Chemical Fingerprinting of *Andrographis paniculata* (Burm. f.) Nees by HPLC and Hierarchical Clustering Analysis

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Abstract

The aim is to develop a simple and specific method for the extraction and chemical fingerprinting of *Andrographis paniculata* (Burm. f.) Nees and to apply the method to this drug from different regions. High-performance liquid chromatographic (HPLC) with gradient elution is used for developing the fingerprints, and liquid chromatography electrospray ionization mass spectrometry (LC–ESI-MS) technique is employed to identify the component of the fingerprints. Nine peaks are selected as common peaks, and six compounds are elucidated by MS data. Twenty-three samples of *A. paniculata* from different regions of China are collected and detected by HPLC fingerprinting. Comparisons of the chromatograms show that there are obvious differences in the content of each component contained between the habitat samples in China. The results of hierarchical cluster analysis show that these samples can be clustered reasonably into three groups, and the growth of *A. paniculata* and their internal quality are related to their habitat. The HPLC fingerprint developed allows simple identification of *A. paniculata* from many natural drugs.

Introduction

Andrographis herb, commonly known as Kalmegh (Chuanxinlian), is the dried aerial part of *Andrographis paniculata*, which is a member of the plant family Acanthaceae. This crude drug has been widely used in China, India, and other countries in Asia for the treatment of digestive problems, common cold, fever, snake bite, and infections ranging from malaria to dysentery (1–2). The therapeutic activity of this herb has been attributed to andrographolide and its related diterpenoid compounds i.e., deoxyandrographolide, 14-deoxy-11,12-didehydro-andrographolide (dehydroandrographolide), and neoandrographolide (Figure 1) (3). These constituents are believed to have immune stimulating, anti-inflammatory, fertility-decreasing, liver-protective, and bile secretion-stimulating actions (4–6). Chuanxinlian is widely distributed and used in China. So, the composition of the various active compounds in Chuanxinlian varies significantly with geographic location, climate conditions, environment, and other factors. Due to the existence of such differences, controlling the quality of this herbal medicine and their derivatives is difficult.

Recently, chromatographic fingerprint technique was regarded as an effective and feasible means for controlling the quality of traditional Chinese medicines (TCM) and their product because this technique emphasizes the systemic characterization of compositions of samples and focuses on identifying and assessing the stability of the plants. Accordingly, fingerprint technology has been introduced and accepted by World Health Organization as a strategy for the quality evaluation of herbal medicines (7). And it is also required by the U.S. Food and Drug Administration to standardize injections made from TCM and their raw materials (8). At present, the methods thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), etc. are usually employed to construct the fingerprint, and HPLC fingerprint analysis is the first choice due to its advantages and popularization (9–11).

Until now, several methods have been reported for the determination of these diterpenoids in *A. paniculata*, including TLC, HPTLC, HPLC, high-speed counter-current chromatography, micellar electrokinetic chromatography (MEKC) (12–18), and so

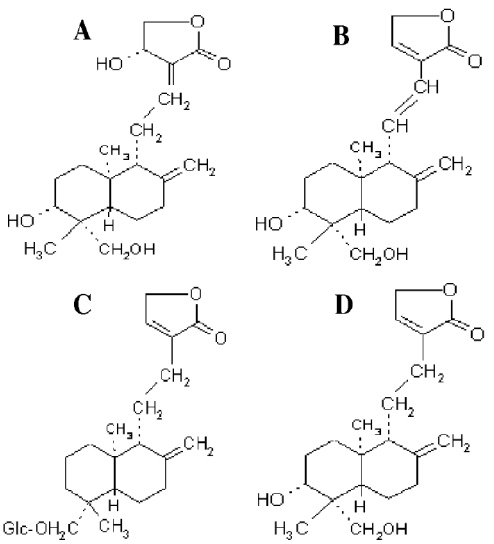

Figure 1. Chemical structures of the four main diterpenoids in *A. paniculata*: andrographolide (A), dehydroandrographolide (B), neoandrographolide (C), and deoxyandrographolide (D).

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on. But these methods cannot control the quality of *A. paniculata* from different regions effectively and systematically. The aim of this study was to develop a characteristic fingerprint of *A. paniculata* using HPLC method for identifying the raw herb and distinguishing them from different regions of China.

