

Quality evaluation of *Dendrobium* based on ultra-performance liquid chromatography (UPLC) and chemometrics

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ABSTRACT

Dendrobium, is one of the most important herbs in the traditional Chinese medicine, which widely distributed throughout Asia, Europe and Australia. Nowadays, the quality and safety of different *Dendrobium* species in usage are still big problems, and few studies on it. In this study, 15 important species (60 samples) of *Dendrobium* were studied based on the compounds, including total polysaccharides, total alkaloids, dendrobine and bibenzyl by ultra-performance liquid chromatography. The results showed that *D. devonianum* has the secondary top contents of polysaccharides and alkaloids among all the species, and erianin is detected only in *D. chrysotoxum*. This investigation provides scientific evidences for quality control and sustained development of *Dendrobium* plants.

INTRODUCTION

Dendrobium (Shihu), a well-known traditional Chinese medicine (TCM) and healthy food with widely using for 3000 years, is regarded as a top-grade drug according to Shen Nong's Herbal Classic. *Dendrobium* presents benefits to the stomach by producing biofluids, which enhances its Yin function and eliminates malignant heat from the human body. It is used to treat disorders such as asthenia, dim vision and retching. Besides, *Dendrobium* has been developed as a superior functional tea for eliminating lung heat and invigorating the spleen in ancient China (Li, 2004). Shihu widely distributed throughout Asia, Europe and Australia, with more than 1100 species. About 74 species and 2 variations of *Dendrobium* plants were found in China, and about 30 species are used in TCM (Pharmacopoeia Commission of Prc, 2010). Four specific *Dendrobium* species were recorded in the Pharmacopoeia of the People's Republic of

China (PPRC, 2010 version), including *D. officinale*, *D. nobile*, *D. fimbriatum* and *D. chrysotoxum*. The latter three species are called Shihu in PPRC, and *D. officinale* is called Tiepi Shihu. Nowadays, numerous *Dendrobium* species exist in medicinal markets in China, including *D. officinale*, *D. nobile*, *D. fimbriatum*, *D. chrysotoxum*, *D. chryseum*, *D. thyrsoiflorum*, *D. chrysanthum*, *D. loddigesii*, *D. devonianum*, *D. moniliforme*, etc. (Fig. 1) Recent studies have showed the big differences of chemical composition between different main origins in the same species (Li *et al.*, 2013). However, due to multiple origins, their active compounds including polysaccharides, alkaloids, dendrobine and bibenzyl were greatly varied. Although there exists the quality differences between species (Bao *et al.*, 2005), few researches have reported the differences between different species. Therefore, it is urgent to evaluate these species to ensure the safety in usage. The present work was carried out to scientifically compare the active compounds of *Dendrobium* from different origins. We collected 15 species (60 samples) of *Dendrobium* from different locations in this investigation, which are the main commercial species in China.

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We investigated the contents of bioactive compounds, including total polysaccharides, total alkaloids, dendrobine and bibenzyls, to evaluate the quality of *Dendrobium* by ultraviolet-visible spectrophotometry (UV-VIS), gas chromatography (GC), UPLC (Xu *et al.*, 2005; Huang and Li, 2007; Lu *et al.*, 2010; Xia *et al.*, 2008), and other technologies (Deng *et al.*, 2016; Chen *et al.*, 2016; Jin *et al.*, 2016). The results of this investigation are significant for scientific cultivation of *Dendrobium* with the purpose of sustained development of resources in China.

