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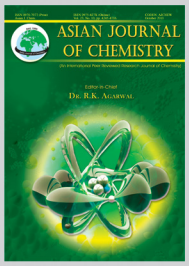

Simultaneous Determination of Ten Active Compounds in the Whole Plant of *Sarcandra glabra* and its Related Traditional Chinese Medicinal Preparations by Ultra High Performance Liquid Chromatography

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A simple, senstive and specific ultra high performance liquid chromatography (UPLC) was established for the simultaneous determination of ten active compounds in *Sarcandra glabra* and its preparations *e.g.*, Qingrexiaoyanning capsule, Zhongjiefeng tablet, Xiekang capsule, Jinsulan liniment and Zhongjiefeng injection. The analysis was performed on acquity UPLC system with acquity UPLC BEH C_{18} column (50 mm × 2.1 mm I.D., 1.7 μm) and gradient elution of acetonitrile and water containing 0.1 % formic acid, and all ten major compounds achieved complete separation. Results indicate that the developed UPLC assay can be successfully utilized as a quality method for simultaneous determination of ten representative compounds in *Sarcandra glabra* and its related preparations.

Key Words: *Sarcandra glabra*, Phenolic compounds, UPLC, Determination.

INTRODUCTION

Sarcandra glabra (Thunb) Nakai (Family: Chloranthacea), one of the most frequently used traditional Chinese medicines (TCMs), is distributed mainly in the south of Asia. The traditional Chinese medicinal preparations just made from *Sarcandra glabra*, such as Qingrexiaoyanning capsule, Zhongjiefeng tablet, Jinsulan liniment, Xiekang perorole, Xiekang capusle and Zhongjiefeng injection, were mainly used to treat influenza, pharyngolaryngitis, thrombocytopenia, pneumonia, cellulites, appendicitis, shigellosis, leukoderma vitiligo, abscess, bone fracture, cancer, *etc.*[1,2]. According to the literature[3], the active constituents of *Sarcandra glabra* can be divided into two groups: phenolic compounds, such as caffeoylquinic acids, flavonoids, coumarins, which are mainly water-soluble and terpenoids, which are mainly lipophilic. Phenolic compounds should be main active constituents, due to its wide range of biological activities, including antioxidant, antiinflamatory, antibacterial, antidepress and antitumor properties[4-7].

Several chromatographic methods, including HPLC-UV[8-11], capillary electrophoresis with electrochemical detection[12], capillary electrophoresis[13], have been published for the analysis of *Sarcandra glabra* and its medicinal preparations. However, these methods proposed in the literature are only concerned with analysis of one or two constituents due to complexity of phenolic compounds in *Sarcandra glabra* and its preparations. Up to data, there have been no reports on the simultaneous determination of multiple water-soluble constituents in *Sarcandra glabra* and its preparations. It is well accepted that the bioactivities of traditional Chinese medicine are not the effect of a single compound or a class of compounds, but the synergistic effects of all active components in the herb. Therefore, it is necessary to develop a method for the rapid identification and quantification of these major compounds. So the aim of this work was the development of a ultra high performance liquid chromatography (UPLC)-UV method for completely separating the major phenolic compounds and simultaneous quantification of these constituents in *Sarcandra glabra* and its preparations. In this study, ten major bioactive phenolic constituents (Fig. 1), were selected for analysis, including 10 batches of samples from different province of China and 5 related preparations only containing *Sarcandra glabra*. These compounds not only have the activities described above, but also are the characteristic constituents of *Sarcandra glabra*. Therefore, the contents of the ten major compounds could be used to evaluate the quality of *Sarcandra glabra* and its related preparations.

EXPERIMENTAL

Isofraxidin (**6**) was purchased from the National Institute for Control of Biological and Pharmaceutical Products (China).

