J Pharm Anal Vol 1, No 2, 113 - 118 (2011)

ORIGINAL ARTICLES

Development of the fingerprints for the quality evaluation of *Viscum coloratum* by high performance liquid chromatography

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Abstract: A high-performance liquid chromatography coupled ultraviolet (HPLC-UV) method was developed for a chemical fingerprint analysis of *Viscum coloratum*. Eighteen peaks were selected as the common peaks and Homoeriodictyol-7-O-β-D-apiosiyl-(1→2)-β-D-glucoside was used as a reference. The relative areas of common peaks were used for hierarchical clustering analysis and similarity calculation. Thirty-seven samples collected from different sources were classified into five groups. The similarities of 21 batches *Viscum coloratum* samples were beyond 0.90. The results obtained suggest that the chromatographic fingerprint can efficiently identify *Viscum coloratum*. Additionally, the fingerprints can then be used to evaluate the correlation between *Viscum coloratum* and hosts.

Keywords: *Viscum coloratum*; quality; high-performance liquid chromatography; fingerprint

1 Introduction

Traditional Chinese medicines (TCMs) and their preparations have a long history in medical practice and health care, especially in Asia. Due to the effective therapeutic performance, TCMs have attracted considerable attention in many fields. According to Chinese medicine theory, the whole components in crude herbs are responsible for the beneficial medicinal effects, which make the quality control of herbal products very difficult. Traditionally, one or two markers or active components in herbs or herbal mixtures are used to assess the authenticity and quality of the complex TCMs. This strategy has been proved to be insufficient for the quality control of TCMs and their preparations because it does not evaluate all chemical components present in the chromatographic profile. With the development of analytical technique, chromatographic fingerprints have been widely used for the authentication and quality control of TCMs [1,2]. Chromatographic fingerprints techniques can be used to characterize both the marker compounds and the unknown components in a complex system, a strategy recommended to assess the quality and consistency of botanical products by US Food and Drug Administration (FDA), the European Medicines Evaluation Agency (EMEA), and State Food and Drug Administration of China (SFDA) [3-5].

Viscum coloratum (Kom.) Nakai (*V. coloratum*) is a perennial evergreen, semi-parasitic plant which grows on branches or stems of deciduous trees. It is known as Hujisheng in Chinese [6]. *V. coloratum*, an important medicinal herb suitable for commercial production, has been used for the treatment of various ailments including cardiovascular diseases, cancer, hepatitis and hemorrhage [7,8]. In clinical use, *V. coloratum* is usually mixed up with *Taxillus chinensis* (DC.) Danser. However, they belong to different genera of Loranthaceae. Hence, the development of the fingerprints is essential for efficiently identify *V. coloratum*. Otherwise, to grow and reproduce *V. coloratum* as a semi-parasitic plant must successfully compete for a share of the host's water, avoid mineral deficiencies, tolerate differences in host xylem sap chemistry and, over time, flower and seed within the host canopy [9]. It acquires most of nutrients and water from their host through a root connection. So *V. coloratum* is restricted by the complicated interaction between *V. coloratum* and its host, and the impact of the host on the quality of *V. coloratum* is critical. So far, a few studies on fingerprints of *V. coloratum* using HPLC have been reported [10,11]. However, the small sample size limits to some extent the generalization of the findings made in the study. In addition, there has been no previous report on the evaluation of *V. coloratum*-host interactions. In the present study, a sensitive and accurate HPLC fingerprints method was developed and validated for evaluating 37 batches of *V. coloratum* growing on different sources (host species not clear) and 5 batches of *V. coloratum* growing on different host species (the same sources). We aimed to

Received 24 March 2011; Accepted 27 April 2011

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provide a powerful way and some insight for the quality control of *V. coloratum* and further to find the relationship between *V. coloratum* and its host.

2 Experimental

2.1 Chemicals and reagents

Acetonitrile was of HPLC grade (Richmond Hill, USA). All the other reagents were of analytical grade from Concord Tech Reagent Company (Tianjin, China). Deionized water was used throughout the study.

