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Full Length Research Paper

Simultaneous determination of five active components in traditional Chinese medicine *Apocynum venetum* L. by RP-HPLC–DAD

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A simple, rapid and specific liquid chromatographic–diode array detector (DAD) method was developed and fully validated for simultaneous quantification of five major active ingredients (markers) from *Apocynum venetum* L. Samples were ultrasonically extracted with 70% ethanol. Then the foreign materials were removed in solid phase extraction column. The chromatographic separation was performed on a Zorbax SB-C_{18} column (250mm×4.6mm i.d., 5μm) with a gradient of acetonitrile and water containing 0.1% glacial acetic acid, at a flow rate of 1.0 ml/min, detected at 360 nm. Five regression equations showed good linear relationships (r^2 > 0.999) between the peak areas of each marker and concentrations. The assay was reproducible with overall intra- and inter-day variation of less than 6.0%. The recoveries, measured at three concentration levels, varied from 97.8% to 102.5%. This assay was successfully applied to the determination of the 5 bioactive compounds in 10 samples. The results indicated that the developed assay method was rapid, accurate, reliable and could be readily utilized as a quality control method for *A. Venetum* L.

Key words: HPLC, *Apocynum venetum* L., chlorogenic acid, hyperoside, isoquercitrin, quercitroside, quercetin.

INTRODUCTION

Apocynum venetum L. is one of the well-known traditional Chinese medicines (TCM), known as “Luobuma” in Chinese medicine. It is a wild plant that grows widely from the middle to northwestern regions of China, where its leaves are commonly used to make tea. They have also been used to treat cardiac disease, hypercholesterolemia (Zuo, 1999; Kim et al., 1998), nephritis, neurasthenia and other diseases (The Pharmacopoeia Committee, 2005; Butterweck et al., 2001). Pharmacological studies have showed that *A. venetum* L. have many strong activities, such as anti-hypertensive (Tagawa et al., 2004; Kim et al., 2000), anti-hyperlipemia, hepatoprotective (Xiong et al., 2000), antioxidant (Inal and kanbak, 2001; Yokozawa et al., 2002), anti-aging (Yu et al., 2006), sedative and anti-convulsion effects.

Clinically, the pharmacological effects of the leaves are usually the summation of the effects of the many flavones constituents, a phenomenon which is very common in TCM. First, the amounts of the flavones usually vary substantially among materials from different sources. Secondly, different flavones seem to have different activities and pharmacological effects, with some low-content compounds having very strong activities. Thirdly, even if compounds are not active themselves, they may affect other bioactive constituents, because of interactions between them. Considering these factors, analysis of one or two constituents alone is not sufficient

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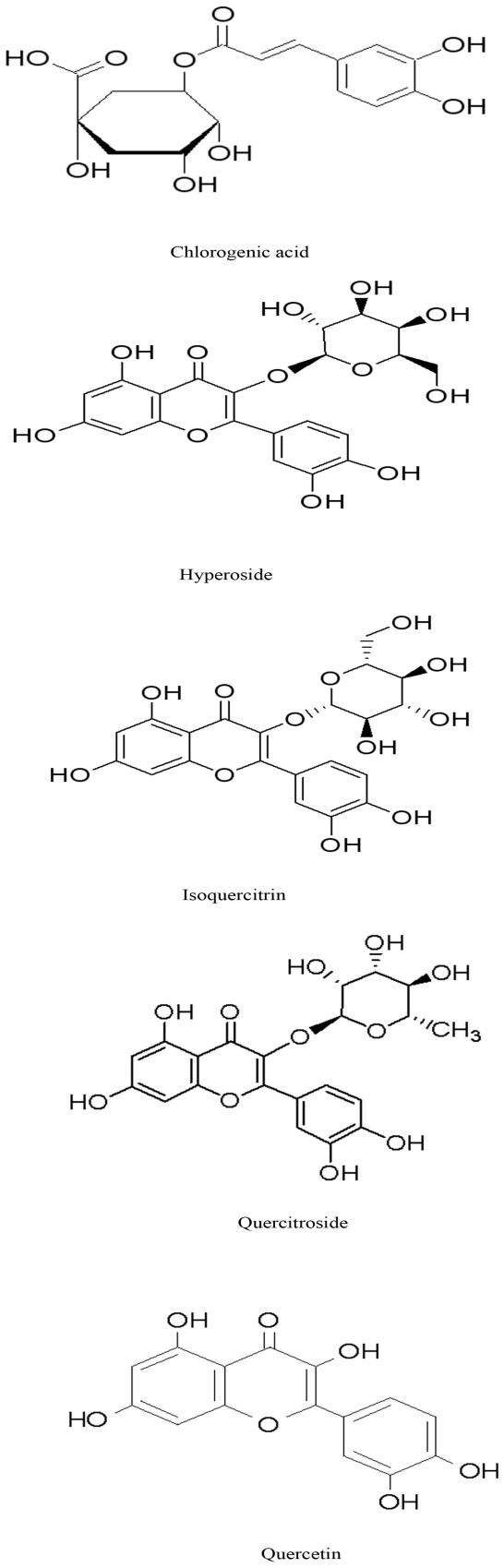

