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Comparative Analysis of the Major Constituents in the Traditional Tibetan Medicinal Plants *Saussurea laniceps* and *S. medusa* by LC-DAD-MS

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Abstract

A liquid chromatography (LC) coupled with diode array detector (DAD) and electrospray ionization mass spectrometry (ESI-MS) method was developed for the qualitative and quantitative comparison of the main constituents in *Saussurea laniceps* (SL) and *S. medusa* (SM), two species of plants used under the name "Xuelianhua" in traditional Tibetan medicine. A method validation including linearity, limit of detection, precision and recovery was performed. The results showed that a good linearity with R^2 > 0.99 was achieved, and the limit of detection of the quantified constituents was reported to be between 0.8 and 3.3 ng. The relative standard deviation (RSD) value was below 3.82% for repeatability, and recovery studies for the quantified compounds were found to be within the range 90.92–103.12%. The unique properties of the present method were evaluated by analyzing twelve related herbal samples including five SL samples and seven SM samples. Twenty-two compounds including phenolic acids, cumarins, lignanoids and flavonoids were identified by online ESI-MS and by comparison with literature data and standard compounds, and seven of them were quantified by LC-DAD simultaneously. The results demonstrated that the common constituents in the two herbs were protocatechuic acid, syringoside, chlorogenic acid, isoquercitroside, 1,5-dicaffeoylquinic acid, apigenin 7-*O*-β-D-glucoside, chrysoeriol 7-*O*-β-D-glucoside, acacetin 7-*O*-β-D-glucoside, apigenin and chrysoeriol. In the present study, it was found that the characteristic constituents were umbelliferone, scopoletin and their glucosides in SL, as well as arctiin and arctigenin in SM. It was feasible to choose these characteristic compounds for the quality evaluation as well as chemical authentication of the two related herbs. The results also support discrimination between the two species when using them in folk medicine.

Keywords
LC-DAD-MS
Traditional Tibetan medicine
Xuelianhua
Saussurea laniceps
Saussurea medusa
Composite

Introduction

Because of biodiversity, related medicinal plants from the same family or genus have been, and are being, used for similar therapeutic purposes in folk medicine. However, the qualitative and quantitative differences of these related plants regarding chemical composition are usually unknown thereby limiting pharmacological research and clinical application. Therefore, detailed analysis of the chemical composition should be performed to enable alternative or discriminating use of plant resources [1].

In our previous study, we reported the major constituents in *Saussurea involucrata*, one species used under the name "Xuelianhua" in traditional Uighur medicine [2]. In this follow-up study, we sought to compare the chemical composition in *Saussurea laniceps* (SL) and *S. medusa* (SM), two other species also used under the name "Xuelianhua" in traditional Tibetan medicine.

SL and SM are medicinal plants of the Composite family, listed in most herbal medicine dispensatory [3-7]. In Tibetan folk medicine, the dried aerial parts of the two plants have been used under the name "Xuelianhua" and prescribed for treatment of chronic diseases such as rheumatism, impotence and menoxenia [5-9]. To date, no study has been able to clarify whether the two herbs have similar chemical composition and/or similar potencies, although SL and SM have both been used as "Xuelianhua" in clinics. One attempt to assess the qualitative and quantitative difference in the two related herbs using conventional methods was not successful.

To solve this problem, some studies have attempted to analyse the constituents in these medicinal plants by using colorimetry [10], thin layer chromatography (TLC) [11, 12] and liquid chromatography (LC) [13]. However, the specificity and precision of colorimetry and TLC were unsatisfactory. The current LC method seeks to determine one or a few marker compounds for quality assessment. Additionally, it is widely accepted that multiple constituents are responsible for the therapeutic effects of herbal products [14, 15]; hence, the current protocol based on determination of a few marker compounds cannot accurately reflect the quality of herbal products [2]. Therefore, to find out the differences of the two related herbs, the necessity for a comparative analysis based on chemical identification of the main constituents is urgent.

