PHYTOCHEMICAL DIVERSITY OF CULTIVATED *DENDROBIUM NOBILE* LINDL. IN SOUTHWESTERN CHINA AND ITS ASSOCIATION WITH ENVIRONMENTAL CONDITIONS

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Abstract. *Dendrobium nobile* Lindl. is an important understory economic plant, which has medicinal and nutritional value. The goal of this study was to reveal the phytochemical diversity of the species and its correlation with environmental conditions using multivariate statistical analysis. All accessions were originally collected from 10 cultivated populations in Southwestern China. It was observed that the content of alkaloid, polysaccharide, flavonoid, tannin, and amino acid showed highly significant differences ($p < 0.01$) among the 10 populations. The 10 populations were classified into two groups, two subgroups and three sub-subgroups using cluster analysis. By Pearson's correlation analysis, it was found that the content of alkaloid showed significant positive correlation with latitude ($p < 0.05$), while that of tannin showed strong significant negative correlation with latitude ($p < 0.01$). Principal components and partial least squares regression analysis indicated that temperature, precipitation, sunshine and frost-free day were considered as key factors influencing chemical synthesis. Low temperature and less accumulated temperature were favorable for enrichment of alkaloid and amino acid. This study is helpful for the screening of high quality and special germplasm resources and provides guidance for the introduction and the formulation of the regional national strategic plan for artificial cultivation of *D. nobile*.

Keywords: *active ingredient contents, climatic factors, geographical factors, multivariate analysis, latitude*

Introduction

Dendrobium nobile Lindl. is an important understory economic plant, which is distributed in humid, warm and ventilated environments, and often attached to the trunk or rock in the forest with humus accumulation. *D. nobile* have been considered commonly as tonic herbs and healthy food in China and in many other southeast Asian countries, with stem being the major parts used medicinally since ancient times. They have special efficacy of moistening lung, generating body fluid, nourishing yin and clearing heat etc. (National Pharmacopoeia Committee, 2020). The modern pharmacological study suggested that the main reason for such high medicinal attribute of *D. nobile* in human health is primarily the important biochemical compound like alkaloid, polysaccharide, flavonoid, tannin and other active compound which have protecting-nerves, improving-cardiovascular function, enhancing-body immunity, anti-cataract and cancerous, reducing-blood glucose and blood lipids (Bhattacharyya et al., 2014; Lam et al., 2015; Wang et al., 2016; Zhou et al., 2017;

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Zhang et al., 2018; Song et al., 2019). Previous study also showed that the species has significant role in the treatment of various degenerative diseases (Yoon et al., 2011). Moreover, such high health care of *D. nobile* was attribute to the content of amino acid (Qu et al., 2018). *D. nobile* has both medicinal and nutritional value. As a result of this, plant of *D. nobile* has been widely artificial cultivated for meeting the need of markets in order to protect wild resource of the species. However, the phytochemical content showed difference when *D. nobile* was planted in various geographical regions. The active ingredient of medicinal plant is important index to evaluate the quality of Chinese medicinal plant (Wu et al., 2018). Moreover, its medicinal efficacy is often the result of the joint action of multiple active ingredients (Huang et al., 2008). Therefore, it is necessary to measure the content of multiple components so as to evaluate comprehensively the quality of *D. nobile*.

Phytochemical diversity is an important part of genetic diversity, including the similarity and difference within and among populations (Xiong et al., 2018). Essentially, the genetic diversity is built upon the information from whole biosphere to summarize all development, variation, similarity and difference (Nei, 1972). It may help understanding quality of resources by comparing similarities and difference of active ingredient content in medicinal plant. In previous the results have been reported that the variation of chemical component in various medicinal plant from different regions result in quality difference, for example: *Gentianae Macrophyllae Radix* (Shi et al., 2010), *Habenaria edgeworthii* (Giri et al., 2017), *Glycyrrhiza uralensis* (Li et al., 2019), and *Paris polyphylla* var. *yunnanensis* (Wang et al., 2019).

It is well known that phytochemical content of medicinal plant is affected by various environment factors (Tao et al., 2003; Cao et al., 2007). Sunshine, altitude and rainfall were considered as key factors that influenced the accumulation of chemical component in *Notopterygii Rhizoma* (Huang et al., 2013). Polysaccharide content in *Lycium barbarum* showed negative correlation with temperature and humidity, and positive correlation with sunshine and altitude (Zeng et al., 2015). Altitude and rainfall were considered as main factors that influenced the accumulation of flavonoid in *Hippophae rhamnoides* subsp. Sinensis (Su et al., 2017). Different chemical component in *Perilla frutescent* showed positively correlated with altitude, sunshine and rainfall, and negatively correlated with longitude, latitude and temperature respectively (Shen et al., 2018).