The standard Homoeriodictyol-7-*O*-*β*-*D*-apiosiyl-(1→2)-*β*-*D*-glucoside was isolated and purified by us from the branches and steaks of *V. coloratum*. The structure was characterized by chemical and spectroscopic methods (UV, IR, NMR, MS) and then the data were compared with those reported by literature [12]. The purities of the isolated compound checked by HPLC-DAD at different wavelengths were above 98.9%.

2.2 Plant materials

Commercial herb samples of *V. coloratum* (host species were not clear, source group) were purchased from various pharmacies around China, while another *V. coloratum* (host species were clear, host group) were collected in Changbai Mountain (CBM, Yanji, Jilin). The collected plant material was dried at room temperature in the absence of light in a well-ventilated room. Professor Qi-Shi Sun authenticated the plant material, and the voucher specimens were deposited in the State Key Laboratory of Traditional Chinese Medicine (Shenyang Pharmaceutical University, China).

2.3 Apparatus and chromatographic conditions

The HPLC system was composed of a Shimadu LC-10AT*vp* series binary pump and a Shimadu SPD-10A*vp* UV detection. A Zhejiang University 2010 Chromatography Data System was used for data acquisition and integration. The analysis was carried out on a Phenomenex Synergi C_{18} column (250 mm×4.6 mm, 4 μm). The mobile phase was 0.5% glacial acetic acid (A) and methanol-tetrahydrofuran (90 : 10) (B) with a gradient elution program of 3% − 20% (B) in 0 − 20 min, 20% − 70% (B) in 20 − 60 min and then re-equilibration of the column with 3% B for 10 min. The injection volume was 20 μL and the flow rate was maintained at 1.0 mL/min throughout the elution. The column temperature was kept at 30 ℃. The wavelength was set at 270 nm.

2.4 Preparation of standard solutions

In a clean, dry 10-mL volumetric flask, reference standard Homoeriodictyol-7-*O*-*β*-*D*-apiosiyl-(1→2)-*β*-*D*-glucoside was accurately weighed and dissolved in methanol to make a reference standard solution (20 μg/mL). The standard solution was stored at 4 ℃ and brought to room temperature before use.

2.5 Preparation of samples

The *V. coloratum* samples were powdered in an electrical blender and then passed through a # 4 mesh sieve. The powder was accurately weighed (1.0 g) into a round-bottom flask and 25 mL of 50% (v/v) methanol was added. The flask was then weighed and heated in a water bath for 30 min. The flask was then cooled and 50% methanol was added in order to compensate for the solvent loss. The extract was mixed well and transferred to a 50 mL centrifuge tube and centrifuged at 10000 rpm for 5 min. The supernatant was filtered through a 0.45 μm millipore filter membrane prior to injection.

2.6 Data processing

2.6.1 Hierarchical clustering analysis (HCA)

Clustering is the art of grouping together pattern vectors that in some sense belong together because of similar characteristics. It provides both a visual representation of complex data and a method for measuring similarity between experiments. The similarity or dissimilarity between samples (objects) is usually represented in a dendrogram for ease of interpretation. Each object is similar to the others within one group but different from those in other groups with respect to a predetermined selection criterion [13]. In this part, source grouping of *V. coloratum* was performed using SPSS statistics software (SPSS for Windows 16.0, SPSS Inc., USA) based on the inter-group linkage method and squared Euclidean distance, respectively.

2.6.2 Similarity analysis

The chromatographic profiles of all extracts were performed by professional software named Similarity Evaluation System for Chromatographic Fingerprint of TCMs, which was developed by Shenyang Pharmaceutical University (China). The software was employed to evaluate the similarities between different chromatograms by calculating the cosine (C_{ir}) values of vectorial angle and correlation coefficient (r_{ir}) among samples [the calculation formula is described in Eq. (1) and Eq. (2)], as well as to compute and generate the mean chromatogram as a representative standard fingerprint.

$$C_{ir} = \frac{\sum_{k=1}^{m} X_{ik} \cdot X_{rk}}{\sqrt{\left(\sum_{k=1}^{m} X_{ik}^2\right)\left(\sum_{k=1}^{m} X_{rk}^2\right)}} \quad (1)$$

$$r_{ir} = \frac{\sum_{k=1}^{m} (X_{ik} - \bar{X}_i)(X_{rk} - \bar{X}_r)}{\sqrt{\sum_{k=1}^{m} (X_{ik} - \bar{X}_i)^2 \sum_{k=1}^{m} (X_{rk} - \bar{X}_r)^2}} \quad (2)$$

where X_{ik} and X_{mk} represent the kth variate of the ith sample and mean vector in common pattern, respectively; X_i and X_r are the mean values of them.