The present study describes the development of an LC-DAD-MS method for comparison of the main chemical constituents in the two herbs. The results revealed that the main constituents in the two herbs were different, which supports discrimination between the two herbs when using them in folk medicine. In the present study, it was first found that the characteristic constituents of SL were umbelliferone, scopoletin and their glucosides in SL, while the constituents of SM also included arctiin and arctigenin. Therefore, it is feasible to choose these characteristic compounds for quality evaluation as well as chemical authentication of the two herbs.

Experimental

Plant Materials

The sources of the tested samples are listed in Table 1. Identity of the herbs was confirmed by Dr. Hubiao Chen (School of Chinese Medicine, Hong Kong Baptist University, Kowloon, Hong Kong), and voucher specimens were deposited in Hong Kong Baptist University.

Reagents and Chemicals

Formic acid of LC grade was purchased from Merck (Darmstadt, Germany). Acetonitrile of LC grade and methanol of analytical grade were purchased from Lab-scan (Bangkok, Thailand). Water was purified using a Milli-Q water system (Millipore; Bedford, MA, USA).

The standard compounds of syringoside, chlorogenic acid and scopoletin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Umbelliferone were purchased from Fluka (Buchs, Switzerland). Protocatechuic acid, quercetin 3-*O*-rhamnoside, 1,5-dicaffeoylquinic acid, apigenin 7-*O*-β-D-glucoside, arctiin, luteolin, acacetin 7-*O*-β-D-glucoside and apigenin were isolated from *Saussurea stella* Maxim. by Prof. Shaoqing Cai and Dr. Tianmin Wang, and their identities were confirmed by the comparison of their respective NMR and MS spectra with the published data. The details of separation and structural elucidation will be reported in another paper. The chemical structures of standards are shown in Fig. 1.

Preparation of Solutions

The preparation of stock and sample solutions was conducted according to the description of the previous report [2]. The stock solutions of seven standards (1000 mg L^{-1}) were prepared in 70% methanol and stored in -4 ^{o}C temperature. Syringoside, chlorogenic acid and arctiin working solutions of 1-100 mg L^{-1}, umbelliferone and scopoletin solutions of 5-125 mg L^{-1}, 1,5-dicaffeoylquinic acid solutions of 0.5-50 mg L^{-1} and apigenin of 0.2-20 mg L^{-1} were prepared by appropriate dilution of the stock solutions, respectively. Plant materials were cut into small pieces and mixed thoroughly. A representative portion of the sample pieces was ground into a powder with a Fargo RT-04 grinder (Century Equipment Co. Ltd., Kowloon, HK), and then the powder was passed through a 20 mesh (0.9 mm) sieve. Herbal sample powder (0.5 g) was extracted with 10 mL of 70% methanol by means of sonication at room temperature for 0.5 h. The operations were repeated two times, and the residue was washed with 4 mL of fresh extraction solvent. Total extracts were combined in a 25-mL volumetric flask, which was filled up to the calibration mark with extraction solvent. The extracts were then filtered through a syringe filter (0.2 μm, Alltech, Beerfield, IL, USA). An aliquot of 10 μL solution was injected for LC-DAD-MS analysis.

LC–DAD–MS Conditions

An Agilent 1100 series LC-DAD system (Hewlett Packard, Palo Alto, CA, USA) hyphenated to a Bruker MicrOTOFQ mass spectrograph by electrospray ionization (ESI) interface (Bruker Daltonics, Bremen, Germany) was used for chromatographic and mass spectrometric analysis. An Alltima C_{18} column (5 μm, 4.6 × 250 mm, Alltech Associates) with a compatible guard column (C_{18}, 5 μm, 4.6 × 7.5 mm) was used for chromatographic separation. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using a gradient program of 7% (B) in 0-5 min, 7-17% (B) in 5-24 min, 17-36% (B) in 24-45 min, and 36-48% in 45-60 min. The solvent flow rate was 1 mL min^{-1} and the column temperature was set to 30 ^{o}C. The effluent from DAD was drained to the MS system with a split ratio of 10:1. The conditions of MS analysis were reported in the previous paper [2].

