Study on HPLC Fingerprint of Dendrobium Nobile Lindl. Flower Based on Chemometrics

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Abstract. Objective: TO establish HPLC fingerprint of Dendrobium nobile Lindl. (D. nobile Lindl.) flower. Method: Agilent SB-C18 (250×4.6mm, 5μm) chromatographic column was used, with the mobile phase of acetonitrile-0.1% phosphoric acid solution, the flow rate was 0.8mL/min, the wavelength was 254nm, and column temperature was 30℃. Similarity evaluation combined with cluster analysis (CA), principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were used to evaluate the HPLC chromatographic fingerprint of 15 batches of D. nobile Lindl. flower. Result: The HPLC fingerprint of D. nobile Lindl. flower was obtained with 26 common peaks, and 3 of them were identified, including protocatechuic acid, chlorogenic acid and rutin. The similarity of 15 batches of D. nobile Lindl. flower was between 0.866 ~ 0.989. By CA, PCA and OPLS-DA analysis, the 15 batches of samples were divided into 2 categories, and 14 components contributed most to the classification were obtained. Conclusion: The method is simple and reproducible, and could provide a reference for the quality evaluation of D. nobile Lindl. flower.

1. Introduction
Dendrobium nobile Lindl. (D. nobile Lindl.), belonging to an orchid species, is native to China distributed in Guizhou, Sichuan, Guangxi, Yunnan and other places [1]. Modern studies have shown that the chemical constituents of D. nobile Lindl. are mainly polysaccharides, alkaloids and other substances, which have the effect of boosting the stomach, engendering liquid, clearing heat and nourishing Yin [2-6]. The chemical components of D. nobile Lindl. flower mainly contain volatile oils, polysaccharides and flavonoids, etc, which can lower blood pressure, anti-inflammatory, bacteriostasis, anti-aging, etc [7-8], it is often used as herbal tea or health tonical. For a long time, the research of D. nobile Lindl. mainly focused on the stem. However, the study on HPLC fingerprint of D. nobile Lindl. flower has not been reported. In order to more comprehensive to control the quality of the D. nobile Lindl. flower, this paper took the flower of D. nobile Lindl. as the research object, established the HPLC fingerprint of D. nobile Lindl. flower, and provided scientific basis for the authenticity identification and quality control of D. nobile Lindl. flower.
2. Materials and methods

2.1. Instruments and reagents

The LC-20AT (Shimadzu, Japan) of HPLC was used to test all samples. Ultrasonic cleaning machine (model: JOYN-10A, Shanghai Qiaoyue Electronic Co, Ltd.), Constant temperature water bath pot (model: DRHH-S8, Shanghai Shuangjie Experimental Equipment Co, Ltd.), electrothermal constant temperature blast drying oven (model: GZX-9070MBE, Shanghai Bosun company Co, Ltd.). The reagent was acetonitrile for the chromatographic purity, and phosphoric acid for the analytic purity, Protocatechuic acid (No.b21614), Shanghai Yuanye Biotechnology Co, Ltd.), chlorogenic acid (No.b20782, Shanghai Yuanye Biotechnology Co, Ltd.), rutin (No. b20771, Shanghai Yuanye Biotechnology Co, Ltd.) and water was pure water. In addition, all data were analyzed by the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012A). The 15 batches of samples were identified as the flower of *D. nobile* Lindl. by vice professor Ke Zhong of Guizhou University of Chinese Medicine. Specific sources are shown in table 1.

