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Simultaneous determination of 12 major constituents in *Forsythia suspensa* by high performance liquid chromatography—DAD method

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Abstract

A high performance liquid chromatographic method was developed to simultaneously determine the 12 major constituents of *Forsythia suspensa*, namely *R*-suspensaside, *S*-suspensaside, *S*-suspensaside methyl ether, (+)-pinoresinol-β-D-glucoside, forsythiaside, (+)-epipinoresinol-4′-*O*-glucoside, suspensaside A, rutin, phillyrin, (+)-pinoresinol, (+)-epipinoresinol and phillygenin. The HPLC assay was performed on a Zorbax XDB C_{18} column with gradient elution of methanol and 0.3% aqueous acetic acid within 55 min. The detection wavelength was 280 nm. All the compounds showed good linearity ($r^2 > 0.9998$). The method was reproducible with intra- and inter-day variation less than 3%. The recovery of the assay was in the range of 91.2–104.9%. The method was successfully applied to the quantification of 12 constituents in 33 *F. suspensa* samples. The results indicated that the developed assay could be considered as a suitable quality control method for *F. suspensa*.

Keywords: RP-HPLC; *Forsythia suspensa*; Determination; Quality control

1. Introduction

The fruit of *Forsythia suspensa* (Thunb.) Vahl (Oleaceae) is a well-known traditional Chinese medicine (TCM), named 'Lianqiao' in Chinese. According to maturity level of the fruits, the commercial drugs could be classified into 'Qingqiao' and 'Laoqiao', both of them are official sources of this TCM. More than 40 Chinese medicinal preparations containing *F. suspansa* are listed in Chinese Pharmacopoeia, such as Shuanghuanglian oral solution, Yinqiao Jiedu tablet and Qinlian tablet, etc. [1].

The crude drug had been widely used as an antipyretic, antidotal and anti-inflammatory agent for the treatment of infections, such as acute nephritis, erysipelas and ulcer [2]. It was also reported that *F. suspensa* could suppress vomiting, resist hepatic injury, inhibit elastase activity, and exhibit diuretic, analegesic, antioxidant, antiendotoxin and antivirial effects [2–7].

A number of compounds including phenylethanoid glycosides, lignans, flavonoids, terpenes, and volatile oils had been isolated from the title plant [8]. Among them, the first three types of compounds were proven to be responsible for the various biological activities of the herb. Some of them showed antibacterial (forsythiaside, suspensaside) [9,10], antioxidant (phillyrin, (+)-pinoresinol, phillygenin and rutin) [11,12], anti-inflammatory (forsythiaside, suspensaside) [13], weight losing (phillyrin) [14], blood pressure reducing (suspensaside, (+)-pinoresinol, (+)-pinoresinol-β-D-glucoside) [15,16] and cyclic adenosine monophosphate (cAMP) phosphodiesterase inhibitory effects (forsythiaside, (+)-pinoresinol, (+)-pinoresinol-β-D-glucoside) [9,17]. Hence, quantification of these types of compounds in *F. suspensa* would be of great significance for the evaluation of the quality of this herb. However, previous studies mainly focused on the quantitative determination of single or a few constituents in *F. suspensa* by TLC [18–20], CE-ED [21], CE-UV [22] and HPLC [23–28]. The current study aimed at developing a simple and feasible method for the simultaneous quantification of 12 major constituents in *F. suspensa* in order to control the quality of this important Chinese herbal medicine.

2. Experimental

2.1. Chemicals and materials

HPLC grade methanol and analytical grade acetic acid were purchased from Beijing Chemical Factory (Beijing, China). The

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deionized water was prepared using Millipore purification system (Millipore, Milford, MA, USA) and filtered with 0.45 μm membranes. Commercial herbal samples were collected from local drug stores in different provinces. Rutin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). *R*-suspensaside, *S*-suspensaside, *S*-suspensaside methyl ether, (+)-pinoresinol-β-D-glucoside, forsythiaside, (+)-epipinoresinol-4′-*O*-glucoside, suspensaside A, phillyrin, (+)-pinoresinol, (+)-epipinoresinol and phillygenin were isolated by the author from the fruits of *F. suspensa*.