Assay Validation and Sample Determination

Linearity of the assay for standards was evaluated by determining the working solutions with five points and by plotting the peak area against the concentrations with linear regression analysis. Repeatability was assessed by determining the same batch of herbal sample three times at different days. Recoveries of all of the analytes were determined using material samples, for which the respective chemical contents had been predetermined, spiked at 50, 100 and 200% of the quantified levels of constituents. Samples were then processed and quantified in accordance with the established procedures. Average recovery at three spiked levels was calculated for the evaluation of method accuracy [2]. All herb samples were analyzed using the present method.

Results and Discussion

Optimization of Sample Solutions

Regarding extraction of herb samples, sonication was chosen because its efficacy and straightforward handling were proven [1]. Methanol and other solvents were evaluated as solvents. The results demonstrated that extraction with methanol was more complete than with other solvents. Therefore, methanol was used as the solvent for sample extraction. The extraction procedure, including solvent consumption, extraction times, extraction periods, and solvent concentration was further optimized. The optimal conditions are presented in detail in the section "*Preparation of Solutions*".

Online ESI-MS Identification of the Major Constituents

Conditions of chromatographic separation and MS analysis were optimized as originally described in literature [2]. Briefly, a wavelength of 280 nm was chosen to represent the profile of the major constituents in the two herbs (Fig. 2), and 0.1% formic acid was used in the mobile phase to promote the formation of quasi-molecular ions $[M+H]^+$ in MS analysis. Based on the comparison of samples with standard compounds, twelve peaks were unambiguously identified as protocatechuic acid (1), syringoside (3), chlorogenic acid (5), umbelliferone (7), scopoletin (8), quercitroside (11), 1, 5-dicaffeoylquinic acid (13), apigenin 7-*O*-β-D-glucoside (14), arctiin (17), luteolin (18), acacetin 7-*O*-β-D-glucoside (19) and apigenin (20). Other ten peaks were tentatively

identified as umbelliferone 7-*O*-β-D-glucoside (2), scopoletin 7-*O*-β-D-glucoside (4), malonyl umbelliferone 7-*O*-β-D-glucoside (6), isoquercitroside (9), luteolin 7-*O*-α-L-rhamnosyl-(1→2)-β-D-glucoside (10), apigenin 7-*O*-α-L-rhamnosyl-(1→2)-β-D-glucoside (12), luteolin 7-*O*-β-D-glucoside (15), chrysoeriol 7-*O*-β-D-glucoside (16), chrysoeriol (21) and arctigenin (22) by comparing their *m*/*z* values and UV spectra with the literature data [16-20]. The chromatographic and spectrometric data of the identified compounds are listed in Table 2.

From the assay results, it was determined that the common constituents in the two herbs were protocatechuic acid, syringoside, chlorogenic acid, isoquercitroside, 1,5-dicaffeoylquinic acid, apigenin 7-*O*-β-D-glucoside, chrysoeriol 7-*O*-β-D-glucoside, acacetin 7-*O*-β-D-glucoside, apigenin and chrysoeriol. The results also demonstrated that SL contained cumarins, including umbelliferone, scopoletin and their glucosides, while SM contained lignanoids, including arctiin and arctigenin,. These differences can be employed for chemical authentication of the two herbs.

Validation of the Analysis Method

Calibration curves showed that there was a linear correlation between peak areas and the concentrations of the standards, and a good linearity with $R^2 > 0.99$ was achieved. Based on visual evaluation with a signal-to-noise ratio of about 3:1, the limit of detection (LOD) of the quantified constituents was reported to be between 0.8 and 3.3 ng. The method's precision was evaluated, and the relative standard deviation (RSD) values were found to be below 3.82%. The accuracy of the method was validated by the determination of recovery. The average recovery of syringoside, chlorogenic acid, umbelliferone, scopoletin, 1, 5-dicaffeoylquinic acid, arctiin and apigenin were 100.76% (RSD 4.29%), 100.08% (RSD 4.88%), 98.46% (RSD 2.36%), 98.38% (RSD 1.55%), 90.92% (RSD 1.08%), 95.84% (RSD 1.83%) and 103.12% (RSD 3.41%), respectively.