3 Results and discussion

3.1 Optimization of chromatographic conditions

The chromatographic conditions were optimized to obtain an ideal fingerprint containing sufficient information of constituents with good resolution of adjacent peaks within a short analysis time. Four types of chromatographic columns, namely, Agilent Zorbax SB-C_{18} column, Phenomenex Luna C_{18} column, Phenomenex Synergi C_{18} column and Dikma Diamonsil C_{18}, were chosen. The extract showed different retention behaviors on these columns. Phenomenex Synergi C_{18} column was more suitable for separation of the constituents of the herb.

According to the UV absorption maxima of compounds on the three-dimensional chromatograms of HPLC-DAD detection, the monitoring wavelength was set at 270 nm. The chromatographic peaks of extracts were identified by comparing the retention time of target compounds and UV spectrum with the chromatograms of standards. Homoeriodictyol-7-*O*-*β*-*D*-apiosiyl-(1→2)-*β*-*D*-glucoside was used as a reference (Figure 1A). Relative retention time (RRT, the ratio between retention time of characteristic peaks to that of reference peak) and relative peak area (RPA, the ratio between peak area of characteristic peaks to that of reference peak) of each characteristic peak to reference were calculated in the chromatograms.

To obtain good separation, methanol-water and acetonitrile-water were investigated as mobile phases. With acetonitrile-water the peaks of short retention time were not separated well. More compounds were separated well by use of methanol-water containing acid and tetrahydrofuran. As a result, 0.5% glacial acetic acid (A) and methanol-tetrahydrofuran (90 : 10) (B) with a gradient elution program was chosen as the eluting solvent system. The chromatogram is shown in Figure 1B.