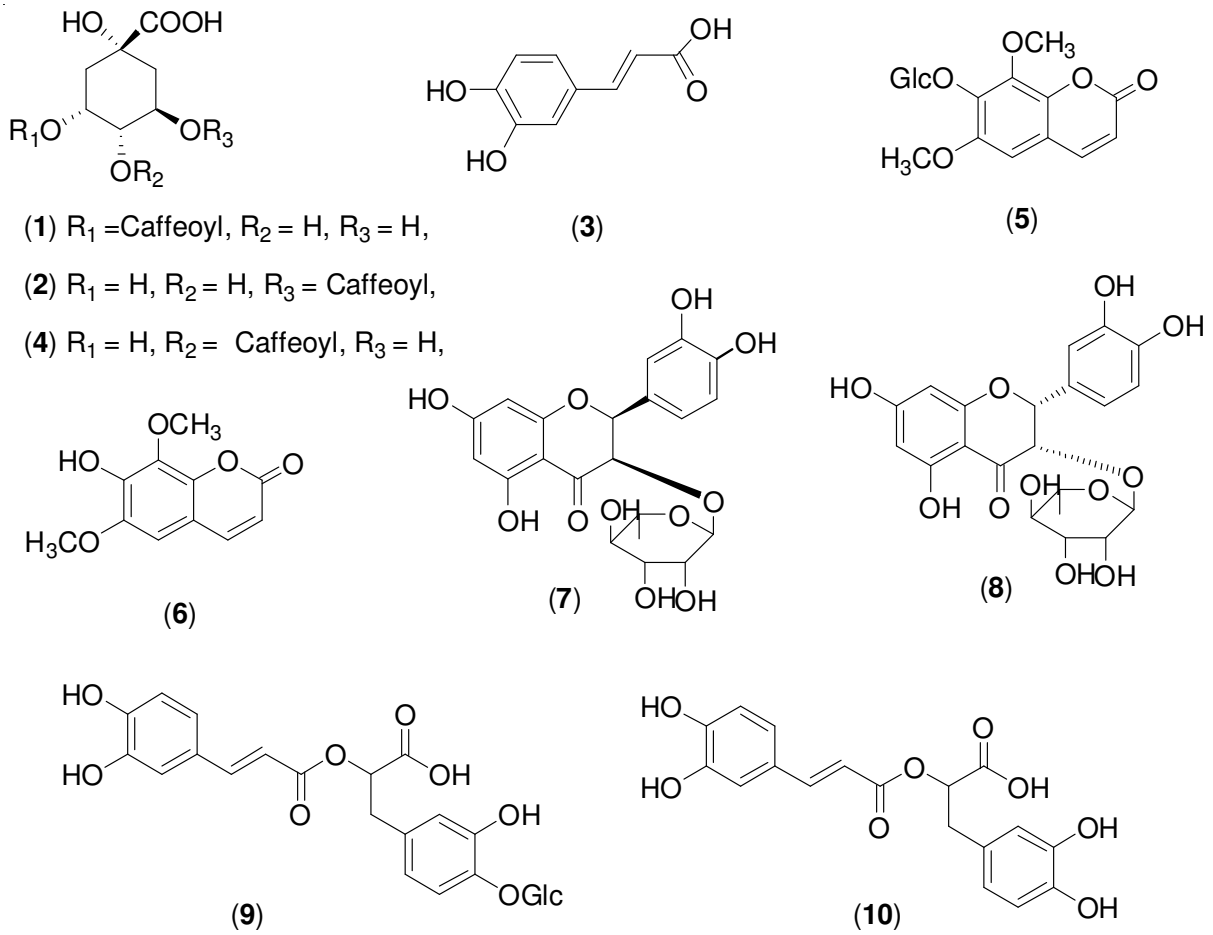

Fig. 1. Chemical structures of the ten active phenolic compounds from *Sarcandra glabra*

3-O-Caffeoyquinic acid or neochlorogenic acid (1), 5-O-caffeoyquinic acid or chlorogenic acid (2), 4-O-caffeoyquinic acid or cryptochlorogenic (**3**) ,caffeic acid (**4**) , isofraxidin 7-O-β-D-glucose (**5**), neoastilbin (**7**), astilbin (**8**), rosmarinic acid 4-O-β-D-glupyrannoside (**9**) and rosmarinic acid (**10**) were isolated from the whole plant of *Sarcandra glabra* by the author. Their identities were confirmed by ^{1}H NMR, ^{13}C NMR and MS spectral analysis and their purity was over 98 % by HPLC analysis.