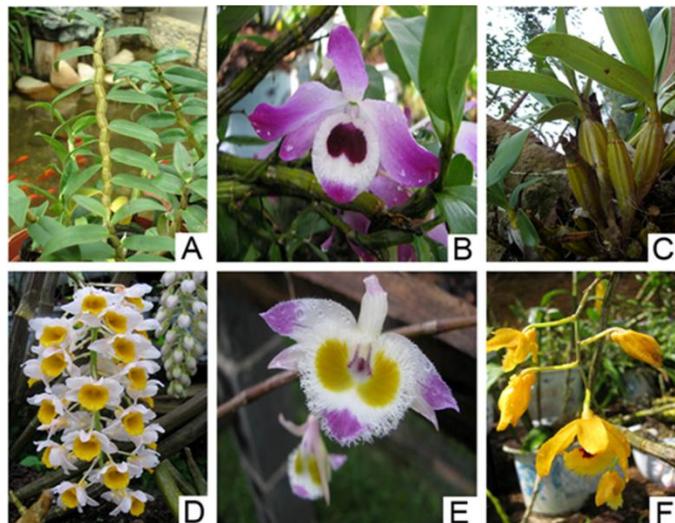


Fig. 1: The major *Dendrobium* of different species. (A: *D. officinale*; B: *D. nobile*; C: *D. chrysotoxum*; D: *D. thyrsiflorum*; E: *D. devonianum*; F: *D. fimbriatum*)

MATERIALS AND METHODS

Chemicals, reagents and materials

Waters Acquity™ UPLC-diode array detector (UPLC-DAD) (Waters Co., Milford, MA, USA); Mettler AB135-S electronic analytical balance (Mettler-Toledo, Greifensee, Zurich, Switzerland); electric thermostatic water bath (Beijing Analyst Co., Beijing, China); KQ-400KDB ultrasonic cleaner (Kunshan Co., Hangzhou, China).

Methanol and acetonitrile (chromatographic grade, Fisher Co., Fairlawn, NJ, USA); ultrapure water (Milli-Q system, 18.2 MΩ, Millipore, Bedford, MA, USA); sulfuric acid (98 %), redistilled phenol, potassium hydrogen phthalate (KHP), formic acid, methanol, NaOH, bromocresol green, absolute ethyl alcohol, petroleum ether and chloroform (analytic grade, Beijing Chemical Co., Beijing, China); dendrobine standard (>98%, Chengdu Beth Reagent Co., Sichuan, China); scoparone, gigantol, erianin and D-anhydrous dextrose standards (>98%, National Institutes for Food and Drug Control, Beijing, China).

All of the medicinal and plant materials were identified by Prof. Lin Yulin (Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) and deposited at the Herbarium of

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (Beijing, China) with 4 °C storage.

Determination of total polysaccharides and alkaloids in *Dendrobium*

Fresh stems of *Dendrobium* were processed at 105 °C for 1 h to inhibit enzymes in the samples. Then, samples were dried at 60 °C, and were pulverized (through 60-mesh sieve). Extracted with alkaline chloroform, the total alkaloids were determined with the bromocresol green colorimetry. The polysaccharides were extracted with 80% ethanol after the impurity was eliminated with petroleum ether, and determined with phenol-concentrated sulfuric acid colorimetry (Lu *et al.*, 2010).

Determination of bibenzyl in *Dendrobium* by UPLC

About 0.25 g of the sample was weighted accurately and extracted with 50 mL 80% methanol. The samples were extracted in an ultrasonic bath for 45 min; and were filtered through a 0.22 μm membrane, then were analyzed by UPLC.

Chromatographic analysis was performed using a BEH C₁₈ column (100 mm × 2.1 mm, 1.7 μm, Waters) at 230 nm by UPLC. The binary gradient elution system consisted of acetonitrile (A) and water (0.005% formic acid) (B); and separation was achieved using the following gradient: 0 min to 7 min, 5% to 40% A; 7 min to 10 min, 40% to 60% A; 10 min to 14 min, 60% to 100% A. The flow rate was 0.25 mL·min⁻¹; and the column and sample temperatures were maintained at 25 °C and 15 °C, respectively. Scoparone, gigantol, and erianin standard solutions were prepared with methanol and stored at 4 °C.