The application of various multivariate mathematical statistical methods is more and more frequent in the elucidation and assessment of the TCM. In this study, a characteristic fingerprint of *A. paniculata* using HPLC had been developed, and main components were identified initially. Significantly, hierarchical cluster analysis was applied to evaluate the fingerprints of the *A. paniculata* from different regions of China. It was regarded as the foundation of quality control of *A. paniculata* completely.

Experimental

Standards and chemicals

Standards of andrographolide and dehydroandrographolide were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) (purity > 98%). Acetonitrile of HPLC-grade was purchased from VWR International Company (Darmstadt, Germany). Formic acid and ethanol of analytical-grade were obtained from Nanjing Chemical Reagent Co. Ltd. (Nanjing, China). Water for HPLC analysis was prepared in our laboratory. Deionized water was distilled before using. Other reagents were used as received.

Crude drugs

Twenty-three samples of crude drugs were collected from the different regions of China (Table I), all of which had been identified as the *A. paniculata* (Burm. f.) Nees by Professor Zhengtao Wang from China Pharmaceutical University.

Sample preparation

A. paniculata were dried at 40ºC and milled to 40 mesh powder as extraction sample. Two grams of sample was accurately weighted and extracted with 40 mL of 90% ethanol in water bath for 4 h at 60ºC three times. All filtrate was collected, and 3 mL filtrate was evaporated under a stream of nitrogen at 40°C. The residue was redissolved in 2.5 mL of 50% methanol and filtered through a 0.45-µm filter membrane to obtain the test solution.

Table I. Samples of *A. paniculata* From Different Regions

Sample No.	Growing regions	Sampling part	Sample No.	Growing regions	Sampling part
1	Bozhou, Anhui	Stem/Leaf	13	Unknown	Stem/Leaf
2	Unknown	Stem/Leaf	14	Zhaoqing, Guangdong	Stem/Leaf
3	Bozhou, Anhui	Stem/Leaf	15	Zhaoqing, Guangdong	Stem/Leaf
4	Guangdong	Leaf	16	Qingyuan, Guangdong	Stem/Leaf
5	Guangdong	Stem	17	Qingyuan, Guangdong	Stem/Leaf
6	Wuzhishan, Hainan	Stem/Leaf	18	Maoming, Guangdong	Stem/Leaf
7	Wuzhishan, Hainan	Stem/Leaf	19	Maoming, Guangdong	Stem/Leaf
8	Yunan, Guangdong	Stem/Leaf	20	Yingde, Guangdong	Stem/Leaf
9	Linquan, Anhui	Stem/Leaf	21	Yingde, Guangdong	Stem/Leaf
10	Yulin, Guangxi	Stem/Leaf	22	Shaodong, Hunan	Stem/Leaf
11	Bozhou, Anhui	Stem/Leaf	23	Shaodong, Hunan	Stem/Leaf
12	Linqua, Anhui	Stem/Leaf			

HPLC conditions for HPLC fingerprinting

The HPLC system consisted of two delivery pumps (Shimadzu LC-10AT, Kyoto, Japan), a UV detector (Shimadzu LC-10AVP, Kyoto, Japan), and a model 7725 manual injector valve with a 20-µL sample loop. The HPLC fingerprint was carried out on a Shimadzu VP-ODS column (150 mm × 4.6 mm i.d., 5 µm) at ambient temperature with sample injection volume of 20 µL, and the wavelength was 254 nm. The mobile phase consisted of 0.2% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using gradient

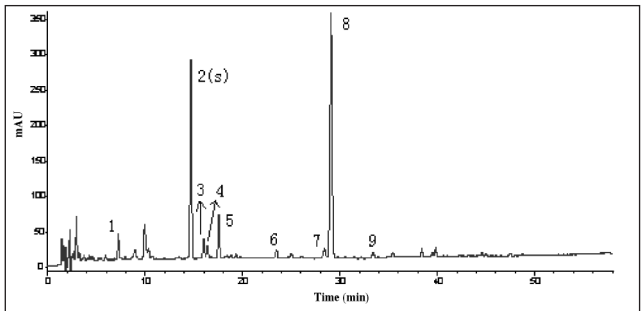

Figure 2. The typical HPLC fingerprint of *A. paniculata* from Anhui of China.