Ten batches of *Sarcandra glabra* were collected from different provinces of China, Sichuan, Zhejiang, Anhui, Jiangxi and Guangdong. All the samples were purchased from various drug stores around China. The whole plant of *Sarcandra glabra* to separate the standard compounds were obtained from Sichuan province. Zhongjie feng injection was procured from Boluo xianfeng Pharmaceutical Corporation, China. Zhongjiefeng tablet was obtained from Shanghai Xinyi Jiahua Pharmaceutical Corporation, China. Qingrexiao yanning capsule was procured from Guangzhou Jingxiutang Pharmaceutical Corporation, China. Xiekang capsule was obtained from Jiangxi Tianshikang Pharmaceutical Corporation, China. Jinsulan liniment was available from Guangdong Hospital of Traditional Chinese Medicine. The voucher species were deposited in the Center for Laboratory, Second Affiliated Hospital, Guangzhou University of Traditional Chinese Medicine.

Ultrapure water from Milli-Q system (Millipore, Bedford, MA, USA) was used in all experiments. HPLC grade acetonitrile (Fisher, USA) were used for the UPLC analysis. All the other reagents are of analytical grade.

Apparatus and chromatographic conditions: UPLC analysis were performed using a Waters Acquity Ultra performance LC (UPLC) system (Waters, MA, USA), including binary solvent manager, sampler manager, column compartment and TUV detector, connected with Waters Empower software. The separation was carried out on an Acquity UPLC^{BEH} C_{18} column (50 mm × 2.1 mm i.d, 1.7 μm, Waters Corp., Milford, MA, USA). The column temperature was maintained at 35 ºC. The standards and samples were separated using a gradient mobile phase consisting of 0.1 % formic acid (A) and acetonitrile (B). The gradient condition was as follows: 0-4.5 min, 4-5.4 % B; 4.5-9.0 min, 5.4 % B; 9.0-10.0 min, 5.4-6.3 % B; 10.0-46.0 min, 6.3-14.5 % B and finally reconditioning the column with 4 % B isocratic for 10 min after washing column with 90 % B for 5 min. The flow rate was set at 0.2 mL min^{-1} and the injection volume was set at 5 μL. The detection wavelength was set at 330 and 289 nm.

Preparation of standard solutions: Stock solutions of individual standards were prepared in appropriate concentration in 30 % acetonitrile (v/v). The solutions were properly mixed and diluted in 30 % acetonitrile (v/v) in various concentrations for the establishment of calibration curves. Each calibration curve was analyzed three times with six different concentrations. The standard stock solutions were all prepared in dark brown calibrated flasks and stored at 4 ºC.

Sample solutions preparation: The dried whole plant of *Sarcandra glabra* samples were powdered to a homogeneous size using a mill and filtered (60 mesh). Each solid powder (0.2 g) was accurately weighed into a 50 mL flask and extracted in an ultrasonic bath with 20 mL 30 % acetonitrile for 0.5 h at room temperature. Then, the extract solutions were prepared by the method of weight relief. As to Jinsulan liniment and Zhongjiefeng injection, 1 mL of liquid sample was diluted to 1/3rd and 1/10th with 10 % acetonitrile. All the solution was filtered through a 0.22 μm nylon membrane filter prior to injection.

Carlibration curves and the limit of detection: The external standard method was used to get the regression equations. Stock solutions of 10 mixed standards in 30 % aqueous acetonitrile were diluted to appropriate concentrations for the establishment of calibration curves. The calibration curves were constructed by plotting the peak areas versus the concentrations of each analyte. The limits of detection (LOD) and quantification (LOQ) for each analyte were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Precision and accuracy: Intra-day and inter-day precision were determined by assaying standard solutions during a single day and on three consecutive days. Recovery was used to evaluate the accuracy of the method. A recovery test was used to evaluate the accuracy of this method. Accurate amounts of 10 standards were added to 0.1 g of *Sarcandra glabra* from sample **1** and then extracted and analyzed as described above. For stability test, the sample solution was analyzed every 24 h in 3 days at the room temperature.