The fruits of *F. suspensa* (5 kg) were extracted with hot 95% EtOH three times, and the solutions were combined and concentrated under reduced pressure. A suspension of the EtOH extract in H_2O was extracted successively with petroleum ether, EtOAc and *n*-BuOH. The EtOAc extracts (170 g) were subjected to repeated column chromatography on silica gel, eluting with petroleum ether–acetone or $CHCl_3$–MeOH gradient solvent system. Further purification was performed by using semipreparative HPLC with MeOH–H_2O solvent system to give phillygenin (18 mg), (+)-epipinoresinol (26 mg), (+)-pinoresinol (264 mg), phillyrin (130 mg), (+)-pinoresinol-β-D-glucoside (112 mg), (+)-epipinoresinol-4′-*O*-glucoside (27 mg), suspensaside A (35 mg) and forsythiaside (135 mg). The *n*-BuOH extracts (100 g) were subjected to column chromatography on AB-8 macroporous resin, eluting with EtOH–H_2O gradient solvent system. Repeated re-chromatography on Sephadex LH-20 and purification with semipreparative HPLC gave *R*-suspensaside (10 mg), *S*-suspensaside (20 mg) and *S*-suspensaside methyl ether (15 mg). All these compounds were identified by direct comparison of their ^{1}H NMR, ^{13}C NMR and MS spectral data with those reported in the literature [10,29–33], and their purities were not less than 95% by HPLC analysis. Structures of the 12 compounds are shown in Fig. 1.

2.2. Apparatus and chromatographic conditions

The analyses were performed using an Agilent 1100 liquid chromatography system, equipped with a quaternary solvent delivery system, an autosampler and a DAD detector. The

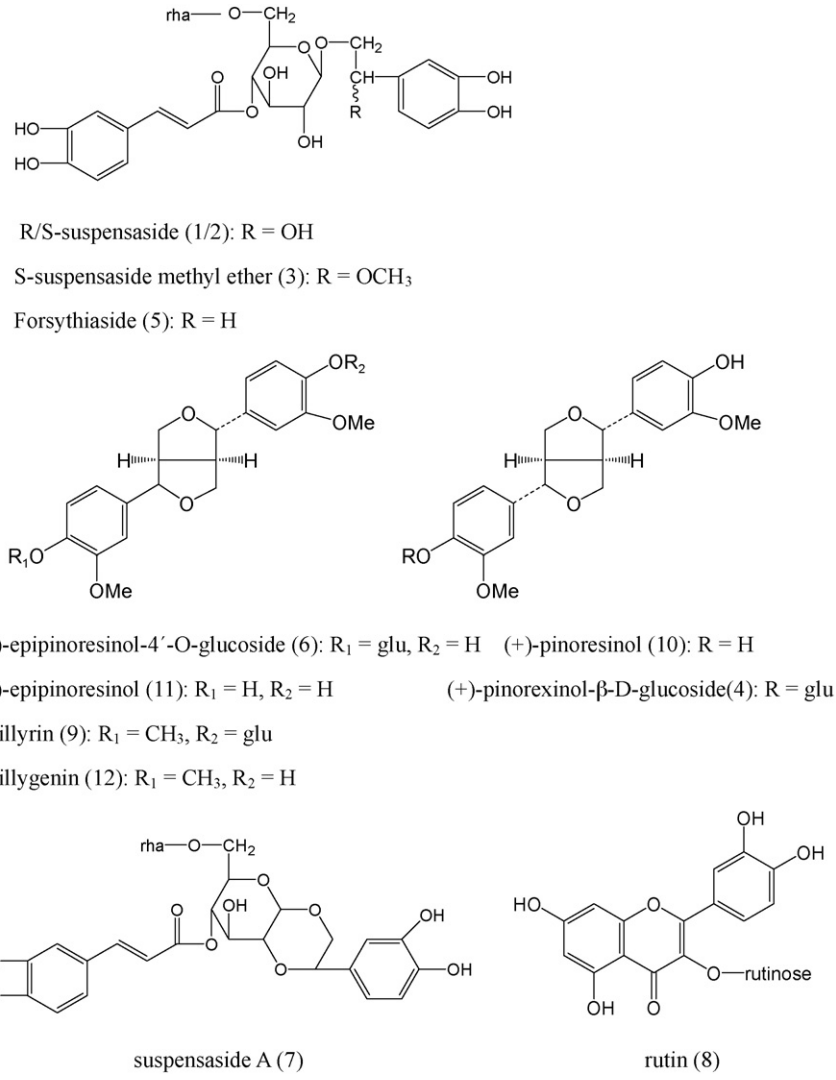

Fig. 1. The structures of 12 major constituents in *F. suspensa*.