Methodological evaluation

The calibration curve, stability, precision, repeatability and recovery rate were performed to validate the above methods (Table 1). Linearity was examined using the standard solutions by calibration curves test. The calibration curves were constructed by plotting the peak area (Y) versus the concentrations (X) of the standard solutions. All calibration curves showed good linear regressions and were within the test ranges. The precision was determined by intra-day and inter-day variations through the analysis of the six replicate samples within one day and three consecutive days, respectively. Six independently prepared solutions from the same sample were analyzed for repeatability testing. In the determination of total polysaccharides and alkaloids, the stability of the samples was analyzed at 0, 10, 20, 40, 60, 80, 100 and 120 min in 4 °C storage, respectively. The results present that this experiment was stable within 2h with RSD<1.29%. In the determination of dendrobine and bibenzyl, the stability of the samples was analyzed at 0, 2, 4, 8, 12, 24, and 36 h with 4 °C storage, respectively. The sample solutions were stable within 36 h with RSD<3.64%. The average recoveries of the four compounds ranged from 93.72% to 99.07% (RSD<2.63%). These validation results indicated that the methods used in the quantitative determination of the target compounds were scientific.

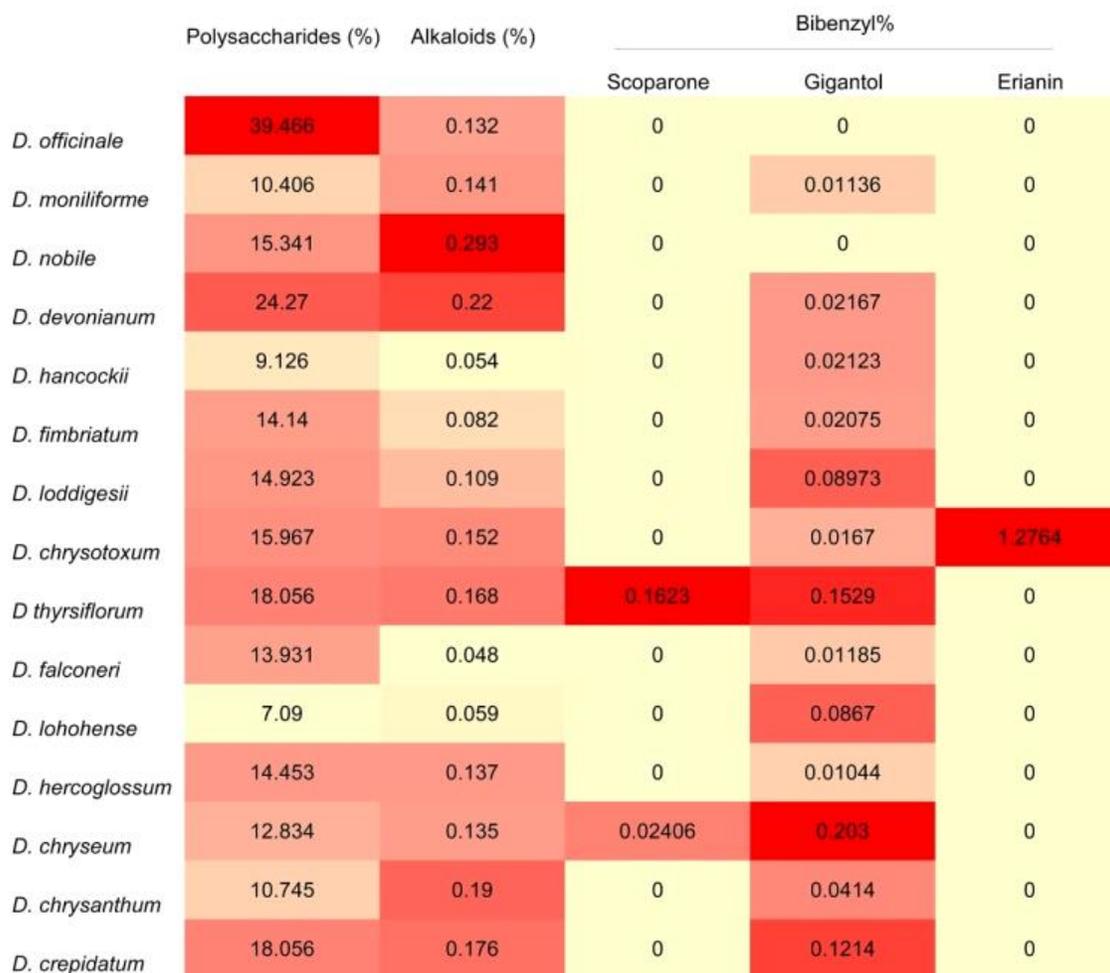
Table 1: Methodological validation.