RESULTS AND DISCUSSION

Sample extraction conditions: In order to obtain quantitative extraction, variables involved in the procedure such as solvent, extraction method and extraction time were optimized. In this study, 70 % methanol, 50 % methanol, 30 % methanol and 50 % acetonitrile, 30 % acetonitrile, were employed as extraction solvents and the best solvent was found to be 30 % acetonitrile. The peak areas of the ten phenolic compounds reached the highest values. Furthermore, the ten compounds were more stable in acetonitrile-water solvents than in methanol-water solvents, therefore, 30 % acetonitrile was selected as the extraction solvent. After comparing several extraction methods such as ultrasonic, soaking and regurgitation, ultrasonic was found to be the most suitable extraction method. Then the optimal extraction time was investigated. Solid sample (0.200 g) was extracted with 20 mL of 30 % acetonitrile for 15, 30, 45 and 60 min, respectively. The peak areas of all the tested compounds reached the highest valves, hence 0.5 h was selected as the optimal extraction time. From above experiment, the most suitable extraction method of the phenolic compounds was ultrasonic extraction by 20 mL of 30 % acetonitrile for 0.5 h.

Optimization of UPLC conditions: In these studies, two different C_{18} columns were tested. One was an Acquity UPLC^{BEH} C_{18} column (50 mm × 2.1mm i.d, 1.7 μm) and the other a Thermo Hypersil Gold C_{18} column (50 mm × 2.1 mm i.d, 1.9 μm). The later proved insufficient to obtain a good chromatographic separation. Therefore, Acquity UPLC^{BEH} C_{18} column was chosen. In order to improve the resolution obtained, an alternative method was tested using acetonitrile instead of methanol as an organic modifier of the mobile phase. The results show that elution with acetonitrile is beneficial for the separation. In the chromatogram obtained with acetonitrile, there is better separation of these compounds, hence acetonitrile was selected for the separation. By optimizing the compositions of mobile phase, 0.1 % (v/v) formic acid and acetonitrile were finally preferred as mobile phase and all 10 compounds could be eluted with baseline separation in 46 min. For the optimization of the elution gradient, it was necessary to make minor changes to the gradient, as well as to the temperature of the column, to improve the separation and get a higher resolution. According to the maximal absorption peaks of the four kinds of phenolic constituents in their UV spectra, the choice of detection at 330 nm for compounds **1-4**, **6**, **9**, **10** and 289 nm for compounds **5**, **7**, **8** provides an optimum S/N for simultaneous analysis of the ten constituents.

Validation of the developed method: The linearity, regression and precision of ten major phenolic constituents were performed using the developed UPLC method. The high correlation ($r^2 > 0.9990$) values indicated good correlations between investigated compounds concentrations and their peak areas within the test ranges and the LOQ and LOD were lower to 0.05- 0.37 and 0.02-0.10 μ/mL, respectively (Table-1). The overall RSD of the intra-day and inter-day were less than 1.0 and 3.7 % for all analytes and the developed method had the accuracy with the overall recovery of 96.24-101.60 % for the

TABLE-1
CALIBRATION CURVES, LOD AND LOQ FOR TEN PHENOLIC COMPOUNDS

Compounds	Retention time (min)	Calibration curves[a]	r^2	Liner ranges (μg/mL)	LOQ[b] (μg/mL)	LOD[c] (μg/mL)
1	3.5	y = 35000x - 648	0.9999	0.90-7.20	0.05	0.02
2	6.5	y = 25800x - 1870	0.9999	1.92-38.40	0.09	0.03
3	7.0	y = 51600x - 1950	0.9999	0.44-8.80	0.06	0.02
4	8.3	y = 29500x - 1430	1.0000	0.66-13.20	0.09	0.03
5	10.5	y = 13600x - 3970	0.9999	1.20-24.00	0.20	0.08
6	20.1	y = 31600x - 5910	0.9999	1.40-28.00	0.20	0.07
7	26.2	y =22400x - 12100	0.9999	3.84-76.80	0.25	0.08
8	28.6	y = 31600x - 5910	0.9999	3.10-62.00	0.22	0.08
9	37.9	y = 23400x - 4230	0.9999	2.20-44.40	0.37	0.15
10	43.1	y =30500x -13000	0.9990	2.20-44.00	0.25	0.10

a: y is the peak area in the UV chromatograms monitored at 330 nm for compounds **1-4**, **6** and **9-10**, 289 nm for compounds **5** and **7-8**. b: LOD refers to the limits of detection. c: LOQ refers to the limits of quantification.