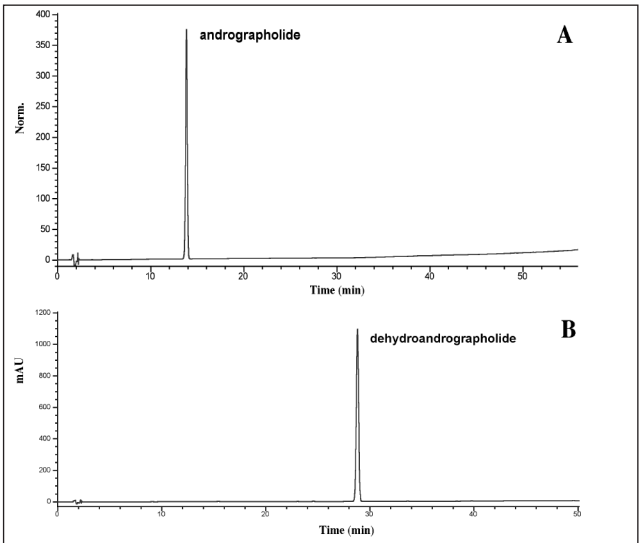

Figure 3. The chromatogram of standards of andrographolide (A) and dehydroandrographolide (B).

Table II. The Mass Data and Compound Names of Six Peaks

Peak*	Mass data	Identification
2	351[M+H]$^+$, 373 [M+Na]$^+$, 389 [M+K]$^+$, 333 [M+H-H_2O]$^+$, 315 [M+H-2H_2O]$^+$, 297 [M+H-3H_2O]$^+$	andrographolide,
3	519 [M+Na]$^+$, 535 [M+K]$^+$, 479 [M+H-H_2O]$^+$	19-glucosyl-deoxyandrographolide
5	349 [M+H]$^+$, 371 [M+Na]$^+$,387 [M+K]$^+$, 331 [M+H-H_2O]$^+$,313 [M+H-2H_2O]$^+$	14-deoxy-11-oxoandrographolide
6	481 [M+H]$^+$,503 [M+Na]$^+$,519 [M+K]$^+$, 319 [M+H-Glu]+,301 [M+H-Glu-H_2O]$^+$	neoandrographolide
7	335 [M+H]$^+$, 357 [M+Na]$^+$, 373 [M+K]$^+$, 317 [M+H-H_2O]$^+$, 299 [M+H-2H_2O]$^+$	deoxyandrographolide
8	333 [M+H]$^+$,355 [M+Na]$^+$,371 [M+K]$^+$, 315 [M+H-H_2O]$^+$, 297 [M+H-2H_2O]$^+$	dehydroandrographolide

* The peak number corresponds to Figure 2.

program of 20–40% (B) in 0–30 min, 40–85% (B) in 30–55 min and 85% (B) in 55–60 min. The flow rate was 1.0 mL/min.

HPLC–ESI-MS analytical conditions

Agilent (Palo Alto, CA) Series 1100 equipped with G1312A Quaternary Pump, G1329A automatic sample injector, G1316A thermostatted column compartment, and G1315A diode array detector was used. The mass spectrometry detector (MSD) was equipped with electrospray ionization source (ESI). One-third of the eluant was introduced into the mass system by using split technique. The MSD parameters were as follows: scan mode, positive; scan range *m/z*, 100–1000; dry gas, 10 L/min; dry gas temperature, 350ºC; nebulizer pressure, 40 psi; capillary voltage, 4000 V; transmission voltage, 70 eV.

Results and Discussion

Selection of extraction methods

According to the references, we know that the therapeutic activity of *A. paniculata* has been attributed to andrographolide and its related diterpenoid compounds i.e., deoxyandrographolide, 14-deoxy-11,12-didehydro-andrographolide, and neoandrographolide. In the experiment, we considered different solvents (methanol, ethanol) and extraction modes (sonication, soakage, regurgitation) according to the chemical property of those compounds to choose the suitable method for extraction. At last, on the basis of the high efficiency of the main compounds in this herb and minor interference, extracted with 40 mL of 90% ethanol in water bath was designed as the extraction method for the sample preparation of HPLC fingerprint.

Optimization of HPLC condition

In the mobile phase, formic acid was added to depress the tailing and improve the separation of diterpenoid compounds. The effect of the concentration of formic acid in mobile phase was investigated. It was found that the 0.2% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B) would achieve better results. Because in constant speed elution these compounds, which have different polarity in the sample, may not have been separated well. We chose linear gradient elution instead. Satisfactory results were obtained within 60 min for the HPLC separation.

The wavelength for the detection of the components in *A. paniculata* was selected by using DAD detector. The maximum number and the height of peaks of active compounds would be obtained, and the baseline of chromatogram was stable at 254 nm. Therefore, 254 nm was chosen as the detection wavelength.