Figure 1. Structures of the investigated compounds.

for the study of *A. venetum* L. or for its quality control. It is very important to investigate the amounts of different constituents to guide the usage of the plant.

Many constituents have been isolated from *A. venetum L.* (Huang et al., 1998; Fan et al., 1999; Murakami et al., 2001), however, the quantity of most constituents is little, so separation and detection are extremely difficult. Although several HPLC (Liu et al., 2004; Ma et al., 2004), CE and spectrophotometric analysis methods are available for analysis of the constituents from *A. venetum* L. (Cao et al., 2001), they have been developed for analysis of one or two constituents only. This is the first report of simultaneous quantification of five most important constituents in *A. venetum* L. Furthermore, we investigated the sample preparation methods, peak confirmation, gradient elution program regarding the complexity of *A. venetum* L.

The aim of this study was to develop a simple, rapid and accurate HPLC method for the simultaneous determination of the five active compounds to evaluate the quality of this medicinal herb.

MATERIALS AND METHODS

Chemicals and materials

HPLC grade acetonitrile was purchased from Tedia Co. Ltd. (United States of America). HPLC grade glacial acetic acid was purchased from Tianjin Concord Tech Reagent Co. Ltd. (Tianjin, China). All the other reagents were of analytical grade and used without further purification. Distilled water, prepared with demineralized water, was used throughout the study. Commercial herb samples of *A. venetum* L. were purchased from drug stores or markets in different provinces of China. Hyperoside, quercitroside, quercetin and chlorogenic acid (Figure 1) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Isoquercitrin was purchased from Sigma (St. Louis, MO, USA). Their purities were all above 98%.

Apparatus and chromatographic conditions

A Shimadzu LC-2010 liquid chromatography system, equipped with a quaternary solvent delivery system, an autosampler and a DAD detector, was used. The LC separation was carried out on a Zorbax SB-C_{18} column (250mm×4.6mm i.d., 5μm) from Agilent Analytical Instruments Co., Ltd. The mobile phase consisted of: (A) acetonitrile and (B) water containing 0.1% glacial acetic acid (v/v). A gradient elution program of 16% A at 0 to 15 min, 16 to 20% A at 15 to 24 min, and 20 to 42% A at 24 to 42 min was used to run the separation. Re-equilibration duration was 18 min between individual runs. Detection wavelength was set at 360 nm. The flow rate was 1.0 $mL.min^{-1}$ and aliquots of 10μl were injected. The column temperature was maintained at 40°C.

Preparation of standard solutions

The reference standards of the target compounds, that is;

(1) chlorogenic acid, (2) hyperoside, (3) isoquercitrin, (4) quercitroside and (5) quercetin were accurately weighted and dissolved in methanol then diluted to appropriate concentration ranges for the establishment of calibration curves. These solutions were stored at 4°C.

