origin of SL and CR ought to be identical, and it was suggested that SL and CR should be classified as the same species, and the botanical name of CR should be *Ligusticum chuanxiong* instead of *Cnidium officinale* [37]. If this is correct, SL and CR samples should belong to the same species and the difference in chemical fingerprints should belong to the intra-species variations. However, further studies should be carried out in order to have a definitive conclusion in the future.

3.4. Identification of chemical compounds in fingerprint chromatograms

In order to quantitatively express the distribution of chemical compounds in the chromatograms, two compounds were unequivocally identified and nine compounds were tentatively assigned based on their on-line APCI–MS data and UV spectra. Their retention time (t_R), APCI–MS and UV data are listed in Table 2.

Peaks 1 and 9 were unequivocally identified as ferulic acid and *Z*-ligustilide by spiking authentic compounds and then comparing the UV and APCI–MS spectra with those of authentic compounds. In the APCI–MS spectra, strong deprotonated molecular ion $[M-H]^-$ peak at 193 (*m*/*z*) for ferulic acid was observed. For *Z*-ligustilide, the strong protonated molecular ion $[M+H]^+$ at 191 (*m*/*z*) was found, and the characteristic fragments corresponding to $[M+H-H_2O]^+$ and $[M+H-H_2O-CO]^+$ at 173 and 145 (*m*/*z*) were also noted. The UV spectra were also compatible with those of reported spectra and/or data in literatures [18,19,25–27].

Owing to the unavailability of authentic compounds, peaks 2–8, 10 and 11 could only be tentatively assigned as senkyunolide I (2), senkyunolide H (3), coniferyl ferulate (4), senkyunolide A (5), butylphthalide (6), *E*-ligustilide (7), *E*-butylidenephthalide (8), *Z*-butylidenephthalide (10) and levistolide A (11) by comparing their APCI–MS and UV spectra in literatures [18,19,25–27].

The protonated molecular ions $[M+H]^+$ were found in the APCI–MS spectra for all compounds except compound **4**. The observed characteristic ions of these seven compounds (**2**, **3**, **5**–**8** and **10**), namely $[M+H-H_2O]^+$, $[M+H-CO]^+$, $[M+H-H_2O-H_2O]^+$ and/or $[M+H-H_2O-CO]^+$, provide further evidence on their chemical structures. For compound **4**, the strong positive fragment ion at 163 (*m*/*z*) was assigned as coniferyl alcohol moiety of coniferyl ferulate. In APCI–MS negative mode, the deprotonated molecular ion $[M-H]^-$ at 355 (*m*/*z*) was relatively weak whilst the negative fragment ion at 193 (*m*/*z*) has the strongest response and was assigned as ferulic acid moiety of the coniferyl ferulate structure. For compound **11**, the characteristic positive fragment at ion 191 (*m*/*z*) was observed and assigned as fragment of one of the two moieties in its structure with molecular mass of 380.

The UV spectra of these compounds were further compared with those reported in literatures. For compounds **4**–**5**, **8** and **10**, their UV spectra were consistent with those reported in literatures [18,19,25–27]. For compounds **2**–**3**, the UV data were consistent with the reported UV data [19,27]. These two compounds were further differentiated by the relative amount in CA and SL samples when comparing with observations reported in literatures, i.e. the content of senkyunolide I was much higher than that of senkyunolide H in CA and SL samples [19,27]. Therefore, peaks 2 and 3 could be generated from senkyunolide I and senkyunolide H, respectively. For compounds **6**–**7**, their UV spectra were consistent with the reported spectra of butylphthalide and *E*-ligustilide in literatures and hence the identities of these two compounds were suggested accordingly [25,26]. Similarly, the UV spectra of compound **11** were consistent with those in literatures and suggested to be levistolide A [18,25]. For unambiguous identification of these compounds, further studies are required by using authentic compounds.

4. Conclusions

Chinese Angelica is a widely used TCM material. Although there are morphological differences and variations in the chemical contents as a result of difference in source of origin and processing methods, the chromatograms of different products were found generally consistent both with respect to correlation coefficient of their chromatograms and relative peak areas of characteristic compounds. The consistency in chromatogram of these representative samples reflects the similar chemical properties of Chinese Angelica, and hence the simulative mean chromatogram of the 40 tested samples could be employed as the standard fingerprint/chromatogram for chemical identification of Chinese Angelica.

The developed HPLC fingerprints can be used to differentiate Chinese Angelica from Japanese Angelicae Root, Szechwan Lovage Rhizome and Cnidium Rhizome. The chemical components in Chinese Angelica and Japanese Angelicae Root were quite different even though they belong to same genus, genus *Angelica*, and both of them are official medicinal materials used in China and Japan, respectively. They could be easily distinguished by either their chromatographic patterns or the content of coniferyl ferulate. For the differentiation of Chinese Angelica, Szechwan Lovage Rhizome and Cnidium Rhizome, despite some chemical compounds commonly found in the samples, the amounts of senkyunoide A in these samples are relatively different and hence the chemical differentiation between these species become possible. Similarly, Japanese Angelicae Root can be distinguished from Szechwan Lovage Rhizome and Cnidium Rhizome using similar approach. However, our results are not conclusive for differentiation of Szechwan Lovage Rhizome and Cnidium Rhizome. It is suggested that *Ligusticum chuanxiong* Hort. and *Cnidium offlcinale* Makino could be considered as a single species in terms of chemotaxonomy.

Analysis of the 3D-plot obtained by HPLC–DAD is a very useful tool for comparing the chemical components in different species with selected characteristic peak and detection wavelength. In our present study, senkyunolide A was chosen as marker compound to distinguish CA from SL and CR using UV at 280 nm as measuring wavelength for the characteristic chromatograms for differentiation.

Eleven compounds were unequivocally determined (peaks 1 and 9) or tentatively identified (peaks 2–8, 10 and 11) in the chromatogram using LC–(APCI)–MS and HPLC–DAD techniques. Furthermore, the *Computer Aided Similarity Evaluation System* was a very useful tool for quantitative studies of the similarity of chromatographic patterns and generation

of the mean chromatogram used as standard chromatogram of samples. The 3D-projection plot of principal component analysis of three principal components can visually show the relationships between the medicinal materials based on the chemical constituents.

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