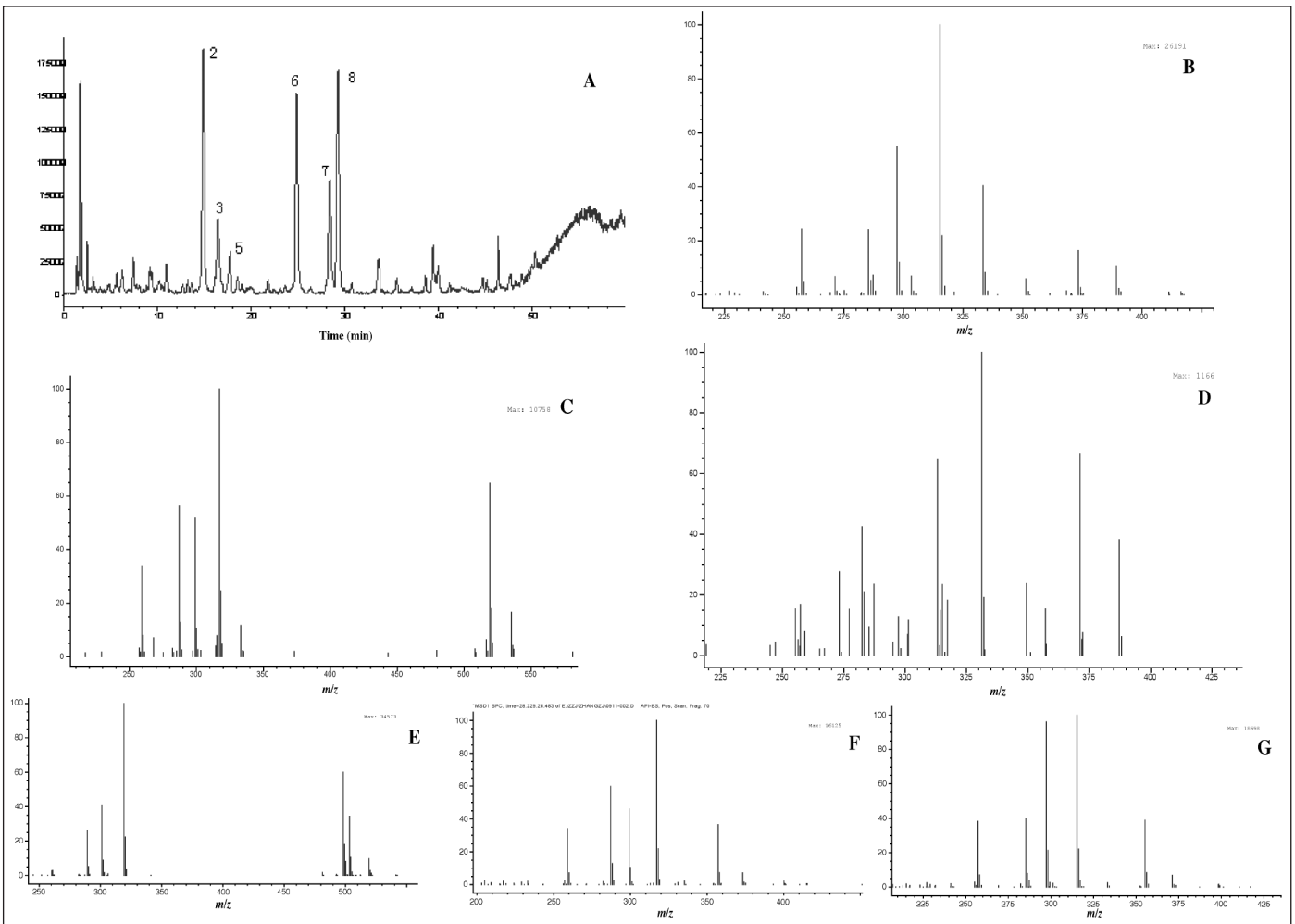

Figure 4. The TIC of *A. paniculata* (A) and the mass spectrum of peaks (B) andrographolide, (C) 19-glucosyl-deoxyandrographolide, (D) 14-deoxy-11-oxoandrographolide, (E) neoandrographolide, (F) deoxyandrographolide, (G) dehydroandrographolide.