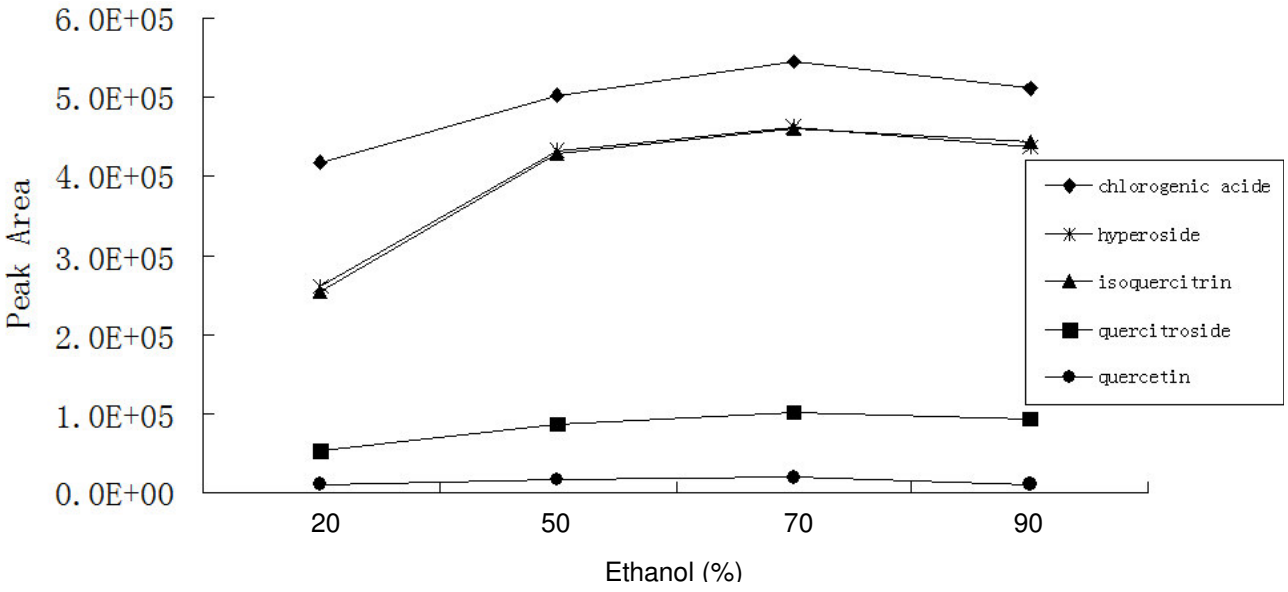

Figure 2. Extraction efficiency of different solvents

Preparation of sample solutions

The powdered samples (about 0.5 g) were suspended in 70% ethanol (50 ml) and extracted in an ultrasonic bath for 60 min. Then the extraction solutions were prepared by the method of weight relief, by which the weight lost in the extraction procedure was compensated. The obtained solution was centrifuged at 12,000 rpm for 3 min (Himac CF 15RX High-Speed Micro Centrifuge, Hitachi Koki Co., Ltd., Japan). The supernatant 5ml was added to C_{18} solid-phase extraction column. The initial filtrate (3 ml) was disposed and the remaining filtrate was filtered through a 0.45 μm Millipore filter (Beijing Sunrise T and D Company, China) before injection. All samples were determined in triplicate.

System suitability

The system suitability was conducted by using the standard solutions and evaluated by making five replicate injections. The system was deemed to be suitable for use if the tailing factor was less than 1.2, the resolution was greater than 1.5 and column plate number was more than 10,000 for each analyte.

RESULTS

Optimization of extraction procedure

In order to obtain optimal extraction efficiency, variables involved in the procedure such as solvent, extraction method and extraction time were optimized. Ultrasonic extraction with ethanol solution was chosen as a preferred method. The effect of ethanol concentration on extraction efficiency was investigated; 20, 50, 70 and 90% ethanol were employed. The extraction efficiency was the highest when 70% ethanol was used (Figure 2). Ultrasonic and reflux extraction were compared, and the ultrasonic method was found to be more suitable.

The foreign materials were removed in solid-phase extraction column. The optimal extraction time was investigated. The peak areas of the constituents obtained by different extraction times (20, 40, 60 and 80 min) are shown in Figure 3. It is seen that the five constituents were almost completely extracted within 60 min.