separation was carried out on a Zorbax XDB C_{18} column (250 mm × 4.6 mm, 5 μm) coupled with a Zorbax XDB C_{18} guard column (12.5 mm × 4.6 mm, 5 μm). The mobile phase consisted of solvent A (methanol) and solvent B (0.3% aqueous acetic acid, v/v). Gradient elution was as follows: initial 0–8 min, linear change from A–B (30:70, v/v) to A–B (33:67, v/v); 8–24 min, linear change to A–B (40:60, v/v); 24–39 min, linear change to A–B (48:52, v/v); 39–55 min, linear change to A–B (64:36, v/v). UV absorption was monitored at 280 nm. The column temperature was set at 25 °C. The flow rate was 0.8 ml min^{-1} and sample injection volume was 5 μl.

2.3. Calibration curve

A 50% methanol stock solution containing all 12 reference standards was prepared by dissolving the reference standards in 50% methanol to final concentration of 552.5 μg/ml for *R*-suspensaside, 641.3 μg/ml for *S*-suspensaside, 276.3 μg/ml for *S*-suspensaside methyl ether, 250 μg/ml for (+)-pinoresinol-β-D-glucoside, 4275 μg/ml for forsythiaside, 270 μg/ml for (+)-epipinoresinol-4′-*O*-glucoside, 213.8 μg/ml for suspensaside A, 700 μg/ml for rutin, 415 μg/ml for phillyrin, 150 μg/ml for (+)-pinoresinol, 165 μg/ml for (+)-epipinoresinol and 165 μg/ml for phillygenin, respectively, then diluted the mixture stock solution to appropriate concentration to establish calibration curves. Each calibration curve consisted of six different concentrations and was performed in triplicate. All calibration curves were constructed from peak areas of the reference standards versus their concentrations.

2.4. Sample preparations

The dried powders of *F. suspensa* samples (0.2 g, 75 mesh) were accurately weighed and extracted by refluxing with 5 ml 50% aqueous methanol solution for 1 h. Then the resultant mixture was adjusted to the original weight and aliquots of the supernatant were filtered through 0.45 μm membrane before HPLC injection.

3. Results and discussion

3.1. Configuration of compounds *1–3*

Suspensaside was previously isolated as a racemate from *F. suspensa*, which exhibited two sets of signals in ^{13}C NMR spectrum [10,29]. In current work, two extremely similar compounds (**1**, **2**) were isolated by semipreparative HPLC. They had almost the same ^{1}H NMR and ^{13}C NMR data, which were in good agreement with the literature data of suspensaside [10,30]. Therefore, they were possibly a pair of isomers. To determine their absolute configuration at C-7 of the 7-hydroxy-phenylethyl moiety, differences of molecular optical rotation (Δ[*M*]) between compounds **1**, **2** and forsythiaside were compared with molecular optical rotation ([*M*]) value of (+)-phenylethane-1,2-diol. It was expected that the compound with Δ[*M*] value nearly equal to [*M*] value of (+)-phenylethane-1,2-diol had the *S* configuration. The results showed that compound **2** was *S*-suspensaside (see Table 1). While compound **1** had contrary Δ[*M*] value, therefore it was identified as *R*-suspensaside. This method was successfully applied previously for the configuration confirmation [34].

Table 1
Difference of molecular optical rotation of several suspensaside related compounds

	$[\alpha]_D$	$[M]_D$	$\Delta[M]_D$
Compound **1** (*R*-suspensaside)	−31.3	−200.3	−79.9
Compound **2** (*S*-suspensaside)	−4.7	−30.1	+90.3
Compound **3** (*S*-suspensaside methyl ether)	−4.7	−30.7	+89.7
Forsythiaside	−19.3	−120.4	
(+)-Phenylethane-1,2-diol	+60.3	+83.2	

The ^{1}H NMR and ^{13}C NMR data of compound **3** were similar to those of compounds **1** and **2** except for the signals of additional methoxyl group. With reference to literature data, it was identified as suspensaside methyl ether [29]. *S* configuration was confirmed with the abovementioned method. Therefore, compound **3** was identified as *S*-suspensaside methyl ether.