analytes (Table-2). For stability test, the analytes were found to be stable within 72 h. These results indicated that this UPLC method was rapid, precise, accurate and sensitive for quanti-ta-tive determination of ten major phenolic compounds.

Sample analysis: The five categories of samples were prepared as described earlier. A volume of 5 µL of the filtered solution of each sample was injected into the instrument. Peaks in the chromatograms were identified by comparing the retention time and on-line UV spectra with those of the standards. Each sample was determined in triplicate. Retention time for compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9** and **10** were 3.5, 6.5, 7.0, 8.3, 10.5, 20.1, 26.2, 28.6, 37.9 and 43.1 min, respectively (Fig. 2). The content of each analyte was calculated from the corresponding calibration curve. The contents of each analyte in the 18 samples including 10 plant material samples from five locations and five different kinds of preparation were shown in Table-3. From which one can see that the ten analytes were found in all of the samples analyzed and the contents of the ten markers varied greatly among the different province and preparations. In addition, compounds **2**, **6-10** were relatively high, while compounds **1**, **3-5** were relatively low in the samples. In this study, the total content of 10 phenolic compounds in *Sarcandra glabra* plant material ranged from1.65-12.00 mg/g of dry weight. The highest total content (12.00 mg/g) of phenolic acids was found in the commercial sample from Sichuan Province. The total content of the ten phenolic compounds from Sichuan province were generally higher than other provinces. The total contents of the sample from Jiangxi province were inferior than those from Sichuan, but the content of rosmarinic acid 4'-O-B-D-glupyrannoside (3.7 mg/g) and rosmarinic acid (3.6 mg/g) from Jiangxi province were the most highest. Samples from Anhui province showed the lowest total content. The variation of contents may be derived from the different growing climate, the difference of production procedure, storage, transportation, *etc*. The solid preparation, Qingrexiaoyanning capsule and Zhongjiefeng tablet displayed high content of the total active phenolic compounds, while the liquid preparation, Jinsulan liniment and Zhongjiefeng injection, show relatively low content of these compounds.

Based on the analysis results above, it is concluded that the essential quality control markers of *Sarcandra glabra* and its preparations were considered to be the many phenolic compounds including caffeoylquinolic acids (compounds **1**, **2** and **4**), coumarins (compounds **5** and **6**), flavonoids (compounds **7** and **8**) and caffeoyl acids (compounds **3**, **9** and **10**) that not only represent most of the components but also can be determined by a developed UPLC assay method. Furthermore, the

TABLE-2
PRECISION, RECOVERY AND STABILITY OF THE TEN ANALYTES

Analyte	Precision						Stability (n = 3)
	Intra-day (n = 5)		Inter-day (n = 3)		Recovery (n = 6)		
	Mean (µg/mL)	RSD (%)	Mean (µg/mL)	RSD (%)	Mean (%)	RSD (%)	RSD (%)
1	1.47 ± 0.01	0.5	1.47 ± 0.01	0.5	99.4 ± 4.4	4.4	0.8
2	3.96 ± 0.03	0.7	3.94 ± 0.02	0.5	98.9 ± 2.4	2.4	1.4
3	0.93 ± 0.01	0.6	0.93 ± 0.01	0.5	97.6 ± 1.8	1.8	4.9
4	1.37 ± 0.01	0.8	1.35 ± 0.02	1.2	96.2 ± 1.2	1.2	0.8
5	2.61 ± 0.03	1.0	2.58 ± 0.01	0.1	101.6 ± 3.4	3.1	1.9
6	2.83 ± 0.03	1.0	2.79 ± 0.05	1.6	100.0 ± 1.9	1.9	0.7
7	8.16 ± 0.04	0.5	8.09 ± 0.07	0.9	98.5 ± 2.8	2.4	3.7
8	6.65 ± 0.04	0.6	6.57 ± 0.10	1.5	97.6 ± 1.2	1.6	0.3
9	4.66 ± 0.04	0.9	4.55 ± 0.17	3.7	98.2 ± 2.0	2.0	1.0
10	4.96 ± 0.04	0.7	4.91 ± 0.06	1.3	98.5 ± 2.8	2.8	1.1