Optimization of chromatographic conditions

The chromatographic conditions were optimized to obtain chromatograms with a good resolution of adjacent peaks within a short analysis time. Different types of chromatographic column were tested. Agilent Zorbax SB-C_{18}, Zorbax XDB and Zorbax Extend-C_{18} column are suitable to different kinds of chemical constituents and in different pH ranges. The sample showed different retention behaviors on these columns. The analysis time did not vary significantly on the three columns, while the resolution of Zorbax SB-C_{18} column was better than that of the rest two. Thus, Zorbax SB-C_{18} column was used for analysis. Different mobile phase compositions (such as $CH_3OH–H_2O–H_3PO_4$, $CH_3OH–H_2O–HOAc$, $CH_3CN–H_2O–H_3PO_4$, and $CH_3CN–H_2O–HOAc$) were also optimized. As a result, acetonitrile and water containing 0.1% glacial acetic acid were chosen as the eluting solvent system since it was not only the desired separation but also less damaged to the column. Under isocratic elution modes, the five compounds could not be separated effectively. Gradient elution mode was therefore used, which can effectively separate the five ingredients.

According to the absorption maximum of five components on the UV spectra with three-dimensional chromatograms of HPLC-DAD, the monitoring

wavelength was performed at 360 nm. It was also suggested that the separation was improved when column temperature was increased to 40℃ and the mobile phase was delivered with the flow rate of 1.0 mL.min−1. Since the flavonoids are abundant in A. venetum L., and their polarity, solubility and other characteristics differ greatly; at least 40 min of elution time was needed for the complete separation of the five target components. Chromatograms of standard mixture (A) and sample (B) were shown in Figure 4.

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Method validation

The method was validated for parameters such as linearity, precision, accuracy and stability following the International Conference on Harmonization (ICH) guidelines.

Linearity

The calibration curve for each compound was performed with five different concentrations by plotting the peak areas versus mass. Linear regression analysis for each compound was performed by the external standard method. The results were presented in Table 2. All the compounds showed good linearity (r2> 0.999) in the concentration range.

Limits of detection and quantification

The limit of detection (LOD) and quantification (LOQ) under the chromatographic conditions were determined by measuring the magnitude of analytical background by injecting blank samples and calculating the signal-to-noise ratio for each compound by injecting series of solutions until the S/N ratio 3 for LOD and 10 for LOQ, then five replicate injections of the solution gave the R.S.D. less than 3%. LOD and LOQ were reported in Table 2 for each compound.

Repeatability

Measurement of intra- and inter-day variability was utilized to determine the repeatability of the method. The intra-day variability was examined on six individual samples within one day, and inter-day variability was determined for three different days. The relative standard deviation (R.S.D.) was calculated as a measurement of method repeatability. The results were shown in Table 3, indicating that the intra- and inter-day R.S.D. values of the five compounds were all less than 5.8%, which showed good reproducibility.

Recovery

Standard addition test was performed to determine the accuracy as well as the extraction recovery. In this test, the mixed standard solutions were prepared with three different concentration levels. The three standard solutions were added to the sample powder (0.25 g, Tianjin). The resultant samples were extracted and analyzed with the proposed HPLC method. The experiments were repeated three times for each level. The ratio of measured and added amounts was used to calculate the recovery. As shown in Table 4, extraction recoveries were in the range of 97, and their R.S.D. values were less than 1.8%. Considering the results of the recovery test, the method is accurate.

Stability

For the stability test, the same sample solution was analyzed at different times within 12 h at room temperature. The R.S.D. values of the peak areas were all lower than 2.0%, suggesting that it is safe to analyze the sample within 1 day.

DISCUSSION

The developed analytical method was successfully applied to the simultaneous determination of (1) chlorogenic acid, (2) hyperoside, (3) isoquercitrin, (4) quercitroside and (5) quercetin in 10 samples of *A. venetum* L., which were obtained from various provinces and cities in China. Each sample was determined in triplicate. Peaks in the chromatograms were identified by comparing the retention time and UV spectra with those of the standards. Retention time for compounds 1 to 5 were 4.06, 11.95, 12.92, 23.65 and 34.68 min,