3.2. Extraction method

In order to obtain satisfactory extraction efficiency, extraction method, extraction solvent and extraction time were investigated. The results suggested that refluxing was better than ultrasonic extraction, so refluxing was used in further experiments. Water, 10% methanol, 30% methanol, 50% methanol, 70% methanol and methanol were performed as extraction solvents to analyze the effect of the solvent on extraction efficiency. The results showed that 50% methanol was the most suitable extraction solvent (see Fig. 2). Then 0.2 g samples were extracted with 5 ml 50% methanol by refluxing for 30, 60, 90 and 120 min, respectively, to determine optimal extraction time. As shown in Fig. 3, the maker compounds were almost completely extracted within 60 min. Hence, 60 min was chosen as optimal extraction time.

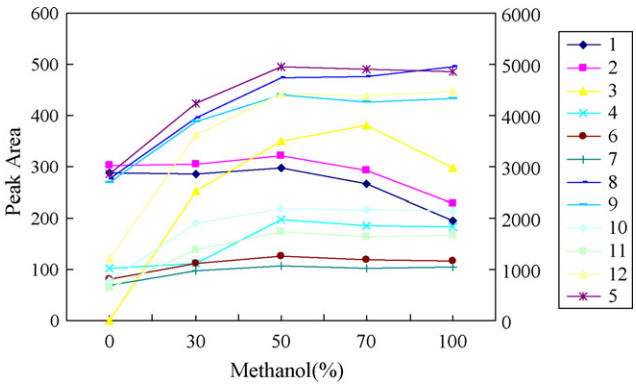

Fig. 2. Extraction efficiency of different solvents: (1) *R*-suspensaside; (2) *S*-suspensaside; (3) *S*-suspensaside methyl ether; (4) (+)-pinoresinol-β-D-glucoside; (5) forsythiaside; (6) (+)-epipinoresinol-4′-*O*-glucoside; (7) suspensaside A; (8) rutin; (9) phillyrin; (10) (+)-pinoresinol; (11) (+)-epipinoresinol; (12) phillygenin.

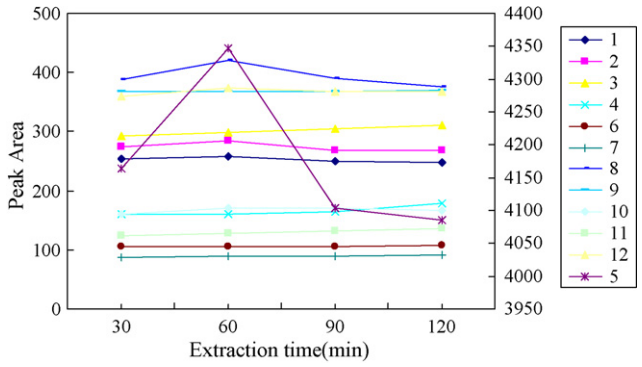

Fig. 3. Extraction efficiency of different extraction time: (1) *R*-suspensaside; (2) *S*-suspensaside; (3) *S*-suspensaside methyl ether; (4) (+)-pinoresinol-β-D-glucoside; (5) forsythiaside; (6) (+)-epipinoresinol-4′-*O*-glucoside; (7) suspensaside A; (8) rutin; (9) phillyrin; (10) (+)-pinoresinol; (11) (+)-epipinoresinol; (12) phillygenin.

3.3. Chromatographic conditions optimization

Different types of chromatographic columns were tested to optimize the separation. Phillyrin and (+)-pinoresinol could not reach a baseline separation on Hypersil C_{18} column and Alltima C_{18} column, while retention times of constituents were too long on Luna C_{18} column. Although *F. suspensa* sample demonstrated similar chromatographic behavior with good separation on Zorbax Extend C_{18} column and Zorbax XDB C_{18} column, the resolution of Zorbax XDB C_{18} column was a little better than the other ones, thus Zorbax XDB C_{18} was used.

The effect of mobile phase composition was also examined. It was found that *R*-suspensaside and *S*-suspensaside could hardly be resolved from each other when acetonitrile was used. However, when acetonitrile was replaced by methanol, the situation was greatly improved and satisfactory resolution was obtained. Addition of acid in mobile phase was found to be useful for the improvement of peak shape, but the type and concentration of acids seem to have no serious effect on the separation.

Since three types of constituents were analyzed by the method, the monitoring wavelength was set at 280 nm, where all the three types of compounds have sufficient absorption. It was also suggested that separation was better when column temperature was kept at 25 °C than 20, 30 and 35 °C.