| NO. | Species | Source | Time  |
|-----|---------|--------|-------|
| S1  | *Dendrobium nobile* Lindl. flower | Wanglong Town, Chishui City, Guizhou Province | 2018.4 |
| S2  | *Dendrobium nobile* Lindl. flower | Wanglong Town, Chishui City, Guizhou Province | 2018.5 |
| S3  | *Dendrobium nobile* Lindl. flower | Wanglong Town, Chishui City, Guizhou Province | 2019.4 |
| S4  | *Dendrobium nobile* Lindl. flower | Wanglong Town, Chishui City, Guizhou Province | 2019.5 |
| S5  | *Dendrobium nobile* Lindl. flower | Wanglong Town, Chishui City, Guizhou Province | 2018.4 |
| S6  | *Dendrobium nobile* Lindl. flower | Wanglong Town, Chishui City, Guizhou Province | 2018.5 |
| S7  | *Dendrobium nobile* Lindl. flower | Wanglong Town, Chishui City, Guizhou Province | 2019.5 |
| S8  | *Dendrobium nobile* Lindl. flower | Wanglong Town, Chishui City, Guizhou Province | 2019.5 |
| S9  | *Dendrobium nobile* Lindl. flower | Changqi Town, Chishui City, Guizhou Province | 2018.4 |
| S10 | *Dendrobium nobile* Lindl. flower | Changqi Town, Chishui City, Guizhou Province | 2018.5 |
| S11 | *Dendrobium nobile* Lindl. flower | Changqi Town, Chishui City, Guizhou Province | 2019.4 |
| S12 | *Dendrobium nobile* Lindl. flower | Changqi Town, Chishui City, Guizhou Province | 2019.5 |
| S13 | *Dendrobium nobile* Lindl. flower | Xishuangbanna Autonomous Prefecture, Yunnan Province | 2019.5 |
| S14 | *Dendrobium nobile* Lindl. flower | Xishuangbanna Autonomous Prefecture, Yunnan Province | 2019.5 |
| S15 | *Dendrobium nobile* Lindl. flower | Xishuangbanna Autonomous Prefecture, Yunnan Province | 2019.5 |

2.2. Chromatographic conditions

The chromatographic column was Agilent SB-C18 (250 × 4.6mm, 5μm). The mobile phase consisted of acetonitrile (A) and water containing 0.1% phosphoric acid (B). The gradient mode was as follows:
The detection wavelength was 254 nm, the volume flow rate was 0.8 ml/min, the column temperature was 30℃, and the injection volume was 10μl.

2.3. Preparation of the standard solution
The proper amount of protocatechuic acid, chlorogenic acid and rutin were separately accurately weighed and dissolved in methanol solution. To produce the mixed standard solution containing 0.24mg/ml protocatechuic acid, 0.42 mg/ml chlorogenic acid and 0.16 mg/ml rutin.

2.4. Preparation of samples
All the samples were crushed into fine powder, accurately weighed 0.4g, put it into conical flask with 20mL methanol, ultrasonic treatment for 120min, and cooled to room temperature. Filter and dried in a water bath pot at a low temperature below 50℃. The residue was dissolved in methanol solution 2mL and moved to a 2mL volumetric flask, and methanol solution was added for constant volume, shake well, filter, take the filtrate over 0.22 micro porous filter membrane.

2.5. HPLC method validation
2.5.1. Precision test. The sample solution of D. nobile Lindl. Flower (S5) was injected for 6 times by the chromatographic method under 2.2 item, and the chromatogram was recorded. The similarity evaluation system of chromatographic fingerprint of traditional Chinese medicine (Version 2012A) was used for analysis. The results show that the RSD of retention time and peak area of each peak are less than 3.0%, indicating that the instrument has good precision.

2.5.2. Stability test. The same sample (S5) was determined by chromatographic method under 2.2 item at 0, 4, 8, 12, 24, 48, respectively, and the chromatogram was recorded. The similarity evaluation system of chromatographic fingerprint of traditional Chinese medicine (Version 2012A) was used for analysis. The results show that the RSD of retention time and peak area of each peak are less than 3.0%, suggesting that the sample was stable within 48h.

2.5.3. Repeatability test. The same six samples (S5) were prepared and determined by the method of 2.3 and 2.2 item, and the chromatogram was recorded. The similarity evaluation system of chromatographic fingerprint of traditional Chinese medicine (Version 2012A) was used for analysis. The results show that the RSD of retention time and peak area of each peak are less than 3.0%, which it showed that the method had good reproducibility.

3. Results
3.1. Establishment of fingerprint of D. nobile Lindl. flower
15 batches of D. nobile Lindl. flower were determined by chromatographic conditions under item “2.2”. The chromatographic data were processed by the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012A) for analysis, and 26 characteristic peaks with good separation effect and large peak area were obtained as common peaks. The fingerprint of D. nobile Lindl. Flower (Fig.1). Compared with the spectogram of reference substances, Peak 4 was protocatechuic acid, peak 7 was chlorogenic acid and peak 23 was rutin. Chromatogram of mixed reference substances (Fig.2) and the common pattern (Fig.3).
Figure 1. Common pattern of HPLC fingerprint of *D. nobile* Lindl. flower.