Sample Analysis

The present method was successfully applied to the quantification of seven components in herbal samples from different localities in China. The results are summarized in Table 1. Table 1 showed that the main constituents in the two herbs were different, which supports discrimination

between the two herbs in folk medicine usage. The data shows, for the first time, that the characteristic and main constituents of SL are umbelliferone, scopoletin and their glucosides, and that SM contains these compounds as well as arctiin and arctigenin,. Therefore, these characteristic compounds can be used as chemical markers for the quality evaluation as well as chemical authentication of the two herbs.

Conclusion

An LC-DAD-MS method was developed for comparative analysis of the main constituents in two herbs of the genus *Saussaurea*, namely *S. laniceps* and *S. medusa*. By comparing chemical composition, the present study supports discrimination between the two herbs when using them in folk medicine.

Acknowledgements

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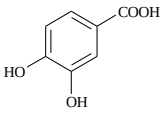
20. Zhou ZW, Yin S, Wang XN, Fan CQ, Li H, Yu JM (2007) Helv Chim Acta 90: 951–956

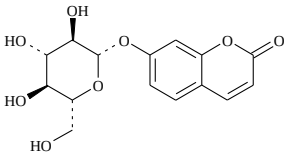
Legends for Figures

Fig. 1. Chemical structures of the compounds identified in the LC chromatograms

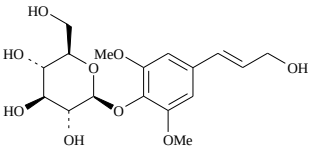
Fig. 2. Typical LC chromatograms of *Saussurea laniceps* (a), *S. medusa* (b) with detection at 280 nm. Key to peak identity as in Fig. 1. (For chromatographic protocol see Experimental section.)



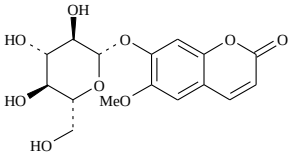
Protocatechuic acid (1)



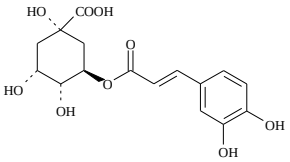
Umbelliferone 7-*O*-β-D-glucoside (2)



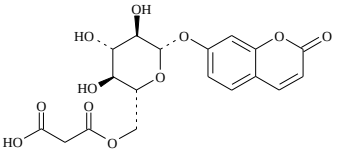
Syringoside (3)



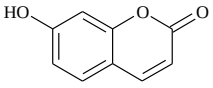
Scopoletin 7-*O*-β-D-glucoside (4)



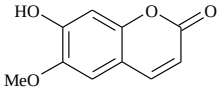
Chlorogenic acid (5)



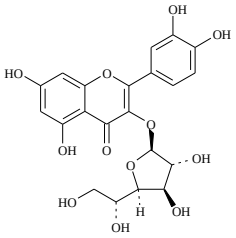
Malonyl umbelliferone 7-*O*-β-D-glucoside (6)



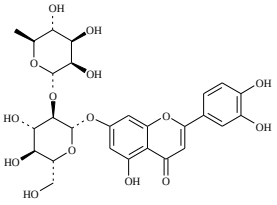
Umbelliferone (7)



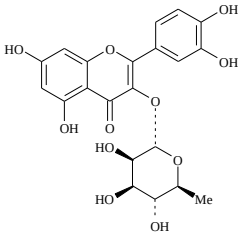
Scopoletin (8)



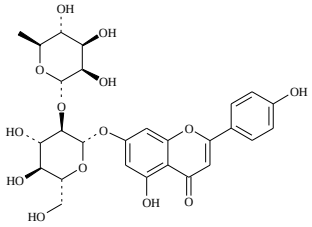
Isoquercitroside (9)



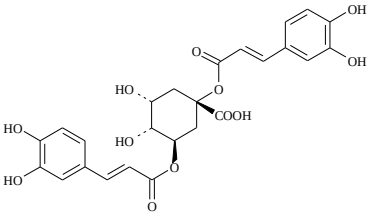
Luteolin 7-*O*-α-L-rhamnosyl-(1→2)-β-D-glucoside (10)



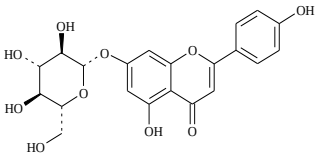
Quercitroside (11)