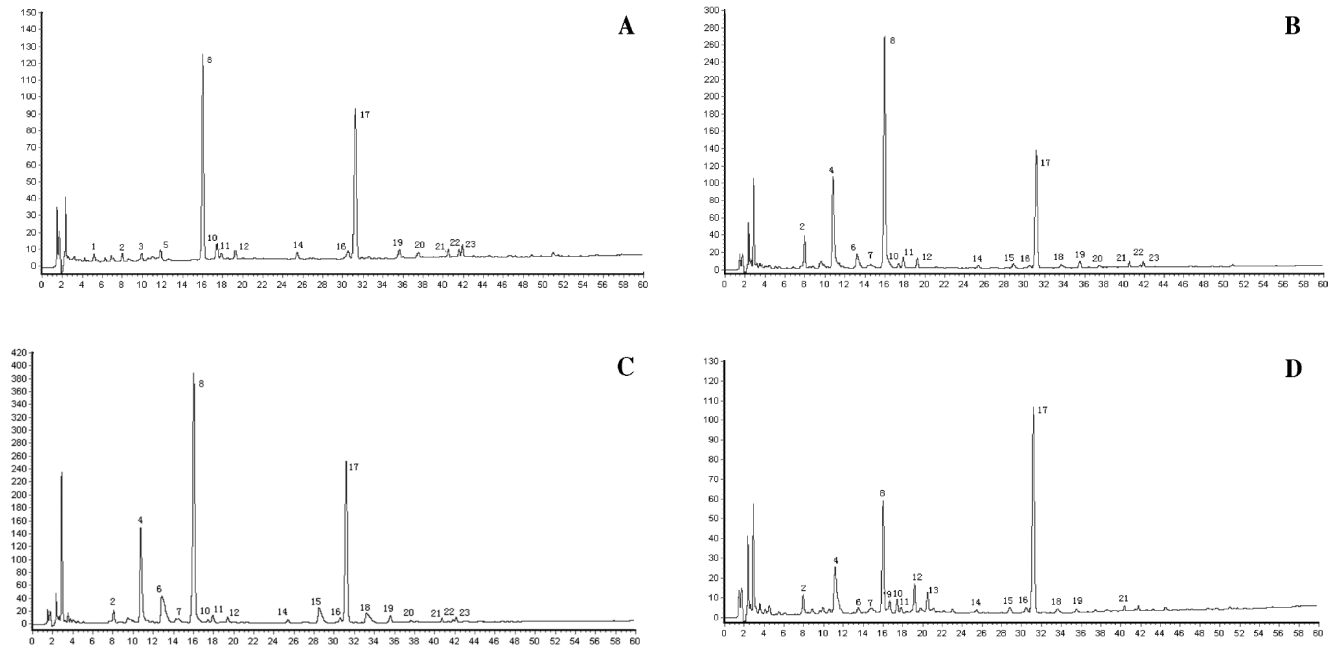

Figure 5. The fingerprint of *A. paniculata* from different regions of China (A) Linquan, Anhui Province; (B) Yunan, Guangdong Province; (C) Wuzhishan, Hainan Province; (D) Shaodong, Hunan Province;.

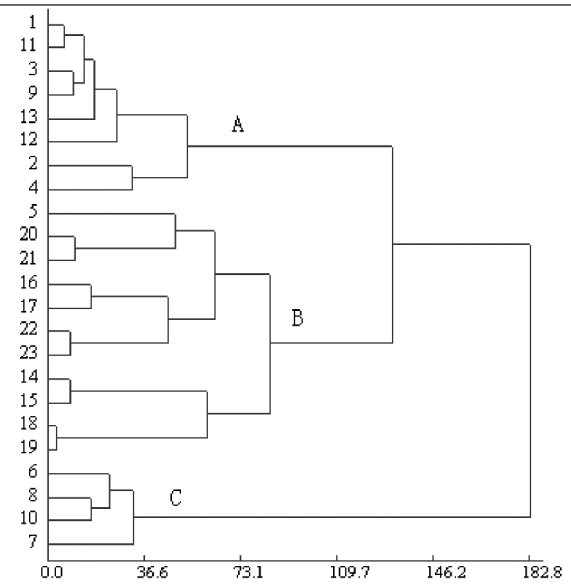

Figure 6. Hierarchical cluster analysis figure of *A. paniculata*. 1 to 23 are different habitats. The samples in group A were from the province of Anhui; the samples in group B were from the province of Guangdong and Hunan; and the samples in group C were from the province of Guangxi and Hainan.