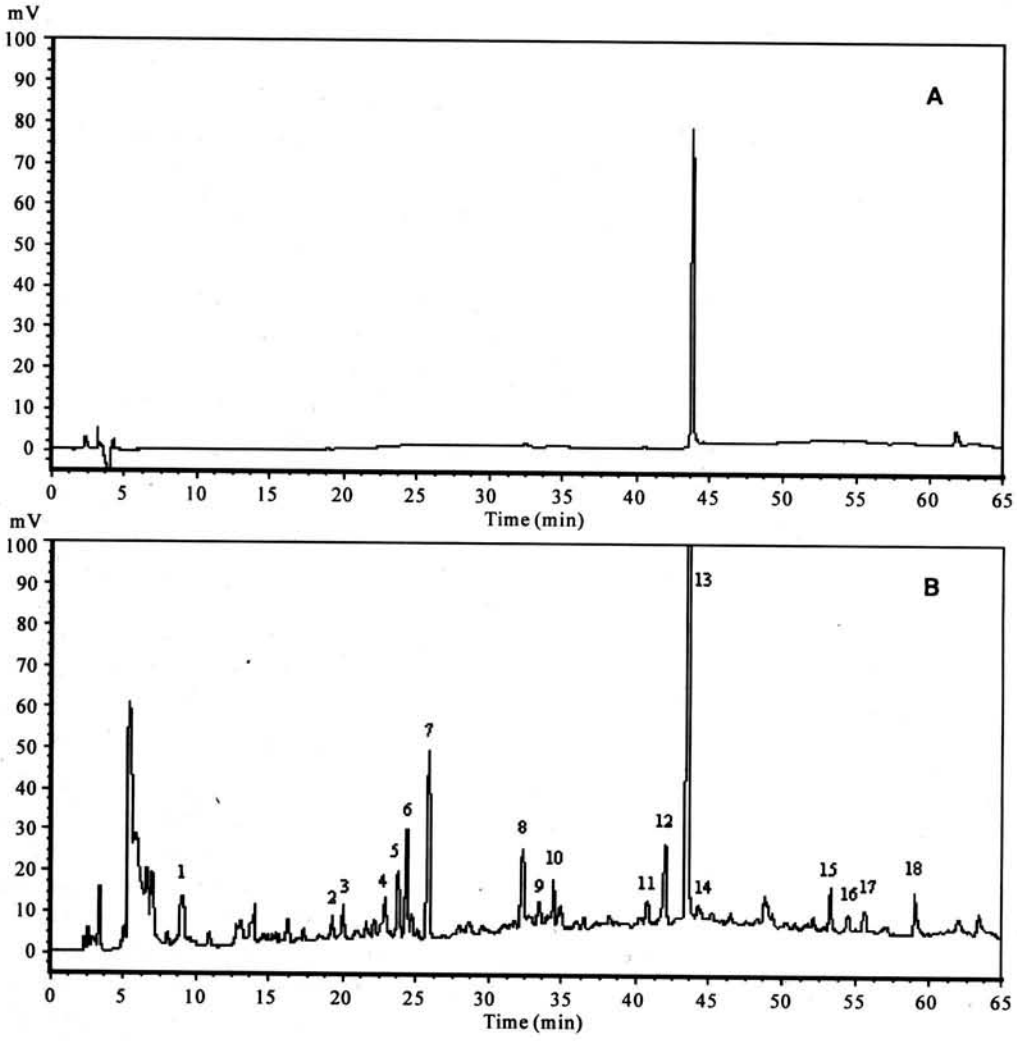

Figure 1 Chromatograms of Homoeriodictyol-7-*O*-*β*-*D*-apiosiyl-(1→2)-*β*-*D*-glucoside standard (**A**) and chromatographic fingerprint of *V. coloratum* from Mudanjiang (No.1) (**B**).

3.2 Extraction procedure

Sample pretreatment conditions were also optimized by investigating the effect of extraction solvents and methods on the extraction efficiencies for different classes of chemical markers used for HPLC fingerprints. In the preliminary studies for the selection of extraction solvents (water, 25%, 50%, 75% and 100 % methanol aqueous solutions; 25%, 50%, 75% and 100% ethanol aqueous solutions), 50% methanol aqueous solution was found to be the most effective solvent for extracting all compounds based on the HPLC results.

Extraction methods including ultrasonication and reflux were then investigated for extraction efficiencies (methanol as the extraction solvent). The HPLC peak areas of the compounds obtained from these two techniques were comparable, and heat reflux was chosen because of high extraction efficiency. Extraction time under reflux was also investigated, and the results showed that the most compounds were extracted within 30 min, and that longer period of reflux did not increase the contents significantly. The optimal extraction conditions for *V. coloratum* used in this study are presented in detail in Section 2.5.

3.3 Methods validation

The method was validated for parameters such as precision, repeatability and stability.

The precision was determined by replicate injection of the same sample solution (No.12) for five times. The relative retention time (RRT) and relative areas (RA) of common peaks were calculated. The relative standard deviations (RSDs) of RRT and RA of common peaks for precision were found in the range of 0.1% − 0.5% and 1.4% − 5.1%, respectively.

The repeatability was determined by injection of five individual sample solutions extracted from the same sample (No.12) in the same way. RRT and RA of common peaks were calculated. The RSDs of RRT and RA were found in the range of 0.1% − 0.2% and 1.3% − 5.3%, respectively.

Stability was tested with one sample solution stored at room temperature for 0, 1, 3, 6, 12 and 24 h, and the areas of the main peaks were found to be stable within 24 h (RSD<5.0%). The RSDs of RRT and RA were found in the range of 0.1% − 1.5% and 1.2% − 4.0%, respectively.

All results indicated that the method was adequate, valid and satisfactory.