TABLE-3
DETERMINATION OF PHENOLIC COMPOUNDS IN HERBAL EXTRACTS OF *Sarcandra glabra* AND ITS PREPARATIONS

Analyte	Mean contents of compounds **1-10**[a] (n = 3)										Preparations								
	Samples from the collected Province																		
	Sichuan		Jiangxi		Zhejiang		Anhui		Guangdong										
	1[d]	2[d]	3[d]	4[d]	5[d]	6[d]	7[d]	8[d]	9[d]	10[d]	11[c]	12[f]	13[f]	14[f]	15[f]	16[g]	17[g]	18[h]	19[i]
1	0.22	0.13	0.27	0.30	0.07	0.13	tr[c]	tr[c]	tr[c]	0.12	0.88	0.97	0.47	0.29	0.53	0.02	0.02	tr[c]	0.45
2	1.43	1.88	1.72	2.11	0.60	0.84	0.13	0.30	0.21	0.69	1.45	2.38	1.03	0.67	1.24	0.02	0.02	0.02	1.00
3	0.11	0.08	0.08	0.08	0.06	0.19	0.09	0.13	0.06	0.05	0.14	nd[b]	nd-	nd[b]	nd[b]	0.02	0.01	0.003	nd[b]
4	0.47	0.54	0.48	0.66	0.14	0.32	0.04	0.07	0.06	0.21	1.22	1.75	0.72	0.43	0.86	0.02	0.02	0.005	0.75
5	0.32	0.44	0.42	0.50	0.26	0.30	0.17	0.13	0.10	0.38	1.37	1.13	0.60	0.90	0.68	0.02	0.02	0.02	0.49
6	0.43	0.48	0.44	0.32	0.28	0.38	0.44	0.52	0.27	0.22	2.20	3.59	2.19	2.84	2.90	0.09	0.09	0.04	3.96
7	1.40	1.34	1.23	1.29	0.22	0.70	nd[a]	nd[a]	nd[a]	0.58	tr[c]	tr[c]	nd[b]	nd[b]	nd[b]	0.01	nd[b]	nd[b]	tr[c]
8	1.87	2.97	2.67	2.39	0.48	1.31	0.40	0.24	0.21	0.61	0.98	1.0	3.21	0.59	1.06	tr[c]	nd[b]	0.01	tr[c]
9	1.97	2.0	1.86	1.88	3.7	1.32	0.14	0.18	0.49	0.81	3.03	4.22	0.93	nd	1.32	0.09	0.09	0.04	2.60
10	1.25	1.77	1.67	2.47	3.6	0.63	0.22	0.45	0.29	0.38	4.20	4.82	3.94	4.85	2.94	0.09	0.08	0.009	2.15
Total	9.47	11.63	10.84	12.0	9.41	6.12	1.65	2.02	1.69	4.05	15.47	19.86	12.69	10.57	11.55	0.38	0.35	0.15	11.40

a: Unit of solid sample is mg/g, unit of liquid sample is mg/mL; b: nd: not detected ; c: tr: trace; d: 1-10 from commercial samples of *Sarcandra glabra* sample; e: 11 from Zhongjiefeng tablet (batch number 11: 090201) ; f: 12-15 from Qingrexiaoyanning capsule (batch number 12: N12015; 13: N08012; 14: N10011; 15: H01001); g: 16-17 from Zhongjiefeng Injection (batch number 16: 090706; 17: 090705); h: 18 from Jinsulan liniment (Batch number 18: 091204); i: 19 from Xiekang capusle (batch number 19: 09090801).