Standard compound	Regression equation	Correlation coefficient (R ² N=6)	Linear range	Stability (RSD%) N=5	Precision (RSD%) N=5	Repeat-ability (RSD%) N=5	Recovery(N=5)	
							Mean(%)	RSD(%)
D-anhydrous dextrose	Y=0.0746x+0.0061	0.9994	0.01-0.06 mg·mL ⁻¹	0.126	2.38	3.93	99.07	1.23
Alkaloid	Y=14.568x+0.4735	0.9991	1.13-5.65 µg·mL ⁻¹	1.29	2.38	3.93	98.16	2.63
Dendrobine	Y=76414x-86.49	0.9997	0.05-0.3 mg	1.63	1.32	1.85	96.47	1.83
Scoparone	Y=46553x-12997	0.9998	2.0-350.0 ng	3.64	2.37	2.58	94.44	1.28
Gigantol	Y=42664x-19172	0.9998	5.0-285.0 ng	3.25	2.29	1.52	93.72	2.46
Erianin	Y=26366x-103254	0.9997	5.0-290.0 ng	2.86	1.60	2.33	96.35	1.39

Table 2: Environmental factors analysis based on Pearson correlation.

Species	compounds	Environmental factors										
		X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	X ₁₀	X ₁₁
<i>D. officinale</i>	Polysaccharides	0.066	0.200	0.085	0.099	0.326	-0.120	-0.291	-0.028	-0.035	0.071	0.150
<i>D. nobile</i>	Alkaloids	-0.001	-0.023	0.049	0.058	-0.098	0.344	-0.195	0.115	0.095	0.095	-0.013
	Dendrobine	-0.275	-0.468	-0.077	0.347	-0.512 ^b	-0.013	0.127	0.128	0.140	-0.201	-0.489
<i>D. chrysotoxum</i>	Gigantol	0.153	0.235	0.088	0.230	0.193	0.035	-0.273	-0.110	-0.136	0.117	0.217
	Erianin	0.820 ^a	0.703 ^b	-0.575	0.218	-0.040	0.387	-0.231	0.704 ^b	0.658	0.732 ^b	0.683 ^b

a Correlation is significant at the 0.01 level (2-tailed); b Correlation is significant at the 0.05 level (2-tailed); X₁, Active accumulative temperature; X₂, Average annual air temperature; X₃, Elevation; X₄, Relative humidity; X₅, Annual sunshine time; X₆, Annual precipitation; X₇, Soil; X₈, Maximum air temperature in July; X₉, Average air temperature in July; X₁₀, Minimum air temperature in January; X₁₁, Average air temperature in January.

**Fig. 2:** Contents of total polysaccharides, alkaloids and bibenzyl in different species of *Dendrobium*.

RESULTS AND DISCUSSION

Determination and analysis of total polysaccharides and alkaloids in *Dendrobium*

The contents of total polysaccharide and alkaloid in 15 species of *Dendrobium* are presented in Fig. 2. The results show that *D. officinale* has the highest content of total polysaccharide among all of the species; and *D. nobile* has the highest content of total alkaloids among all the species, while, *D. devonianum* has the secondary top contents both in polysaccharides and alkaloids.

Polysaccharides are one of the most important compounds in TCM, and the content of polysaccharide is the main criterion in evaluating the quality of *D. officinale* in the PPRC (Luo *et al.*, 2000; Huang *et al.*, 1996). Luo *et al.* has separated four polysaccharides with higher scavenging activity of hydroxyl radical and DPPH radical from *D. nobile* and *D. denneanum* (Luo *et al.*, 2010; Fan *et al.*, 2010; Luo *et al.*, 2009; Huang *et al.*, 2016). Wang, *et al.* has separated nine polysaccharides with higher antitumor activities against Sarcoma 180 *in vivo* and HL-60 *in vitro* (Wang *et al.*, 2009).