3.4. Linearity and the limit of detection

Linear regression analysis for each of the 12 compounds was performed by external standard method. Under current chromatographic conditions, all 12-calibration curves showed good linearity ($r^2 > 0.9998$). The results are given in Table 2. The limits of detection (LOD) ranged from 0.08 to 0.13 μg/ml for the 12 compounds.

3.5. Precision and accuracy

The intra- and inter-day variabilities were measured to determine the precision and reproducibility of the method. The intra-day variability was examined on five individual samples within 1 day, and inter-day variability was determined for 3 independent days. In both tests three different concentration levels (high, middle and low) were prepared. The results are presented in Table 3. The relative standard deviations were 0.12–2.30 and 0.12–2.86%, respectively.

To further evaluate the accuracy of the method, recovery test was performed by spiking known quantities of the mixed standard solution to known amounts of *F. suspensa* samples. The resultant samples were then extracted and analyzed with the described HPLC method. The added standard solutions were prepared in three different concentration levels (high, middle and low) and triplicate experiments at each level. The accuracy was calculated with the value of detected versus added amounts. The recovery of the method was in the range of 91.2–105.0%, with R.S.D. less than 5.11%. Considering the results, the method was deemed to be accurate.

3.6. Repeatability and ruggedness

Seven samples of *F. suspensa* from the same source were extracted and analyzed with the above-established method. The R.S.D. value was calculated as a measurement of method

Table 2
Calibration curves of the 12 constituents in *F. suspensa*

Compound	Regression equation	r^2	Linear range (μg ml^{-1})	LOD (μg/ml)
1	$y = 4.3504x + 8.1935$	0.9998	22.10–552.50	0.09
2	$y = 4.7453x + 10.1700$	0.9998	25.65–641.25	0.13
3	$y = 5.7997x - 0.9827$	1.0000	11.05–276.25	0.11
4	$y = 3.2453x + 0.2731$	0.9998	10.00–250.00	0.11
5	$y = 5.0861x + 28.5170$	0.9998	171.00–4275.00	0.11
6	$y = 3.6373x + 1.5768$	0.9999	10.80–270.00	0.12
7	$y = 5.2783x - 2.6077$	1.0000	8.55–213.75	0.11
8	$y = 4.7045x + 9.9288$	0.9999	28.00–700.00	0.10
9	$y = 4.2097x + 1.1061$	1.0000	16.60–415.00	0.09
10	$y = 5.4047x + 0.1110$	1.0000	6.00–150.00	0.08
11	$y = 4.6110x + 2.3296$	0.9998	6.60–165.00	0.09
12	$y = 5.2141x + 0.3154$	0.9999	6.60–165.00	0.09

y, peak area; *x*, concentration of compound (μg ml^{-1}); limit of detection, S/N = 3.