Figure 2. Chromatogram of mixed reference substances 4- protocatechuic acid 7- chlorogenic acid 23- rutin.

Figure 3. HPLC fingerprint of 15 batches of *D. nobile* Lindl. flower.
3.2. Similarity analysis
The chromatograms of 15 batches of *D. nobile* Lindl. flower samples were imported into the chromatographic fingerprint similarity evaluation system of traditional Chinese medicine (Version 2012A), and the similarity was calculated. The results are shown in Table 2. The similarity of 15 batches of *D. nobile* Lindl. flower was between 0.866 and 0.989, the similarity of the samples of S13, S14, S15 was worse than others. The similarity of other 12 batches of *D. nobile* Lindl. flower was above 0.9, showing good similarity.

| NO. | Similarity | NO. | Similarity |
|-----|------------|-----|------------|
| S1  | 0.981      | S9  | 0.988      |
| S2  | 0.988      | S10 | 0.988      |
| S3  | 0.987      | S11 | 0.987      |
| S4  | 0.987      | S12 | 0.989      |
| S5  | 0.981      | S13 | 0.868      |
| S6  | 0.982      | S14 | 0.869      |
| S7  | 0.980      | S15 | 0.866      |

3.3. Cluster analysis
The relative peak areas of 26 common peaks in 15 batches of *D. nobile* Lindl. flower were analyzed by SPSS19.0 statistical software. The square euclidean distance is selected as the measure for clustering analysis. The results are shown in Fig. 4. According to the clustering results, The 15 batches of *D. nobile* Lindl. flower can be divided into two categories: category one including S1–S12; category two including S13, S14 and S15. The result of cluster analysis was consistent with similarity analysis, which indicated that the quality of *D. nobile* Lindl. flower from the same origin was relatively uniform.

![Cluster analysis dendrogram of 15 batches D. nobile Lindl. flower.](image)

3.4. Principal component analysis
On the basis of cluster analysis, the quantified data was imported into SPSS19.0 software for principal component analysis. Eigenvalues values and contribution rate (Table 3) and Principal component load matrix (Table 4). Taking the eigenvalue greater than 1 as the extraction principle, the first three principal components were extracted, and the accumulating contribution rate was 97.547%. These three principal components can basically reflect the information of *D. nobile* Lindl. flower. Principal
Principal component 1 mainly reflects the information of chromatographic peaks No. 2, 3, 7, 13, 15, 19, 20, 21, 22, 24 and 25; Principal component 2 mainly reflects the information of chromatographic peaks No. 1, 5, 9 and 12; Principal component 3 mainly reflects the information of chromatographic peaks No. 4, 11, 14 and 23. The PCA score was calculated by IMCA-P14.0 software, and the results are shown in Figure 5. The 15 batches of *D. nobile* Lindl. flower can be divided into two categories: S13, S14 and S15 were divided into one category, the other 12 batches of samples were divided into one category, the results were consistent with cluster analysis.

### Table 3. Eigenvalues values and contribution rate.

| component | Eigenvalues | Contribution rate | Accumulating contribution rate |
|-----------|-------------|-------------------|-------------------------------|
| 1         | 17.075      | 65.673            | 65.673                        |
| 2         | 6.792       | 26.122            | 91.795                        |
| 3         | 1.495       | 5.751             | 97.547                        |