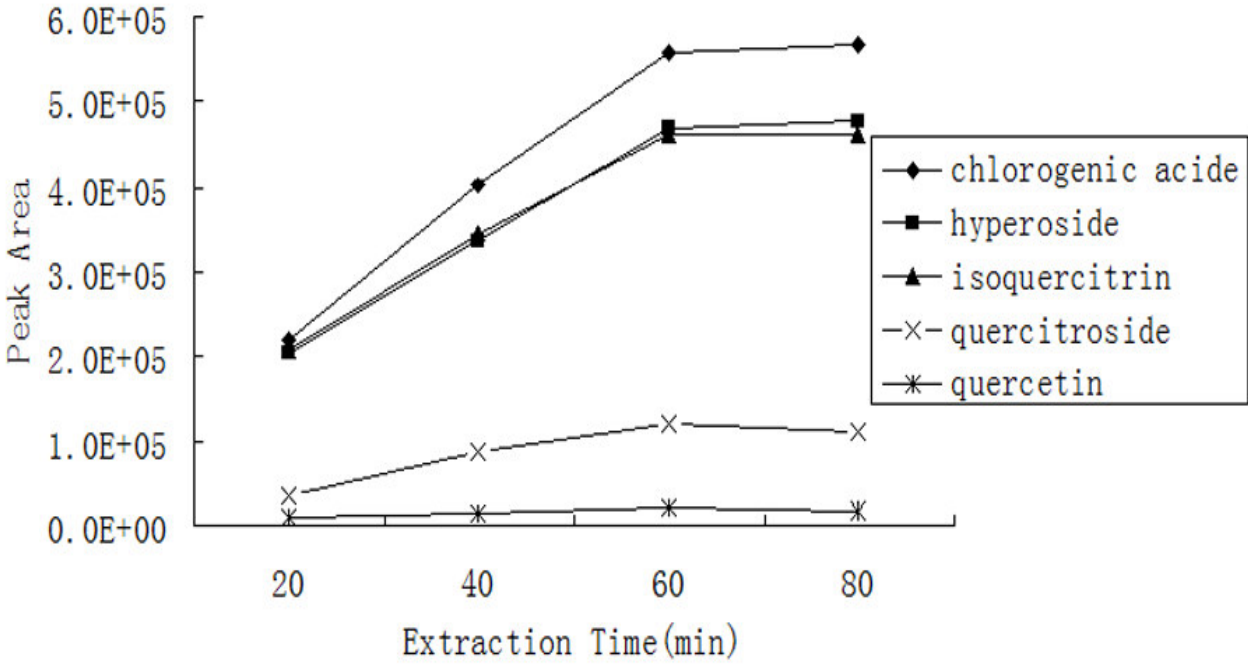

Figure 3. Extraction efficiency at different extraction time.

Table 1. System suitability data.

Compound	t_R(min)	*T*	*Rs*	*N*
Chlorogenic acid	4.06	0.98	8.15	8251
Hyperoside	11.95	0.99	3.44	12167
Isoquercitrin	12.92	0.97	2.04	12537
Quercitroside	23.65	0.99	4.54	36252
Quercetin	34.68	1.02	28.01	315825

T: tailing; R_s: resolution; *N*: thereotical plates.

Table 2. Calibration data for compounds 1–5 (n = 6).

Compound	Regression equation ($y = ax+b$)[a]	r^2	Linear range (μg)	LOD (μg)	LOQ (μg)
Chlorogenic acid	$y = 6.311\times10^{5}x-4.677\times10^{3}$	0.9994	0.1646～2.058	0.02	0.06
Hyperoside	$y = 2.058\times10^{6}x+3.157\times10^{3}$	0.9999	0.04868～0.6085	0.10	0.31
Isoquercitrin	$y = 1.883\times10^{6}x-2.086\times10^{3}$	0.9999	0.06720～0.8400	0.14	0.43
Quercitroside	$y = 1.944\times10^{6}x+1.618\times10^{3}$	0.9996	0.01043～0.1304	0.003	0.01
Quercetin	$y = 7.075\times10^{6}x-1.107\times10^{3}$	0.9997	0.001263～0.01579	0.0004	0.001

[a] *y* is the peak area, *x* refers to the mass of compound (μg); *a* and *b* are the slope and the intercept of the regression line, respectively; r^2 is the correlation coefficient of the equation.

respectively (Figure 4).