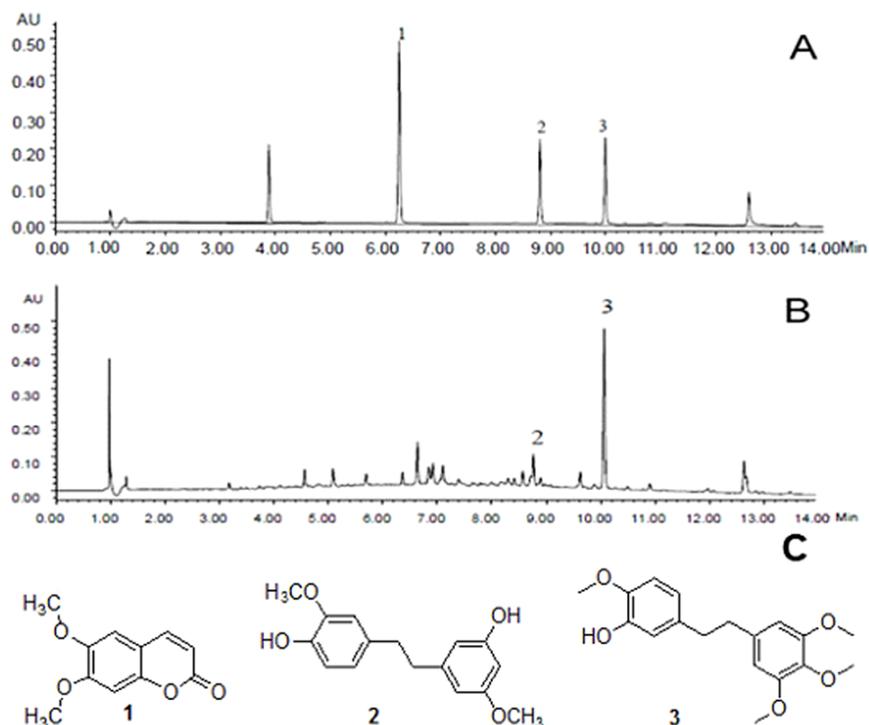


Fig. 3: UPLC chromatography and the structure of three compounds (A. UPLC chromatography of standards. B. UPLC chromatography of *D. Chrysotoxum*. C. The structure of three compounds, 1. Scoparone; 2. Giganto; 3. Erianin.)

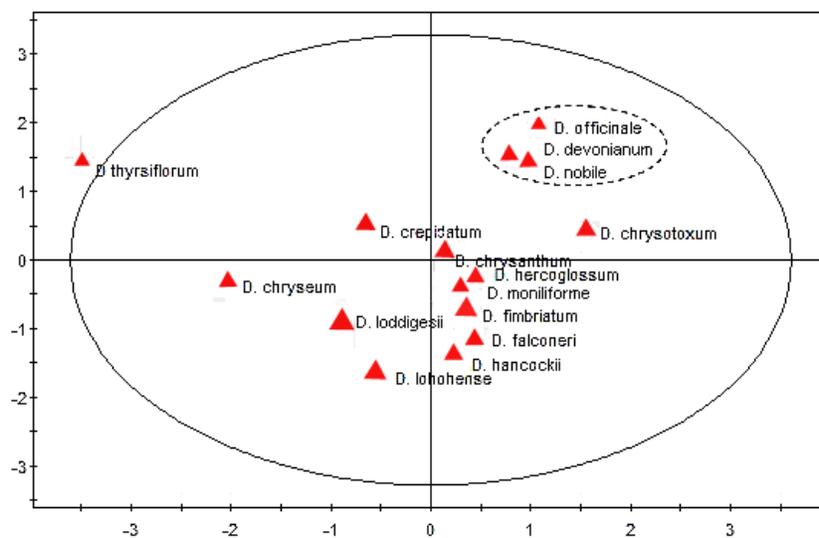


Fig. 4: Scatter diagram of *Dendrobium* species based on polysaccharides and alkaloids.