### Table 4. Principal component load matrix.

| Common peak | Principal component 1 | Principal component 2 | Principal component 3 |
|-------------|------------------------|------------------------|------------------------|
| 1           | -0.094                 | 0.967                  | 0.166                  |
| 2           | 0.991                  | -0.034                 | 0.120                  |
| 3           | 0.938                  | 0.331                  | 0.084                  |
| 4           | -0.127                 | -0.806                 | 0.510                  |
| 5           | 0.681                  | 0.723                  | 0.036                  |
| 6           | -0.830                 | 0.480                  | 0.212                  |
| 7           | 0.979                  | 0.198                  | 0.014                  |
| 8           | -0.304                 | -0.878                 | 0.362                  |
| 9           | 0.099                  | 0.981                  | 0.013                  |
| 10          | -0.771                 | 0.617                  | 0.099                  |
| 11          | -0.857                 | 0.225                  | 0.455                  |
| 12          | 0.691                  | 0.710                  | 0.091                  |
| 13          | 0.951                  | 0.111                  | 0.229                  |
| 14          | -0.752                 | 0.403                  | 0.434                  |
| 15          | 0.984                  | 0.175                  | -0.021                 |
| 16          | -0.913                 | 0.331                  | 0.203                  |
| 17          | 0.715                  | -0.666                 | 0.199                  |
| 18          | 0.770                  | 0.587                  | 0.153                  |
| 19          | 0.903                  | -0.351                 | 0.217                  |
| 20          | 0.982                  | 0.144                  | 0.111                  |
| 21          | 0.942                  | -0.034                 | 0.267                  |
| 22          | 0.989                  | 0.145                  | 0.000                  |
| 23          | -0.813                 | 0.241                  | 0.463                  |
| 24          | 0.959                  | 0.252                  | 0.026                  |
| 25          | 0.928                  | 0.023                  | 0.224                  |
| 26          | 0.839                  | -0.493                 | 0.207                  |
3.5. Orthogonal partial least squares discriminant analysis

The relative peak areas of 26 common peaks in 15 batches of *D. nobile* Lindl. flower were imported into SIMCA-P14.0 software for orthogonal partial least squares discriminant analysis. The score chart is shown in Figure 6. The 15 batches of *D. nobile* Lindl. flower can be divided into two categories, the results were consistent with cluster analysis and principal component analysis. Combined with the variable importance projection (VIP) method, and the results were shown in Figure 7. According to the criterion of VIP than 1, and 14 components contributed most to the classification were obtained. The degree of influence was peak 15 > peak 22 > peak 7 > peak 24 > peak 20 > peak 3 > peak 2 > peak 13 > peak 21 > peak 25 > peak 16 > peak 18 > peak 11 > peak 19.

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**Figure 5.** Score chart of PCA.

**Figure 6.** Score chart of OPLS-DA.
4. Conclusion

The fingerprint similarity of 15 batches of *D. nobile* Lindl. flower was between 0.866 and 0.989. Among them, the similarity of the samples of S13, S14, S15 from Yunnan Province was worse than others. The similarity of the 12 batches of *D. nobile* Lindl. flower from Guizhou Province was 0.980-0.989, all reaching above 0.9, indicating that the chemical types of the *D. nobile* Lindl. flower samples from Guizhou were basically the same. The 15 batches of *D. nobile* Lindl. flower were divided into two categories by CA and PCA analysis, category one including 12 batches of samples from Guizhou Province; category two including three batches of samples from Yunnan Province. It may be caused by the difference in ecological environment between the two places, such as temperature and climate.

Through OPLS-DA analysis, 14 components contributed most to the classification were obtained, which showed that the differences among 15 batches of *D. nobile* Lindl. flower were mainly reflected in the chromatographic peaks No. 15, 22, 7, 24, 20, 3, 2, 13, 21, 25, 16, 18, 11, 19. In this study, the HPLC fingerprint of *D. nobile* Lindl. flower was established, which can be widely used in the quality control and authenticity identification of *D. nobile* Lindl. flower.

5. Discuss

In this experiment, ethanol, 30% methanol, 50% methanol and 80% methanol were used as extraction solvents. The results showed that the extraction rate of methanol was the highest and chromatographic peaks were more. Four mobile phase systems of methanol-water, acetonitrile-water, acetonitrile-0.1% glacial acetic acid and acetonitrile-0.1% phosphoric acid were investigated. When the mobile phase system was acetonitrile-0.1% phosphoric acid, which the separation degree was better, the baseline was stable. The wavelength at 220nm, 254nm, 270nm and 330nm was investigated. There were many chromatographic peaks and the baseline was stable at 254nm. The flow rates of 0.8mL/min and 1.0mL/min were compared. When the flow rate was 0.8mL/min, and the chromatogram had good separation degree and peak shape.

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