Phytochemical diversity existing among 6 natural populations of *D. nobile* present in Northeast India were assessed and the results revealed that a high degree of phytochemical variation among the populations in relation to bioclimatic and geographic locations of populations (Bhattacharyya et al., 2015). The correlation of phytochemical variation with ecological factors was analyzed in *Dendrobium officinale*, and *Dendrobium chrysotoxum* (Li et al., 2013). The results suggested that the polysaccharide content of *D. officinale* showed significant positive correlation with soil type, the content of alkaloid in *D. nobile* showed significantly positive correlated with annual precipitation, and the content of erianin in *D. chrysotoxum* was mainly affected by temperature.

Nonetheless, little is known about phytochemical variability and the association of this variability with environmental conditions in cultivated *D. nobile*. In addition, environmental factors including the accumulated temperature and extreme temperature were often ignored. Hence, in the present study, a total of accessions from 6 regions of Sichuan, Guizhou, and Yunnan provinces in southwestern China were collected, and 10

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environmental variables including accumulated temperature and extreme temperature were obtained. The characteristic of phytochemical content, environmental factors and the relationship between them were analyzed by multivariate statistical methods. The objectives of this study were (1) to assess the level of phytochemical diversity of *D. nobile*, (2) to reveal the key environmental factors affecting the accumulation of the 5 phytochemical content, (3) to evaluate the utility of systematic evaluation method based on multivariate statistical analysis.

Materials and methods

Plant materials

In this study, all accessions of *D. nobile* were originally collected from 10 imitation-wild cultivated populations located in 6 different regions of Sichuan, Guizhou and Yunnan provinces of Southwestern China (*Fig. 1*). The GPS coordinates of the 10 populations, the date and numbers of the sampling, and the national meteorological station in six regions were recorded in *Table 1*. The 2-year-old stems which were healthy and disease-free were harvested from plant and then they were taken back to the laboratory. The species collected was authenticated by authors.

Leaves, membrane sheaths were removed and the stems were washed with running tap water. The surface moisture of tissue was dried at room temperature, then the stems were sliced and dried (constant temperature drying oven, 60°C), finally the samples were powdered using small high-speed powder machine respectively. Powdery samples of *D. nobile* whose size should be less than 60 mesh were stored in the refrigerator at 4°C until being used for phytochemical profiling.

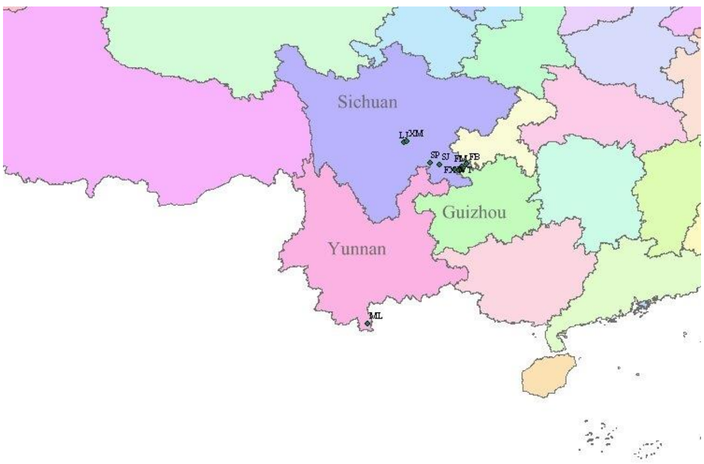

Figure 1. *A map indicating the location of the sampling sites*

Determination of phytochemical content

In this study, the main instruments included DHG-9030 Electric Thermostaticdrying Oven (Shanghai Yihen, China), JS703C Analytical Balance (Mettler-Toledo, Switzerland), A91 Gas Chromatograph (Changzhou Pannuo, China), uv-2550 Ultraviolet Spectrophotometer (Shimadzu, Japan) and S433D ProteinSequencer (Sykam, Germany).

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Table 1. *Geographical location of the 10 populations of D. nobile*

No.	Code	Location	Sample size	Harvest time	GPS coordinate		Altitude (m)	Region of the national meteorological station
					Longitude	Latitude		
1	WL	Wanglong, Chishui, Guizhou	11	2019.05.15 2020.08.01	105.876729	28.513397	536	Chishui city
2	FX	Fuxing, Chishui, Guizhou	10	2019.05.16 2020.08.01	105.764249	28.450668	388	
3	WT	Wutong, Hejiang, Sichuan	13	2019.05.17 2020.07.31	105.563139	28.511375	377	Hejiang county
4	FM	Fengming, Hejiang, Sichuan	13	2019.05.18 2020.07.29	105.882899	28.651125	430	
5	FB	Fubao, Hejiang, Sichuan	10	2019.05.19 2020.07.30	106.114938	28.741050	579	
6	SP	Sipo, Yibin, Sichuan	10	2019.05.25 2020.05.30	104.446465	28.808419	407	Yibin city
7	SJ	Songjia, Yibin, Sichuan	10	2019.09.14 2020.08.05	104.852593	28.735028	368	
8	LJ	Liujiang Hongya, Sichuan	10	2019.06.04 2020.05.17	103.244297	29.725433	615	Hongya county,
9	XM	Xiema, Jiajiang, Sichuan	12	2020.04.15 2020.05.16	103.376492	29.781799	465	Jiajiang county
10	ML	Mengla, Yunnan	10	2019.08.28 2020.09.01	101.578611	21.496388	640	Mengla county