Table 3
Intra- and inter-day variability for the assay of the 12 constituents

Compound	Concentration ($\mu g\,ml^{-1}$)	Intra-day ($n=5$)			Inter-day ($n=3$)		
		Found	R.S.D.[a] (%)	Accuracy[b] (%)	Found	R.S.D. (%)	Accuracy (%)
1	110.50	111.44 ± 0.57	0.51	100.85	111.57 ± 0.33	0.30	100.97
	221.00	222.31 ± 0.69	0.31	100.59	221.82 ± 0.46	0.21	100.37
	331.50	328.02 ± 2.44	0.74	98.95	329.08 ± 4.21	1.28	99.27
2	128.25	129.68 ± 0.38	0.29	101.11	129.57 ± 0.55	0.42	101.03
	256.50	258.01 ± 0.77	0.30	100.59	257.59 ± 0.62	0.24	100.43
	384.75	382.21 ± 2.48	0.65	99.34	383.08 ± 4.95	1.29	99.57
3	55.25	55.25 ± 0.11	0.20	99.99	54.86 ± 0.61	1.11	99.29
	110.50	109.00 ± 0.13	0.12	98.64	109.62 ± 0.52	0.47	99.20
	165.75	164.65 ± 0.91	0.55	99.33	164.70 ± 0.95	0.57	99.36
4	50.00	50.77 ± 0.40	0.78	101.54	50.52 ± 0.37	0.73	101.04
	100.00	100.48 ± 0.45	0.45	100.48	99.80 ± 0.81	0.82	99.80
	150.00	149.48 ± 0.56	0.38	99.65	149.39 ± 1.17	0.78	99.60
5	855.00	871.91 ± 5.32	0.61	101.98	865.27 ± 7.92	0.92	101.20
	1710.00	1681.54 ± 21.87	1.30	98.34	1670.37 ± 13.42	0.80	97.68
	2565.00	2417.52 ± 37.50	1.55	94.25	2439.91 ± 69.84	2.86	95.12
6	54.00	54.25 ± 0.18	0.34	100.47	54.34 ± 0.71	1.31	100.63
	108.00	108.67 ± 0.44	0.40	100.62	108.98 ± 0.66	0.60	100.91
	162.00	164.60 ± 0.35	0.21	101.61	164.31 ± 1.06	0.65	101.43
7	42.75	43.19 ± 0.14	0.32	101.03	43.45 ± 1.09	2.51	101.64
	85.50	87.10 ± 1.01	1.16	101.87	87.35 ± 0.91	1.05	102.17
	128.25	135.17 ± 1.11	0.82	105.40	133.67 ± 1.57	1.17	104.22
8	140.00	143.09 ± 2.43	1.70	102.20	140.50 ± 3.83	2.72	100.36
	280.00	279.48 ± 0.73	0.26	99.81	277.51 ± 4.84	1.74	99.11
	420.00	420.11 ± 1.93	0.46	100.03	416.20 ± 4.19	1.01	99.09
9	83.00	82.94 ± 0.24	0.29	99.93	83.15 ± 0.49	0.59	100.18
	166.00	166.94 ± 0.47	0.28	100.57	166.97 ± 0.06	0.04	100.58
	249.00	251.47 ± 1.45	0.57	100.99	250.46 ± 2.14	0.86	100.59
10	30.00	29.94 ± 0.07	0.25	99.80	29.93 ± 0.04	0.12	99.78
	60.00	59.98 ± 0.10	0.17	99.96	59.70 ± 0.51	0.86	99.50
	90.00	89.83 ± 0.44	0.49	99.81	89.61 ± 0.56	0.63	99.56
11	33.00	32.79 ± 0.19	0.58	99.36	33.01 ± 0.14	0.44	100.03
	66.00	66.44 ± 0.18	0.27	100.66	66.44 ± 0.32	0.48	100.66
	99.00	103.34 ± 2.37	2.30	104.39	100.43 ± 0.41	0.41	101.44
12	33.00	32.70 ± 0.11	0.33	99.10	32.86 ± 0.10	0.30	99.57
	66.00	65.94 ± 0.17	0.26	99.91	66.15 ± 0.29	0.44	100.23
	99.00	99.69 ± 0.35	0.35	100.70	99.50 ± 0.33	0.33	100.51

[a] R.S.D. (%) = (S.D./mean) × 100.
[b] Accuracy (%) = [(mean of measured concentration − spiked concentration)/spiked concentration] × 100.

repeatability. R.S.D. values of 12 compounds were from 0.41 to 3.79%, which showed high repeatability of the method.

The ruggedness of the method was evaluated by applying the developed procedures to assay the same *F. suspensa* sample using different instruments by two different analysts. With R.S.D. less than 4.82%, the method could be considered robust.

3.7. Sample analysis

The developed analytical method was then applied to simultaneously determine the 12 constituents in 33 *F. suspensa* commercial samples obtained from different provinces. All 12 compounds were detected in herbal samples. Their identities were confirmed by comparing the UV spectra and retention times with those of the authentic compounds. Representative chromatograms are shown in Fig. 4. The content of each compound in 33 samples was, respectively, quantified.

The results showed that the total amounts of the 12 constituents determined in 33 herbal samples varied from 16.86 to 74.55 mg/g, with four-fold variation. As far as single constituent was concerned, at least three-fold variation was found. For example, the content of rutin was 0.79 mg/g in sample no. 28 while 2.29 mg/g in sample no. 30, with three-fold variation. The similar situation was noticed for phillyrin with eight-fold variation. While for *S*-suspensaside methyl ether, even 25-fold