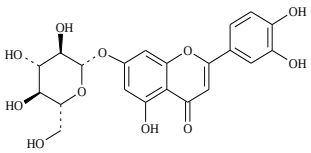
Apigenin 7-*O*-α-L-rhamnosyl-(1→2)-β-D-glucoside (12)



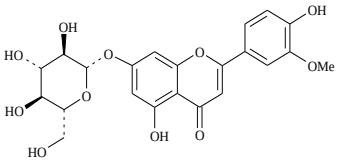
1, 5-Dicaffeoylquinic acid (13)



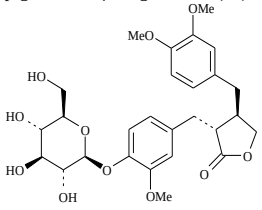
Apigenin 7-*O*-β-D-glucoside (14)



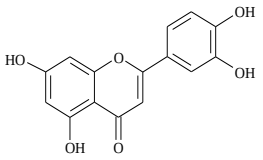
Luteolin 7-*O*-β-D-glucoside (15)



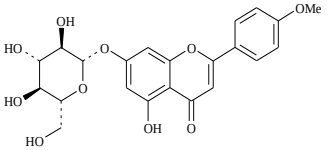
Chrysoeriol 7-*O*-β-D-glucoside (16)



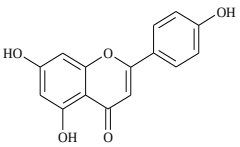
Arctiin (17)



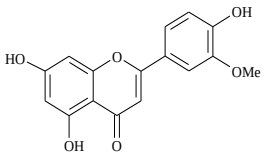
Luteolin (18)



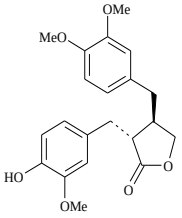
Acacetin 7-*O*-β-D-glucoside (19)



Apigenin (20)



Chrysoeriol (21)



Arctigenin (22)