3.4 Establishment of chromatographic fingerprint and similarity evaluation

3.4.1 Source group samples analysis

To standardize the fingerprint, 37 batches of samples (source group) were analyzed with the HPLC-UV analysis procedure. Peaks which existed in all batches of samples were assigned as common peaks. It was shown that there were 18 common peaks. Homoeriodictyol-7-*O*-*β*-*D*-apiosiyl-(1→2)-*β*-*D*-glucoside was chosen to calculate RRT and RPA. HCA procedure could find natural cluster of samples according to RPA. In this case, inter-group linkage was introduced and the squared Euclidean distance was calculated. HCA result is shown in Figure 2. Depending on the distance, it was clear that the source group samples could be classified into five clusters (Cluster Ⅰ: 32 batches; Cluster Ⅱ − Ⅴ: 7 batches). The average chromatogram of the 32 batches of commercial samples was taken as the standard characteristic fingerprint.

SFDA suggests that all herbal chromatograms should be evaluated in terms of similarity by calculating the correlation coefficient and/or angle cosine value of original data. The results are listed in Table 1. The cosine and correlation coefficient values of 21 batches samples were more than 0.90 as Group Ⅰ, 11 batches of samples ranged from 0.7 to 0.9 as Group Ⅱ, and 5 batches of samples were less than 0.7 as Group Ⅲ. Relatively higher similarity values indicated the consistency of *V. coloratum* which ensured the stability of preparations. Therefore, if 0.90 is set as an appropriate threshold, it is easy to identify *V. coloratum* based on the chromatographic fingerprint. The fingerprint patterns of different samples are different, and by comparing each fingerprint pattern with the mean chromatogram, we can obtain the similarity value of each fingerprint pattern, which can help us evaluate the quality of different samples.

3.4.2 Host group samples analysis

The correlation between *V. coloratum* and its host was also investigated. The chromatogram of *V. coloratum* showed drastic differences from that of the host by direct visual inspection (Figure 3). So in order to distinguish *V. coloratum* from different hosts, the similarities between the entire chromatographic profiles of 5 batches of *V. coloratum* (hosts were clear) and the standard characteristic fingerprint were evaluated. The results suggested that the fingerprints of the *V. coloratum* samples from different hosts were similar (Table 1). The relationship among the *V. coloratum* and their host species needs to be clarified. Further studies are currently in progress in our laboratory.

4 Conclusion

A simple, reliable and accurate HPLC-UV method has been developed for the fingerprint analysis of the major compounds in *V. coloratum*. The fingerprint of the *V. coloratum* sample showing eighteen common peaks represents the characteristics of the herb's constituents and provides an accurate method for the quality control of *V. coloratum*. HCA suggested that 37 batches of source group samples could be divided into five groups, and it revealed that there was a considerable variation between chemical constituents and plant populations. Meanwhile, the cosine and correlation coefficient values of 21 batches of samples

were more than 0.90. If 0.90 is set as an appropriate threshold, it is easy to identify *V. coloratum* based on the chromatographic fingerprint. Relatively higher similarity values indicated the consistency of *V. coloratum* which ensures stability of preparations.