Total alkaloid content in *D. nobile* was determined by gas chromatography method (Li et al., 2018). Alkaloid reference substance was purchased from National Institute for Food and Drug Control (Lot: 111876-201704). Quantification for total alkaloid content was calculated on the basis of alkaloid reference substance standard curves prepared, the equation of regression was $y = 2.0423x - 12.487$, $R^2 = 0.9998$, where y was the chromatographic peak area and x was the alkaloid content, with good linear relationship.

Total polysaccharide content in *D. nobile* was determined by phenol-sulfuric acid method (National Pharmacopoeia Commission, 2020). Reference substance was d-anhydrous glucose (Lot: G7528, Sigma-Aldrich, St Louis, MO, USA). Quantification for total polysaccharide content was done on the basis of d - anhydrous glucose standard curves prepared, the equation of regression was: $y = 0.006x + 0.0634$, $R^2 = 1$, where y was the absorbance, x was the concentration of glucose and the absorbance at 490 nm.

Total flavonoid content in *D. nobile* was determined by method described by Li et al. (2018). Reference substance was rutin (Lot: CAJ3545, Wako Pure Chemical Industries, Ltd, Japan). Quantification for total flavonoid content was calculated on the basis of rutin standard curves prepared, the regression equation was: $y = 0.0322x - 0.0059$, $R^2 = 0.9997$, where y was the absorbance, x was the concentration and the absorbance at 420 nm.

Total tannin content in *D. nobile* was determined according to the method described by Chen et al. (2013). Reference substance was gallic acid (Lot: BCBS2938v, Sigma-Aldrich, St Louis, MO, USA). Quantification for total tannin content was done on the basis of gallic acid standard curves prepared, the regression equation was: $y = 4.6196x + 0.0632$, $R^2 = 0.9985$, where y was the absorbance, x was the concentration and the absorbance at 760 nm. Firstly, the content of total and not adsorbed phenol was calculated respectively, then tannin content = amount of total phenol - amount of phenol that was not adsorbed.

Total amino acid content was conducted by method (National Health and Family Planning Commission of the People´s Republic of China and National Food and Medical Products Administration, 2016). Instrument chromatographic conditions: chromatographic column: 150 mm*4.6 mm cationic resin, detection wavelength: 570 mm, 440 mm, eluent flow: 0.45 ml/min, reaction solution flow: 0.25 ml/min, column temperature: 57-74℃ gradient temperature, reaction temperature: 130℃. An appropriate amount of sample was taken, hydrochloric acid was added at 10 ml of 6 mol/L, and hydrolyzed at 110℃ for 22–24 h after exhausting the air, a certain amount of hydrolysate was taken after constant volume filtration, hydrochloric acid was removed in vacuum. Finally, it was dissolved with 0.02 mol/L hydrochloric acid, and was test on the machine after constant volume. All parameters of the 5 phytochemicals were calculated as $mg \cdot g^{-1}$ of the dry weight.

Collection data of environmental factors

To evaluate the importance of environmental factors on the phytochemical variation of *D. nobile*, the geographical information of the 10 different populations were recorded by GPS (G138BD, UniStrong, Beijing), including longitude, latitude, altitude during samples were collected.

All of the 10 climate variables (annual mean temperature, annual mean maximum temperature, annual mean minimum temperature, annual extreme maximum temperature, annual extreme minimum temperature, annual mean relative humidity, annual precipitation, annual sunshine hour, frost-free day, and active accumulated temperature ≥ 10℃) between 2007 and 2021 were obtained from the national meteorological station in six regions, including Chishui city, Hejiang county, Yibin city, Hongya county, Jiaqiang county and Mengla county.

Statistical analysis

Analysis of phytochemical was conducted using ten biological replications for each population. All of the information related to phytochemical data and environmental factors were processed using Microsoft Excel 2019 software and then multivariate analysis was run by DPS (Version18.10) data processing software. Descriptive statistical analysis was calculated to obtain the mean value ± standard deviation and coefficient of variation (CV). The original data of various variables were standardized prior to analysis. Multiple comparisons were done by Ducan’s multiple range test (DMRT). Analysis of variance (ANOVA) was performed with a complete randomized block design model to test the significance of differences within and among the populations based on phytochemical content. Simpson (*D*) and Shannon-Wiener diversity index (*H´*) were computed to evaluate the genetic diversity of *D. nobile* among the 10 populations based on phytochemical content. A cluster analysis was conducted by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to evaluate the phytochemical similarity of the 10 sampled populations. All climatic factors above mentioned were subjected to a Principal Components Analysis (PCA) to select the variables that characterize the regions. The matrix obtained was subjected to ordination techniques to derive the characteristic Eigen-values, contribution rate, cumulative contribution rate and Eigen-vectors in principal components. The climatic variable plot of PCA was graphed, which reflected the relationship of different climatic regions and the bi-dimensional space. The correlation of geographical factors with the 5

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phytochemical contents was assessed using Pearson's correlation analysis. Eventually, partial least squares regression (PLSR) was performed to estimate the association between 5 phytochemical contents and climatic factors using SIMCA version 14.1 software (Umetrics, MKS Instruments Inc., Umea, Sweden).