From the results presented in Table 5, it was found that the contents of ingredients 1 to 5 varied greatly among the different samples. For example, the contents of quercitroside (compound 4) varied from 0.50 to 1.33 $mg.g^{-1}$, with almost 2 fold variation. In the majority of cases hyperoside (compound 2) and isoquercitrin (compound 3) were main components, whose contents

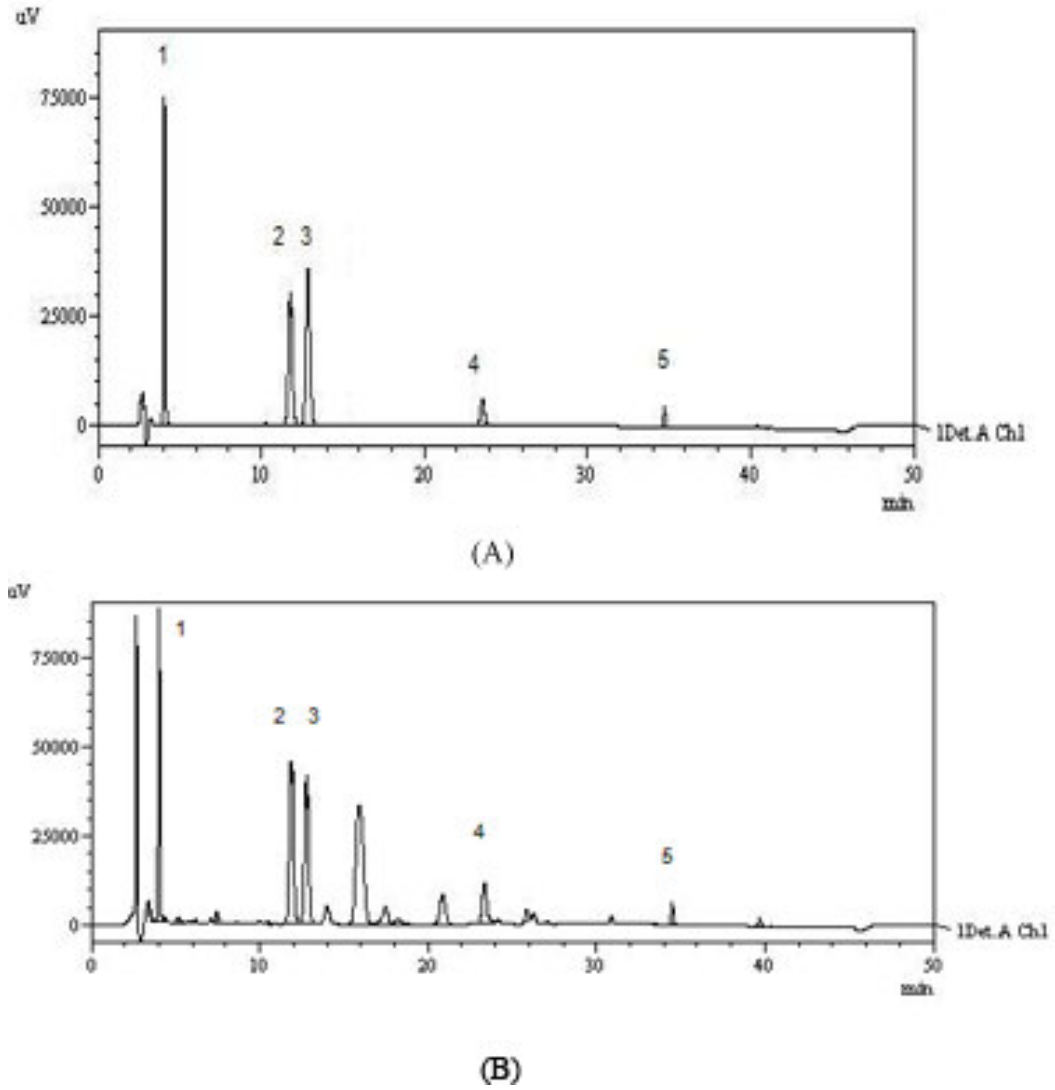

Figure 4. HPLC chromatograms of standard mixture (A) and *Apocynum venetum* L. leaves sample (B); 1) Chlorogenic acid, 2) hyperoside, 3) isoquercitrin, 4) quercitroside and 5) quercetin.

Table 3. Analytical results of intra- and inter-day variability for compounds 1–5 in Apocynum Venetum L. leave.