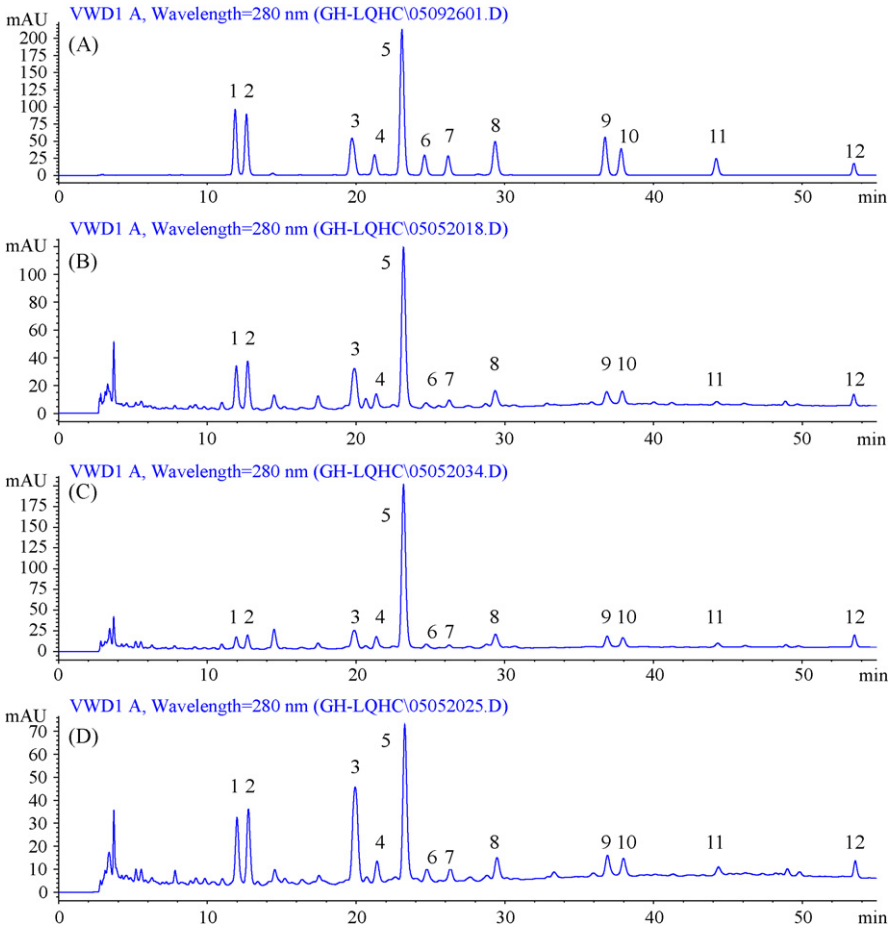

Fig. 4. HPLC chromatograms of: (A) standard mixture; (B) *F. suspensa* (Zhuhai, Guangdong, China); (C) *F. suspensa* (Haerbin, Heilongjiang, China); (D) *F. suspensa* (Shanxi Laoqiao, China). (1) *R*-suspensaside; (2) *S*-suspensaside; (3) *S*-suspensaside methyl ether; (4) (+)-pinoresinol-β-D-glucoside; (5) forsythiaside; (6) (+)-epipinoresinol-4′-*O*-glucoside; (7) suspensaside A; (8) rutin; (9) phillyrin; (10) (+)-pinoresinol; (11) (+)-epipinoresinol; (12) phillygenin.

variation was found. The variation might be the consequence of a number of aspects, such as plant source, harvesting time, processing and storage conditions, etc. The variation in contents of constituents could certainly lead to the variation of therapeutic effects; hence, each procedure involved should be standardized.

From the results, it was easy to note that forsythiaside was the most dominant compound in all herbal samples. Its content ranged from 5.15 to 55.78 mg/g, with the percentage of 27.5–75.9 to the total amounts. Since a number of pharmacological activities of forsythiaside were previously reported, it certainly played an important role in the quality of *F. suspensa*. However, only phillyrin was selected as a quantitative constituent for *F. suspensa* in Chinese Pharmacopoeia. It was suggested that both forsythiaside and phillyrin should be determined in *F. suspensa* at least so as to evaluate the herb properly.

The results of sample nos. 4 and 5 indicated that the contents of the bioactive compounds, such as forsythiaside, phillyrin and rutin, were higher in Qingqiao than those in Laoqiao, which is in agreement with previous studies [35]. Nowadays, Laoqiao is common to be prescribed in clinical practice, hence, it might be advantageous to replace Laoqiao with Qingqiao for better therapeutic effects.

4. Conclusion

A simple, rapid and accurate method was developed for the determination of bioactive constituents in *F. suspensa* by HPLC. This was the first report on the simultaneous quantification of 12 major constituents in *F. suspensa*. The results demonstrated that the developed method could be applied as a reliable and sensitive quality control procedure for *F. suspensa*.

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