Results

Variation of phytochemical content

In this study, the content of total alkaloid, total polysaccharide, total flavonoid, total tannin, and total amino acid in *D. nobile* was determined. The results revealed a wide range of phytochemical variation among the 10 imitation-wild cultivated populations of *D. nobile* (*Table 2*). Analysis of variance showed highly significant difference in terms of total tannin content (P < 0.01) within populations. Additionally, highly significant differences (P < 0.01) were observed in the 5 active ingredient contents among the 10 populations. It implied that phytochemical had higher diversity. In addition, the results showed that the highest content of total alkaloid (5.89 $mg \cdot g^{-1}$), total polysaccharide (76.55 $mg \cdot g^{-1}$), total flavonoid (2.52 $mg \cdot g^{-1}$), total tannin (4.87 $mg \cdot g^{-1}$), and total amino acid (63.99 $mg \cdot g^{-1}$) was found in XM, LJ, SP, ML and SJ population respectively. In contrast, the lowest value of which was measured in ML (3.28 $mg \cdot g^{-1}$), SP (12.75 $mg \cdot g^{-1}$), XM (1.34 $mg \cdot g^{-1}$), LJ (4.01 $mg \cdot g^{-1}$), and ML (9.69 $mg \cdot g^{-1}$) population respectively.

Table 2. *Assessment of 5 phytochemical contents in the 10 populations of D. nobile*

Populations	TAC ($mg \cdot g^{-1}$, DW)	TPC ($mg \cdot g^{-1}$, DW)	TFC ($mg \cdot g^{-1}$, DW)	TTC ($mg \cdot g^{-1}$, DW)	TAAC ($mg \cdot g^{-1}$, DW)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
WL	4.90 ± 0.69ABC	68.58 ± 12.12ABC	1.44 ± 0.38D	4.20 ± 0.55B	20.16 ± 4.27D
FX	5.39 ± 1.09ABC	37.94 ± 7.38D	1.67 ± 0.37CD	4.13 ± 0.30B	31.09 ± 2.28BC
WT	4.29 ± 0.47BCD	57.19 ± 5.85C	2.38 ± 0.60AB	4.05 ± 0.29B	22.83 ± 0.81D
FM	5.14 ± 0.61ABC	66.46 ± 10.83ABC	1.89 ± 0.29BC	4.27 ± 0.37B	29.92 ± 4.15C
FB	5.64 ± 0.53AB	72.49 ± 14.38AB	1.73 ± 0.34CD	4.31 ± 0.30B	34.61 ± 3.94B
SP	4.05 ± 0.83CD	12.75 ± 2.35F	2.52 ± 0.18A	4.27 ± 0.11B	34.86 ± 3.50B
SJ	4.90 ± 0.92ABC	25.23 ± 3.86E	2.36 ± 0.35AB	4.26 ± 0.15B	63.99 ± 8.73A
LJ	5.37 ± 0.99ABC	76.55 ± 21.62A	1.74 ± 0.46CD	4.01 ± 0.41B	35.43 ± 1.46B
XM	5.89 ± 1.48A	58.72 ± 13.31BC	1.34 ± 0.33D	4.08 ± 0.53B	28.47 ± 1.85C
ML	3.28 ± 0.92D	35.55 ± 9.73D	2.44 ± 0.29A	4.87 ± 0.53A	9.69 ± 1.47E
Mean	4.88 ± 0.85	51.15 ± 10.14	1.95 ± 0.36	4.24 ± 0.35	31.11 ± 3.29
F (within population)	0.702	2.147	1.053	3.141**	1.288
F (among population)	8.176**	58.724**	13.308**	4.686**	159.973**

TAC, total alkaloid content; TPC, total polysaccharide content; TFC, total flavonoid content; TTC, total tannin content; TAAC, total amino acid content; DW, dry weight
Different capitals in the same column indicate the significant difference at 0.01 levels
F value: **, P < 0.01

In the meantime, the investigations also revealed a wild range of phytochemical variation among the 6 different regions of *D. nobile* (*Fig. 2*). Chishui, Hejiang, Hongya and Jiajiang regions showed a higher amount of total alkaloid content (>5.00 $mg \cdot g^{-1}$)

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and total polysaccharide content (>50.00 mg·g^{-1}). Among other regions, representatives from Yibin showed a higher mean value of total amino acid content (49.22 mg·g^{-1}), while from Mengla showed a higher amount of total tannin content and a less amount of total alkaloid content.