Compound	Intra-day		Inter-day	
	Mean ±S.D.[a]	R.S.D (%)	Mean ± S.D.[a]	R.S.D (%)
Chlorogenic acid	8.65 ± 0.23	1.7	8.57 ± 0.17	2.6
Hyperoside	2.42 ± 0.04	2.0	2.29 ± 0.03	4.2
Isoquercitrin	2.47 ± 0.02	1.5	2.56 ± 0.04	5.8
Quercitroside	0.51± 0.02	1.9	0.52 ± 0.01	5.6
Quercetin	0.03 ± 0.00	1.9	0.03 ± 0.00	4.0

[a] Data were mg compound per gram crude drug, and S.D. were calculated using SPSS (Statistical Package for the Social Science).

varied from 2.18 to 3.01 $mg.g^{-1}$ and 2.44 to 3.38 $mg.g^{-1}$ in 10 samples, with almost 1.5-fold and 1.5-fold variation. Similar variation could also be found for the other components. The reasons for the variation of the contents may be due to the difference in plant origin, the effect of environment and some other factors, such as season of

Table 4. Analytical results of recoveries (*n*=3).

Compound	Added (mg)	Measured (mg) [a]	Recovery[b]	R.S.D (%)
Chlorogenic acid	1.975	2.004	101.4	1.01
	2.304	2.345	101.7	0.54
	3.292	3.373	102.5	1.61
Hyperoside	0.3651	0.3572	97.8	1.84
	0.4868	0.4856	99.6	1.08
	0.9736	0.9496	100.9	1.54
Isoquercitrin	0.5040	0.5099	101.2	1.21
	0.6720	0.6627	98.6	0.25
	1.2432	1.2410	99.8	1.27
Quercitroside	0.0887	0.0881	99.3	1.08
	0.1095	0.1105	100.9	1.19
	0.2139	0.2190	102.4	1.65
Quercetin	0.0062	0.0063	98.1	0.51
	0.0082	0.0082	100.0	1.30
	0.0139	0.0138	99.5	1.22

[a] Calculated by subtracting the total amount after spiking from the amount in the herb before spiking. Data were means of three experiments. [b] Calculated as detected amount/added amount×100%. Data were mean of three experiments.

Table 5. Contents of chlorogenic acid, hyperoside, isoquercitrin, quercitroside and quercetin in Apocynum Venetum L. leave (*n* = 3).

Sample no.	Origin[a]	Content(mg.g^{-1})[b]				
		Chlorogenic acid	Hyperoside	Isoquercitrin	Quercitroside	Quercetin
1	Jilin	8.79±0.02	3.01±0.04	3.38±0.02	1.18±0.03	0.09±0.01
2	Tianjin 2	8.26±0.02	2.33±0.02	2.68±0.01	1.33±0.02	0.04±0.02
3	Neimenggu 1	9.00±0.03	2.35±0.05	2.89±0.03	0.50±0.05	0.05±0.03
4	Neimenggu 2	9.38±0.04	2.50±0.07	3.02±0.02	0.51±0.08	0.05±0.03
5	Jiangxi	7.42±0.02	2.18±0.05	2.44±0.05	0.96±0.04	0.04±0.01
6	Guiyang	8.26±0.03	2.91±0.02	3.34±0.02	1.17±0.03	0.14±0.04
7	Hebei 1	9.96±0.02	2.33±0.03	2.83±0.01	0.54±0.03	0.05±0.03
8	Tianjin 1	9.24±0.01	2.64±0.01	2.51±0.06	0.62±0.05	0.03±0.04
9	Hebei 2	8.58±0.02	2.61±0.06	2.46±0.04	1.27±0.01	0.03±0.02
10	Tianjin 3	8.69±0.01	2.48±0.02	2.72±0.01	1.11±0.02	0.05±0.03

[a] Commercial samples were purchased from various drug stores or market in China, and the original plants of samples were identified as Apocynum Venetum L. leave. [b] Data were expressed as mean±S.D. of three experiments.

collection, drying process and storage conditions, etc (Wei et al., 2008).

Because variations of the active component content in herb may influence the quality and potency of the medicinal herb, it was necessary to develop an effective qualitative and quantitative method to evaluate the quality of *A. venetum* L. The assay of one or two constituents cannot give a complete assessment of the herb.

Thus, in this paper, five major compounds in *A. venetum* L. were analyzed simultaneously to evaluate its quality.

Conclusion

Method validation data indicated that the present method was a reliable, repeating and accurate HPLC method. So the method could be used as a routine measurement to provide a safe application for good manufacture practices quality control of the medicine.

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