Zhao *et al.* have got a polysaccharides, named as DCLP, from *D. chrysotoxum*. And they believe that DCLP has antioxidative, hypoglycemic and immune stimulating effects *in vitro* and *in vivo* (Zhao *et al.*, 2007). Zha, *et al.* showed that polysaccharides of HPS-1B23, which was separated from *D. huoshanense* possessed potent stimulating functions on IFN- γ and TNF- α production in the culture medium of splenocytes and macrophages, respectively (Zha *et al.*, 2007). Due to the complexity of these polysaccharides in *Dendrobium*, measuring the total polysaccharides in combined with chemometrics becoming an effective way for evaluating qualities comprehensively.

Based on the content of polysaccharides, it indicates that *D. officinale* and *D. devonianum* have the higher qualities compared to other species. And it is the reason that why *D. officinale* is recorded as an official *Dendrobium*.

Alkaloids are other important compounds in *Dendrobium*. The alkaloids from *Dendrobium* presented neuroprotective effects on rat cortical neurons injured by oxygen-glucose deprivation and reperfusion (Tong *et al.*, 2016; He *et al.*, 2016; Wang *et al.*, 2010). Besides, its alkaloids have inhibitory effects on memory impairment (Li *et al.*, 2010). And alkaloids from *D. nobile* have the bioactivity of prevention on diabetic cataract in rat lens (Wei and Long, 2008).

In the determination of alkaloids, it shows that *D. nobile* has the highest content. This result indicates that *D. officinale* and *D. nobile* have the different pharmacological values.

PCA of total polysaccharides, alkaloids and bibenzyl in *Dendrobium*

PCA uses an N-dimensional vector approach to separate samples on the basis of the cumulative correlation of all metabolite data and then identifies the vector (eigenvector) that yields the greatest separation among samples without requiring prior knowledge of the data sets.

The contents of total polysaccharides, alkaloids in different species of *Dendrobium* were analyzed by PCA. As shown in Fig. 4, *D. officinale*, *D. devonianum* and *D. nobile* cluster together. It indicates the higher similarities among these three major species. Both *D. officinale* and *D. nobile* have been recorded in China Pharmacopoeia except *D. devonianum*.

This species is widely used in southwest China (Ding, 2004; Cui *et al.*, 2004), but has not attracted attentions in academic. Fig. 2 also presents that *D. devonianum* has the secondary top contents of polysaccharides and alkaloids among all the species. This result confirms the usage value of *D. devonianum* in folk. It also indicates that *D. devonianum*, *D. officinale* and *D. nobile* have significant differences with other species based on chemical components. Besides, they have genetic relationship based on morphology. In taxonomy, these three species belong to Sect *Dendrobium* (Flora of China, 1999). Therefore, it is suggested that *D. devonianum* could be recorded as an official *Dendrobium* in pharmacopoeia of China to alleviate stressing *Dendrobium* resources in markets.

Determination and analysis of bibenzyl in *Dendrobium* by UPLC

Experiments were conducted to optimize the sample preparation and chromatographic conditions. UPLC chromatographic conditions were developed to consider factors such as mobile phase, modifier and flow rate. With the optimal conditions, the analyzed peaks were well separated from each other. This newly explored chromatographic condition allows the simultaneous quantitative determination of 3 bibenzyl (scoparone, gigantol and erianin) in *Dendrobium*. The extraction investigation demonstrates that 80% methanol and 45 min ultrasonic extraction were the optimal preparation methods. The chromatographic profiling of represented sample and standard compounds are presented in Fig. 3.

15 species of *Dendrobium* were tested in this section. The determination of 3 bibenzyl in species of *Dendrobium* is shown in Fig. 2. The experiments show that only *D. chrysotoxum* contains gigantol and erianin; only *D. chryseum* and *D. thyrsoiflorum* contain scoparone and gigantol; the species including *D. moniliforme*, *D. devonianum*, *D. hancockii*, *D. fimbriatum*, *D. loddigesii*, *D. falconeri*, *D. lohohense*, *D. hercoglossum*, *D. chrysanthum* and *D. crepidatum* contain only gigantol; while *D. officinale* and *D. nobile* have no bibenzyl. Gigantol exists in all the species of *Dendrobium* except for *D. officinale* and *D. nobile*.