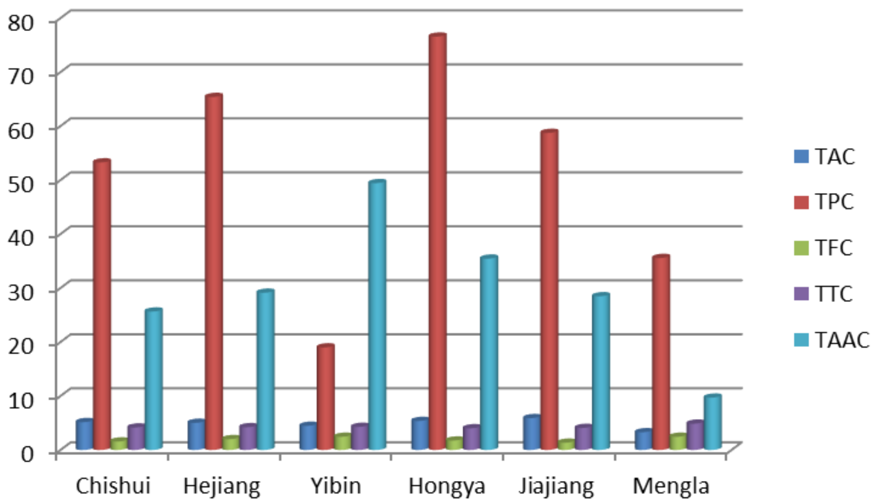

Figure 2. *Assessment of 5 phytochemical contents in the different regions of D. nobile (TAC, total alkaloid content, mg·g^{-1}; TPC, total polysaccharide content, mg·g^{-1}; TFC, total flavonoid content, mg·g^{-1}; TTC, total tannin content, mg·g^{-1}; TAAC, total amino acid content mg·g^{-1})*

Analysis of CV

CV is an important index to reflect the degree of variation. The CV of 5 phytochemical contents presented in *Table 3*. The findings showed that the largest mean value of CV of 5 phytochemical contents was obtained in ML population (18.69%), while the smallest was found in WT population (11.38%). In general, the average values of CV of the 10 populations were greater than 10%, which also implied the high phytochemical diversity. Moreover, for different phytochemical, the CV of total polysaccharide content was the largest (19.55%); followed by that of total flavonoids content (19.27%), total alkaloid content (17.74%), and total amino acid content (10.68%), all of them were greater than 10%; but that of tannin content was the smallest (8.34%), which was less than 10%. It indicated that the content of tannin was relatively stable among different populations. Basing on the above analysis, the mean value of CV of 5 phytochemical contents in the 10 populations was 15.12%, it showed that the abundant variation was relatively existed.

Analysis of diversity index

D and *H′* were calculated based on active ingredient contents to compare diversity among the 10 populations of *D. nobile*. As shown in *Table 4*, the results showed that the basic consistency of diversity index of 5 phytochemicals was found among the 10 populations. For *H′*, all of values were greater than 2.0, with mean value of 2.290. For *D*, the mean values ranged from 0.899 to 0.948 among different phytochemicals, and that from 0.915 to 0.920 among different populations, with mean value of 0.917. It suggested that the same level of diversity was observed not only among different active ingredient contents but also among different populations in this study.

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Table 3. *CV of 5 phytochemical contents in the 10 populations of D. nobile*

Populations	TAC	TPC	TFC	TTC	T AAC	Mean (%)
	CV (%)	CV (%)	CV (%)	CV (%)	CV (%)	
WL	14.05	17.68	26.31	13.14	21.20	18.48
FX	20.16	19.46	22.05	7.34	7.32	15.27
WT	10.89	10.22	25.07	7.21	3.53	11.38
FM	11.92	16.29	15.13	8.67	13.88	13.18
FB	9.37	19.83	19.46	6.97	11.39	13.40
SP	20.50	18.46	6.97	2.49	10.03	11.69
SJ	18.85	15.28	14.93	3.51	13.64	13.24
LJ	18.47	28.24	26.40	10.25	4.12	17.50
XM	25.14	22.67	24.49	12.97	6.50	18.35
ML	28.08	27.37	11.94	10.83	15.21	18.69
Mean (%)	17.74	19.55	19.27	8.34	10.68	15.12

TAC, total alkaloid content; TPC, total polysaccharide content; TFC, total flavonoid content; TTC, total tannin content; TAAC, total amino acid content