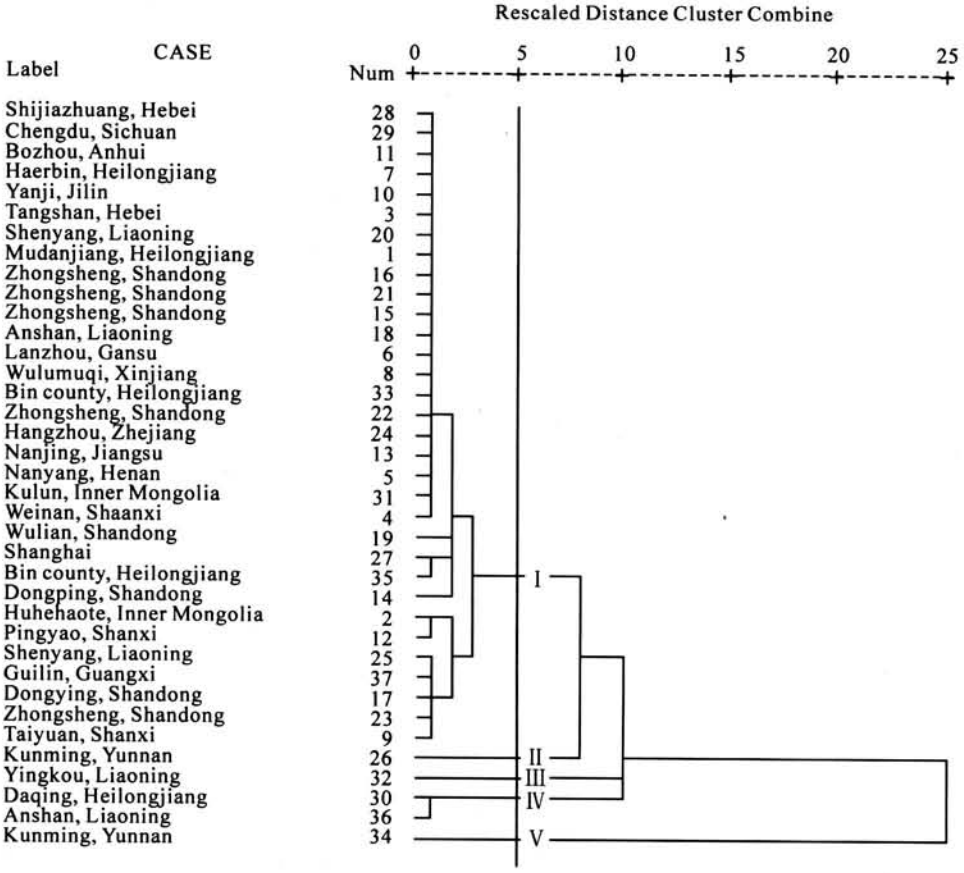

Figure 2 Results of hierarchical cluster analysis of 37 batches of *Viscum coloratum* samples from different sources

Table 1 Evaluation of the similarity of *V. coloratum* from different origins and different host species

No.	Origin	Similarity		No.	Origin	Similarity	
		Cosine	Correlation coefficient			Cosine	Correlation coefficient
1	Mudanjiang, Heilongjiang	0.99	0.99	22	Zhongsheng, Shandong	0.97	0.95
2	Huhehaote, Inner Mongolia	0.78	0.71	23	Zhongsheng, Shandong	0.91	0.87
3	Tangshan, Hebei	0.99	0.99	24	Hangzhou, Zhejiang	0.97	0.96
4	Weinan, Shaanxi	0.94	0.92	25	Shenyang, Liaoning	0.88	0.83
5	Nanyang, Henan	0.99	0.99	26	Kunming, Yunnan	0.57	0.44
6	Lanzhou, Gansu	0.98	0.97	27	Shanghai	0.90	0.86
7	Haerbin, Heilongjiang	0.99	0.99	28	Shijiazhuang, Hebei	0.99	0.98
8	Wulumuqi, Xinjiang	0.98	0.97	29	Chengdu, Sichuan	0.98	0.99
9	Taiyuan, Shanxi	0.86	0.80	30	Daqing, Heilongjiang	0.54	0.40
10	Yanji, Jilin	0.99	0.99	31	Kulun, Inner Mongolia	0.96	0.95
11	Bozhou, Anhui	0.99	1.00	32	Yingkou, Liaoning	0.68	0.52
12	Pingyao, Shanxi	0.84	0.76	33	Bin county, Heilongjiang	0.96	0.95
13	Nanjing, Jiangsu	0.96	0.94	34	Kunming, Yunnan	0.43	0.28
14	Dongping, Shandong	0.85	0.79	35	Bin county, Heilongjiang	0.84	0.76
15	Zhongsheng, Shandong	0.98	0.98	36	Anshan, Liaoning	0.51	0.37
16	Zhongsheng, Shandong	0.98	0.98	37	Guilin, Guangxi	0.87	0.81
17	Dongying, Shandong	0.85	0.78	H1	*Populus ussuriensis* Kom.	0.93	0.94
18	Anshan, Liaoning	0.97	0.96	H2	*Tilia amurensis* Rupr.	0.91	0.89
19	Wulian, Shandong	0.89	0.86	H3	*Betula platyphylla* Suk.	0.91	0.91
20	Shenyang, Liaoning	0.99	0.98	H4	*Ulmus pumila* L.	0.85	0.76
21	Zhongsheng, Shandong	0.97	0.97	H5	*Pyrus ussuriensis* Maxim	0.92	0.89