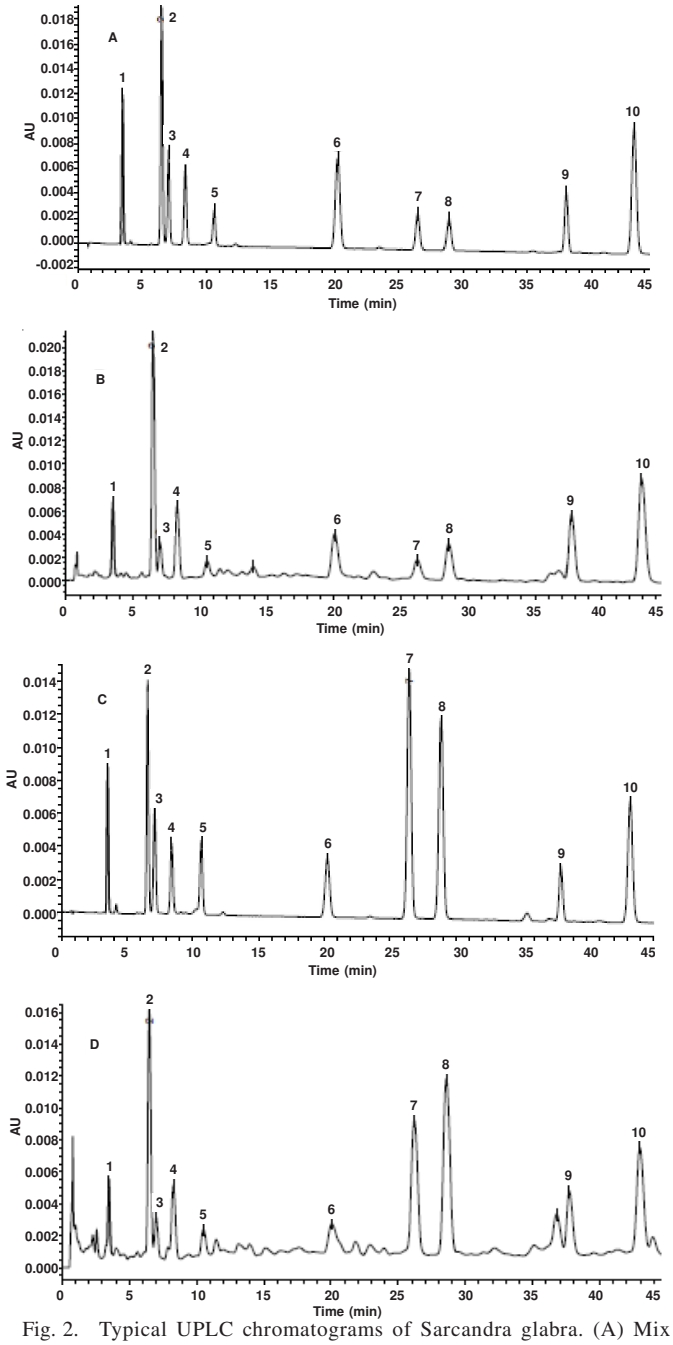

Fig. 2. Typical UPLC chromatograms of Sarcandra glabra. (A) Mix standards detected at 330 nm; (B) *Sarcandra glabra* sample from Sichuan Province detected at 330 nm; (B) *Sarcandra glabra* sample from Sichuan Province detected at 330 nm; (C) Mix standards detected at 289 nm; (D) *Sarcandra glabra* sample from Sichuan Province detected at 289 nm. neochlorogenic acid (**1**), chlorogenic acid (**2**), cryptochlorogenic (**3**), caffeic acid (**4**) , isofraxidin 7-O-β-D-glucose (**5**), isofraxidin (**6**), neoastilbin (**7**), astilbin (**8**), rosmarinic acid 4-O-β-D-glupyrannoside (**9**) and rosmarinic acid (**10**)

obtained chemical profiles can be used as fingerprints for identification of the origin of *Sarcandra glabra* samples and it should be reasonable to have relative fixed regions for the medicinal plant. The established method has been successfully applied to the determination of the ten phenolic compounds in *Sarcandra glabra* and its four related preparations.

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