Table 4. *D and H′ of the 10 populations based on 5 phytochemical contents*

Populations	***D***					Mean
	TAC	TPC	TFC	TTC	TAAC	
WL	0.917	0.899	0.961	0.920	0.900	0.919
FX	0.913	0.899	0.953	0.922	0.902	0.918
WT	0.920	0.901	0.934	0.922	0.904	0.916
FM	0.917	0.899	0.948	0.921	0.901	0.917
FB	0.915	0.898	0.952	0.921	0.901	0.917
SP	0.919	0.904	0.937	0.922	0.902	0.917
SJ	0.916	0.901	0.938	0.922	0.900	0.915
LJ	0.914	0.894	0.948	0.922	0.902	0.916
XM	0.910	0.897	0.967	0.921	0.903	0.920
ML	0.921	0.896	0.937	0.918	0.907	0.916
Mean	0.916	0.899	0.948	0.921	0.902	0.917
Populations	***H′***					**Mean**
	TAC	**TPC**	**TFC**	**TTC**	**TAAC**	
WL	2.294	2.287	2.272	2.295	2.282	2.286
FX	2.284	2.285	2.280	2.300	2.300	2.290
WT	2.297	2.298	2.273	2.300	2.302	2.294
FM	2.296	2.291	2.292	2.299	2.294	2.294
FB	2.299	2.284	2.285	2.300	2.297	2.293
SP	2.284	2.287	2.300	2.302	2.298	2.294
SJ	2.286	2.292	2.293	2.302	2.295	2.294
LJ	2.288	2.266	2.271	2.298	2.302	2.285
XM	2.272	2.281	2.272	2.295	2.301	2.284
ML	2.267	2.268	2.296	2.297	2.292	2.284
Mean	2.287	2.284	2.283	2.299	2.296	2.290

D, Simpson diversity index; H′, Shannon-Wiener diversity index; TAC, total alkaloid content; TPC, total polysaccharide content; TFC, total flavonoid content; TTC, total tannin content; TAAC, total amino acid content

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Analysis of cluster

UPGMA dendrogram based on the 5 phytochemical contents was constructed using the Euclidian distances. According to the dendrogram, the 10 populations were divided into two main groups at D = 4.0 (*Fig. 3*), named group I and group II. Group I consisted of only one ML population, which was highly separated from the others. Group II was the largest group, consisting of the rest of the populations and could be further divided into two subgroups at D = 3.0, named IIa and IIb. The subgroup IIa consisted of two populations of SJ and SP. The subgroup IIb could be further classified into three distinct sub-subgroups at D = 2.0, of which sub-subgroup IIb1, with also only one WT population; sub-subgroup IIb2 with 6 populations consisting of XM, FX, LJ, FB, FM, and WL.

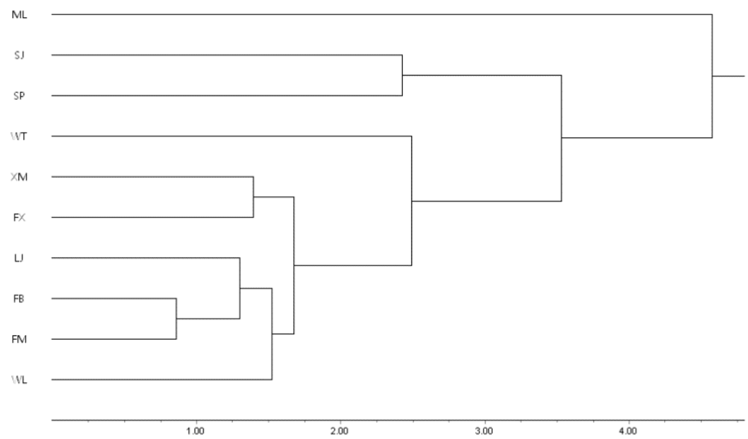

Figure 3. *UPGMA dendrogram of the 10 populations based on 5 phytochemical contents*

Characterization of climate

In this study, mean value, standard deviation of 10 climatic factors from 6 different regions were conducted by DPS software and visualized in *Figure 4*. As shown in *Figure 4*, annual mean temperature changed from 17.00°C to 22.16°C, with an average of 18.68°C; the range of annual mean maximum temperature was 21.14°C–30.0°C, with an average of 23.42°C; annual mean minimum temperature ranged between 14.20°C and 18.13°C, with an average of 15.68°C; annual extreme maximum temperature ranged from 36.09°C to 40.53°C, with an average of 38.22°C; the range of annual extreme minimum temperature was - 0.91°C–5.84°C, with an average of 1.25°C; annual mean relative humidity ranged between 76.90% and 82.10%, with an average of 79.65%; the range of annual precipitation was 157.58–1482.54 mm, with an average of 1265.10 mm; annual sunshine hour varied from 965.86 h to 2053.56 h, with an average of 1237.19 h; frost-free day ranged from 325.5 d and 365.3 d, with an average of 355.15 d; active accumulated temperature ≥10°C ranged between 5621.52°C and 8089.46°C, with an average of 6398.25°C. In the 10 climate variables, it showed that the largest value of CV was observed in annual extreme minimum temperature (100%), whereas the lowest value of that was found in frost-free day (2.27%).