Standardization of fingerprint

The method validation of fingerprint analysis was performed based on the relative retention time (the ratio of peak retention time of sample constituents to the reference standard) and the relative peak area (the ratio of peak area of sample constituents to the reference standard). Among these active components, andrographolide indicates a proper retention time and higher content; therefore, we chose it as the reference substance. All common peaks' relative retention time and relative peak area were obtained on the basis of this substance. The precision of the proposed method, on the basis of analyzing six replicate samples, were both below 3% for relative standard deviation (RSD) of relative retention time and relative peak area of all peaks, respectively. The reproducibility test was performed with six sample solutions extracted from one batch of plant. The RSD of the relative retention times and the relative peak areas were both less than 3%. The stability test was performed with sample solutions for 24 h. RSDs of both the relative retention times and the relative peak areas were less than 3%. The result indicates that this method is adequate, valid, and applicable. To standardize the fingerprint, 10 batches of *A. paniculata* samples from Anhui, one of the main habitats of *A. paniculata*, were analyzed with the developed procedure. Peaks that existed in all 10 batches of samples were assigned as "common peaks" for *A. paniculata*. There were 9 "common peaks" in the fingerprint. The typical chromatogram is shown in Figure 2.

HPLC-MS analysis for components identification

HPLC–ESI-MS had been employed to the components analysis in *A. paniculata* crude drugs. First, the standards of andrographolide and dehydroandrographolide were used to determine these two peaks in the fingerprint (Figure 3A and 3B). Then, in an ESI-MS experiment, we obtained molecular weight of each peak and a series of the fragments. Comparing with references, we had deduced six possible structures of nine common peaks. Because the ionization mode was positive, most of the m/z data were $(M+H)^+$, $(M+Na)^+$, and $(M+K)^+$. In this ionization mode, andrographolide and these diterpenoid components would form a series of peaks of dehydration. So their m/z data were often $(M+H-H_2O)^+$, $(M+H-2H_2O)^+$, and $(M+H-3H_2O)^+$. The mass data and compound names of the six peaks are given in Table II. The total ionization chromatogram (TIC) and the mass spectrum of peaks are shown in Figure 4.

Comparison and hierarchical cluster analysis of fingerprint from different regions

The HPLC fingerprints of 23 sources of samples were obtained by using the method, which we established earlier. Figure 5 shows the typical HPLC profile of 23 tested samples, which were the *A. paniculata* in Anhui, Hainan, Guangdong, Hunan, etc. Though the "common peak" appearing in each sample came from different area, the peak areas of the "common peak" and the "non-common peak" were obviously different. The quality of herbal medicine was closely related to the concentrations of their chemical constituents. Although it was possible to visually differentiate the fingerprints of different regions, the process was subjective and not quantitative. In addition, minor differences amongst very similar chromatograms might be missed.

In this study, the chemometric methods hierarchical cluster analysis was used to classify the samples of *A. paniculata* from the different regions. Twenty-three characteristic peaks were selected, and the relative retention times of these constituents were calculated with respect to the reference peak at retention time ~ 16.0 min (this peak is andrographolide). The relative areas of the 23 characteristic peaks were calculated by using the area of the reference peak as a reference standard. Relative areas of the 23 constituents of 23 samples formed a matrix of 23 × 23. The hierarchical cluster analysis was performed using DPS (Data Processing System) software (DPS 3.01, developed by Professor Qiyi Tang, Zhe Jiang University, Hangzhou, China). The dendrogram is presented in Figure 6. From the dendrogram, these samples could be grouped into groups A, B, and C according to their different geographical origins. The samples in group A were from the province of Anhui; the samples in group B were from the province of Guangdong and Hunan; and the samples in group C were from the province of Guangxi and Hainan. The hierarchical cluster analysis results showed that the growth of *A. paniculata* and their internal quality were related to their habitat.

Conclusion

In conclusion, the HPLC fingerprint developed in this paper allowed the identification and comparison of the *A. paniculata* and can be utilized to assess the quality of the *A. paniculata* from different habitats. This method had been validated for precision, reproducibility, and stability. Meanwhile, the mathematical statistical method HCA in our study was used for quantitative comparison of *A. paniculata*. We can conclude that the main classification pattern was caused by their native geographical distribution. The results indicated that this method was feasible for comprehensive quality evaluation of *A. paniculata*, and the habitat of crude drug should be fixed when the preparation based on the *A. paniculata* was produced.

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