Erianin is detected only in *D. chrysotoxum*. *D. chrysotoxum* is a special plant among all *Dendrobium* species with the special compound--erianin. Therefore, the marker component of *D. chrysotoxum* in PPRC is erianin. Erianin can induce apoptosis in tumor cells (Li *et al.*, 2001), thus generating increased attention recent years. Confusarin and chrysotoxene, which have anti-tumor activities (Wang *et al.*, 2001; Ma *et al.*, 1994), are also found in *D. chrysotoxum*. Therefore, with the high potential in antitumor, we suggest that *D. chrysotoxum* should be recorded independently in the next edition of the PPRC. This result presents that only *D. chryseum* and *D. thyrsoiflorum* contain scoparone; and a significant difference was found between these two species. The content of scoparone in *D. chryseum* ranged from 0.13% to 0.59%, whereas that in *D. thyrsoiflorum* ranged from 0.011% to 0.027%. Scoparone exhibits vasorelaxant and immunosuppressive effects (Huang and Chu, 1994). Scoparone can induce neurite outgrowth by stimulating the upstream steps of ERK, PKA, PKC and CaMK II in PC12 cells (Yang *et al.*, 2008). Besides, *D. chryseum* and *D. thyrsoiflorum* also contained gigantol, which exists in most of the species of *Dendrobium*. In our previous investigation, we found that there is significant difference between fingerprint profiling of these species of *Dendrobium*. Therefore, *D. chryseum* and *D. thyrsoiflorum* have the medical values and they could be used in the market of medicinal *Dendrobium*.

All of the *Dendrobium* species had gigantol except for *D. officinale* and *D. nobile*. Gigantol has inhibitory abilities against the growth of human leukemia cell lines K562 and HL-60, human lung adenocarcinoma A549, human hepatoma BEL-7402, and human stomach cancer SGC-7901 (Chen *et al.*, 2008). 13 species contain gigantol and these species could not be distinguished by

gigantol only although there is a significant difference in different species and locations of *Dendrobium*. But it could be considered as the marker compound to authenticate *Dendrobium* except for *D. officinale* and *D. nobile*. The active compounds from different species or different locations of *Dendrobium* were first compared. The results showed that *D. officinale* and *D. nobile* were considered as important species because of the highest polysaccharide and alkaloid contents, respectively. *D. devonianum* contains high contents of both polysaccharides and alkaloids, as well as its production scale has expanded continuously and the potential market is huge. Therefore, *D. devonianum* is a promising resource and it could be recorded as an official *Dendrobium* and an alternative medicine for *Dendrobium*. *D. chrysotoxum* could be distinguished from 4 species recorded in PPRC for big differences with other species, including the proprietary compound—erianin, appearance and biological characteristic. Its medicinal part is fat and shaped like a drum (Gong *et al.*, 2008), in contrast, other species' are slim, Besides, *D. chrysotoxum* has the compound erianin and it can be the chemical marker, while the others don't have. Indeed, our previous study also showed that the *D. chrysotoxum* is different with other species using specific PCR molecular identification (Zheng *et al.*, 2013). Thus, we suggest that *D. chrysotoxum* should be recorded independently in the next edition of PPRC. Ecological analysis showed that sunlight hours, precipitation and temperature influence the accumulation of the main compounds in *D. officinale*, *D. nobile*, and *D. chrysotoxum*, respectively. This investigation provides valuable information and scientific evidences for the quality controlling, cultivation expanding, sustainable development of *Dendrobium* resources and variety identification.

CONCLUSIONS

D. devonianum has the secondary top contents of polysaccharides and alkaloids among all the species, which can replaced *D. officinale* in medicinal usage. Erianin is a chemical marker for identifying between different species. *D. chrysotoxum* could be distinguished from 4 species recorded in PPRC according to the differences in chemistry and molecular. Furthermore, we can make much effort on *D. chrysotoxum* due to the anti-cancer effect of erianin. This is helpful to protect the valuable *Dendrobium* resources and is the guidance of clinical reasonable use.

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