PCA analysis of climatic variables

The composition matrix, eigenvalue, contribution rate, and cumulative contribution rate of the principal components were obtained by use of PCA based on 10 climate

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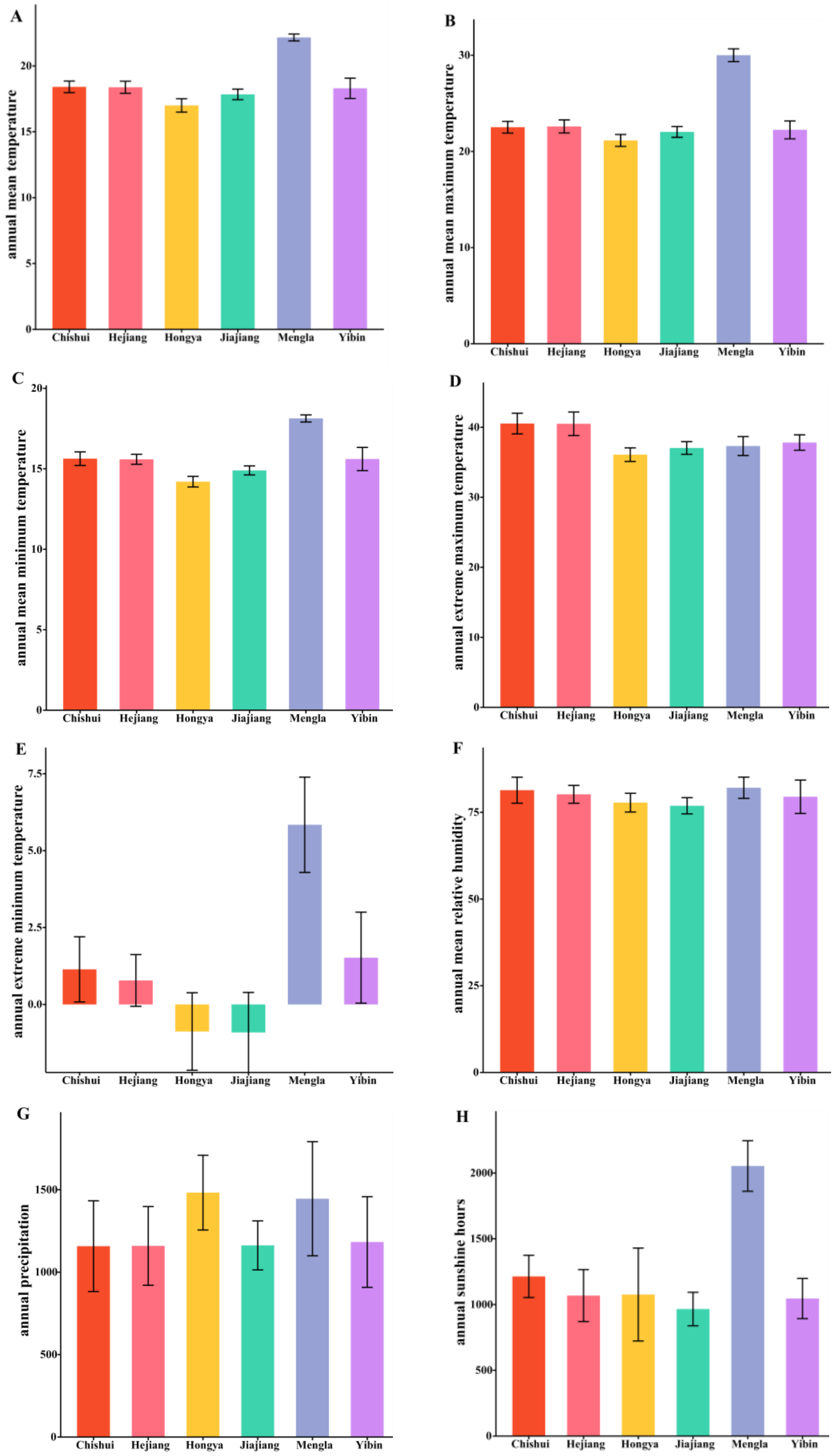
variables in 6 regions, in order to determine the principal variables in all climate variables. As was shown in *Table 5,* the contribution rates of the first three eigenvalues were 67.854%, 18.894% and 11.182% respectively, and the cumulative contribution rate was up to 97.930%. Moreover, the individual eigenvalues were all greater than 1.0. Therefore, the first three components could be analyzed to get the load level of each variable and the component matrix.



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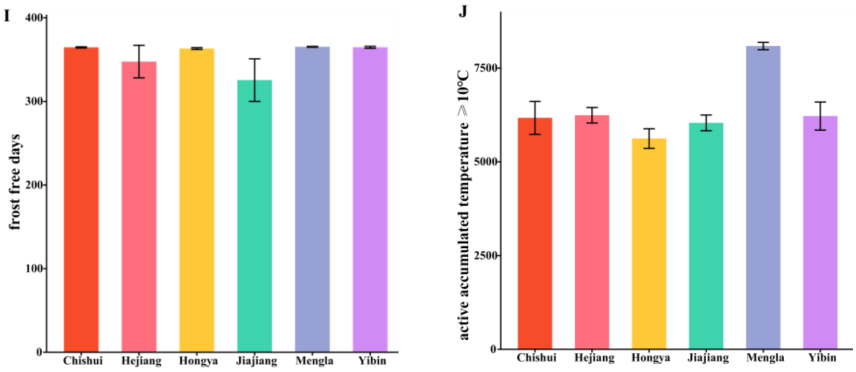