Fig. 1

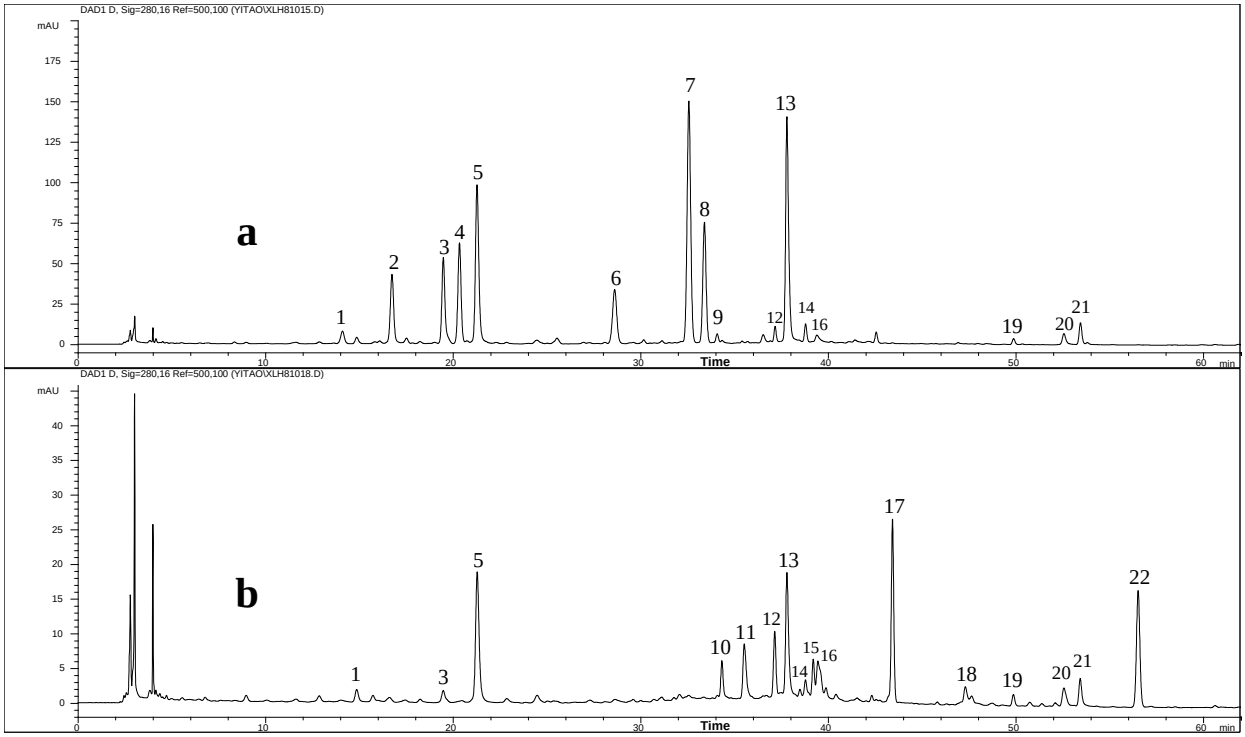

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Fig. 2

Legends for Tables

Table 1 Abundance of seven constituents in the herbal samples

Table 2 Chromatographic and spectrometric data of the identified compounds in the LC chromatograms

Table 1

Materials [a]	Source and year of harvest	Abundance of seven compounds (mg g^{-1}) [b]						
		Syringoside (3)	Chlorogenic acid (5)	Umbelliferone (7)	Scopoletin (8)	1,5-Dicaffeoyl quinic acid (13)	Arctiin (17)	Apigenin (20)
SL-01	Lhasa, Tibet (2007)	1.13	3.75	6.24	6.80	1.50	- [c]	0.32
SL-02	Lhasa, Tibet (2005)	2.53	6.97	6.59	7.78	3.00	-	0.23
SL-03	Lhasa, Tibet (2005)	1.84	4.74	4.79	5.34	1.95	-	0.29
SL-04	Lhasa, Tibet (2005)	1.51	2.97	7.03	9.76	1.90	-	0.44
SL-05	Lhasa, Tibet (2007)	0.61	2.30	3.16	4.32	0.88	-	0.26
SM-01	Aba, Sichuan (2007)	0.03	0.39	-	-	0.13	1.67	0.07
SM-02	Qinghai, China (2007)	0.03	0.32	-	-	0.10	0.55	0.12
SM-03	Shiqu, Sichuan (2003)	0.21	1.74	-	-	0.34	0.49	0.04
SM-04	Lhasa, Tibet (2005)	0.04	1.03	-	-	0.26	4.11	0.11
SM-05	Lhasa, Tibet (2005)	0.02	0.33	-	-	0.12	0.62	0.20
SM-06	Aba, Sichuan (2007)	0.35	2.73	-	-	0.68	0.95	0.18
SM-07	Lhasa, Tibet (2007)	0.02	0.31	-	-	0.14	1.28	0.13

[a] SL-01 to SL-05 are samples of *Saussurea laniceps*, and SM-01 to SM-07 are samples of *Saussurea medusa*, respectively.

[b] Values shown are mean of three independent experiments ($n = 3$).

[c] Undetected

Table 2

Peak	Retention time (min)	Identification	Other peak (*m/z*)	$[M+H]^+$ (*m/z*)	$[M+Na]^+$ (*m/z*)	λ max (nm)
1	14.2	Protocatechuic acid	-	155	177	206, 260
2	16.8	Umbelliferone 7-*O*-β-D-glucoside	163	325	347	198, 318
3	19.5	Syringoside	193, 211	373	395	220, 264
4	20.4	Scopoletin 7-*O*-β-D-glucoside	193	355	377	228, 340
5	21.3	Chlorogenic acid	163, 217	355	377	218, 326
6	28.6	Malonyl umbelliferone 7-*O*-β-D-glucoside	163, 359	411	433	208, 276
7	32.6	Umbelliferone	-	163	185	198, 324
8	33.4	Scopoletin	-	193	215	228, 344
9	34.1	Isoquercitroside	303	465	487	244, 322
10	34.3	Luteolin 7-*O*-α-L-rhamnosyl-(1→2)-β-D-glucoside	287, 449	595	617	254, 346
11	35.5	Quercetin 3-*O*-rhamnoside	303	449	471	254, 348
12	37.2	Apigenin 7-*O*-α-L-rhamnosyl-(1→2)-β-D-glucoside	271, 433	579	601	244, 328
13	37.8	1,5-Dicaffeoylquinic acid	499, 163	517	539	218, 328
14	38.8	Apigenin 7-*O*-β-D-glucoside	271	433	455	268, 334
15	39.2	Luteolin 7-*O*-β-D-glucoside	287	449	471	268, 336
16	39.4	Chrysoeriol 7-*O*-β-D-glucoside	301	463	485	246, 326
17	43.4	Arctiin	355, 373	535	557	228, 280
18	47.3	Luteolin	-	287	309	252, 348
19	49.9	Acacetin 7-*O*-β-D-glucoside	285	447	469	268, 330
20	52.6	Apigenin	187, 234	271	293	268, 336
21	53.5	Chrysoeriol	-	301	323	204, 304
22	56.5	Arctigenin	-	373	395	238, 280