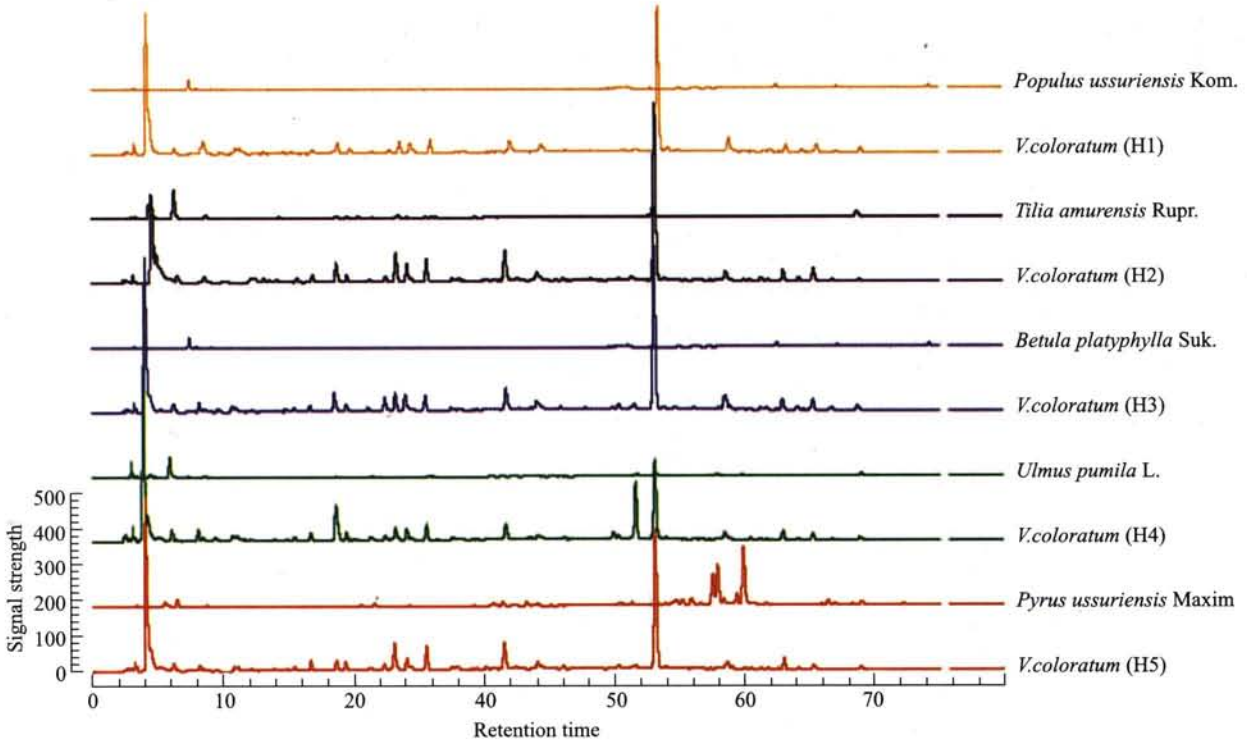

Figure 3 Chromatographic fingerprints obtained from 5 batches of *V. coloratum* from different hosts

Acknowledgments

This work was partly supported by the National Natural Science Foundation of China (No. 30901967). We are thankful to the Scientific Research Foundation for the Doctors of the Liaoning Provincial Department of Science and Technology (No. 20091078) and the Science and Technology Research Projects of the Educational Commission of Liaoning Province (No. 2009A684) for financial support. We are also thankful for the postdoctoral workstation of Pharmaland Technology Development Co., Ltd.

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