Supporting information

Table 1 Linearity calibration curve factors and LOD of seven constituents

Compound	Concentration (mg L^{-1})	Slope (*A*)	Intercept (*B*)	R^2	LOD (ng)
Syringoside	1-100	16.210	- 1.2846	0.9999	1.4
Chlorogenic acid	1-100	12.865	- 2.6343	0.9998	2.8
Umbelliferone	5-125	16.410	+ 3.2	0.9997	1.1
Scopoletin	5-125	6.4137	- 1.1462	1	3.3
1,5-Dicaffeoylquinic acid	0.5-50	42.536	+ 1.2859	1	0.8
Arctiin	1-100	5.1922	+ 0.1389	1	3.2
Apigenin	0.2-20	23.140	- 3.4725	0.9998	1.9

Table 2 Method repeatability

Compound	First day	Third day	Fifth day
	Calculated content (mg/g) [a]	Calculated content (mg/g)	Calculated content (mg/g)
Syringoside	1.13 ± 1.71	1.15 ± 2.39	1.12 ± 2.45
Chlorogenic acid	3.75 ± 1.93	3.73 ± 3.41	3.39 ± 2.64
Umbelliferone	6.24 ± 2.05	6.26 ± 2.11	6.16 ± 1.48
Scopoletin	6.80 ± 2.10	6.83 ± 2.20	6.70 ± 1.60
1,5-Dicaffeoylquinic acid	1.50 ± 0.84	1.48 ± 3.40	1.31 ± 2.61
Arctiin	1.67 ± 2.18	1.68 ± 2.13	1.75 ± 1.61
Apigenin	0.32 ± 1.58	0.34 ± 3.82	0.38 ± 1.62

[a] The value is mean ± RSD (n=3).

Table 3 Recovery study of seven constituents

Compound	Added amount (mg) and recovery (%) at three spike levels						Mean recovery (%)
	50%		100%		200%		
Syringoside	0.28	103.48 ± 0.75 [a]	0.56	103.02 ± 1.29	1.12	95.78 ± 1.61	100.76 ± 4.29
Chlorogenic acid	0.94	94.54 ± 1.70	1.88	103.75 ± 1.47	3.75	101.94 ± 1.14	100.08 ± 4.88
Umbelliferone	1.5	96.04 ± 2.19	3.0	98.68 ± 1.45	6.0	100.67 ± 1.09	98.46 ± 2.36
Scopoletin	3.0	99.55 ± 1.29	6.0	98.94 ± 0.87	12.0	96.66 ± 0.81	98.38 ± 1.55
1,5-Dicaffeoylquinic acid	0.38	90.64 ± 1.02	0.75	90.10 ± 2.43	1.5	92.01 ± 1.14	90.92 ± 1.08
Arctiin	0.425	96.75 ± 1.15	0.85	93.82 ± 1.46	1.7	96.94 ± 1.69	95.84 ± 1.83
Apigenin	0.08	106.05 ± 1.30	0.16	99.22 ± 1.11	0.32	104.09 ± 1.41	103.12 ± 3.41

[a] The value is mean ± RSD (n=3).