***Figure 4.** Mean ± error bar (standard error) plot of climatic factors in different regions. A, Annual mean temperature (°C) (17.00°C–22.16°C); B, Annual mean maximum temperature (°C) (21.14°C–30.0°C); C, Annual mean minimum temperature (°C) (14.20°C–18.13°C); D, Annual extreme maximum temperature (°C) (36.09°C–40.53°C); E, Annual extreme minimum temperature (°C) (- 0.91°C–5.84°C); F, Annual mean relative humidity (%)(76.90%–82.10%); G, Annual precipitation (mm) (157.58–1482.54 mm); H, Annual sunshine hour (h) (965.86–2053.56 h); I, Frost-free day (d) (325.5–365.3 d); J, Active accumulated temperature ≥10°C (5621.52°C–8089.46°C)*

***Table 5.** Matrix, eigenvalues and cumulative contribution rates of PCA*

Climate factors	Principal component 1	Principal component 2	Principal component 3
Annual mean temperature	0.376	0.014	-0.193
Annual mean maximum temperature	0.373	-0.082	-0.190
Annual mean minimum temperature	0.373	0.103	-0.157
Annual extreme maximum temperature	0.011	0.701	0.126
Annual extreme minimum temperature	0.380	0.047	0.002
Annual mean relative humidity	0.312	0.354	0.292
Annual precipitation	0.159	-0.589	0.303
Annual sunshine hour	0.374	-0.123	-0.021
Frost-free day	0.185	-0.020	0.813
Active accumulated temperature ≥10°C	0.373	-0.018	-0.219
Eigenvalues	6.785	1.889	1.118
Contribution rates (%)	67.854	18.894	11.182
Cumulative contribution rates (%)	67.854	86.748	97.930

In the first principal component, the influence variables were: annual mean temperature, annual mean maximum temperature, annual mean minimum temperature, annual extreme minimum temperature, annual mean relative humidity, annual sunshine hour and active accumulated temperature ≥10°C. Their factor loadings were: 0.376, 0.373, 0.373, 0.380, 0.312, 0.374 and 0.373 respectively. The factor loadings were similar and had little difference. In the second principal component, the most influential variables were annual extreme maximum temperature, annual mean relative humidity and annual precipitation, and their factor loadings were 0.701, 0.354 and - 0.589 respectively. The factor loadings of annual extreme maximum temperature and annual

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mean relative humidity were positive, and the factor loading of annual precipitation was negative. For the third principal component, the climate variable that had the greatest impact was: annual frost-free day, whose factor loading was the most prominent, with a maximum of 0.813, followed by annual precipitation, whose factor loading was 0.303. According to the results, all the 10 climate variables were included in the three principal components. Therefore, all of the 10 meteorological variables were used for subsequent PLSR analysis.

Pearson's correlation analysis

Pearson's correlation analysis was carried out between the five phytochemical contents and three geographical factors, elucidating the relationships between two of them. As detailed in *Table 6*, the content of total alkaloid showed significant positive correlation with latitude ($p < 0.05$), indicating that the content of total alkaloid increase with the increase of latitude, while the content of total tannin showed strong significant negative correlation with latitude ($p < 0.01$), implying that the lower the latitude, the higher the content of total tannin. The content of total polysaccharide, total flavonoid, and total amino acid showed no significant correlation with longitude and altitude (368–640 m).

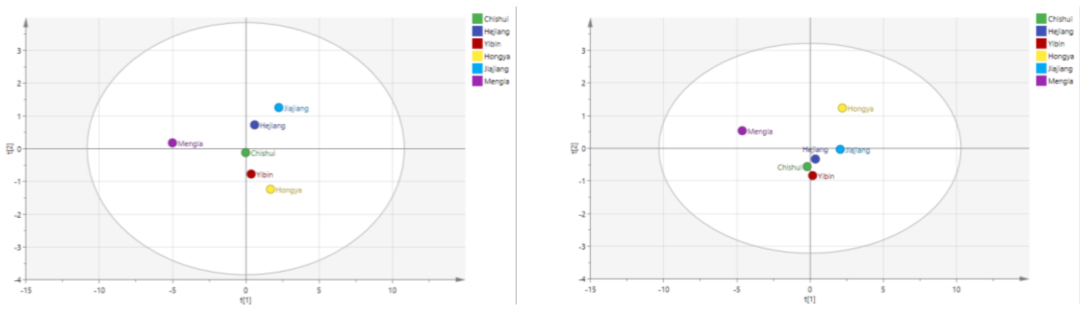
Table 6. *Correlation coefficients between geographical factors and 5 phytochemical contents*

Geographical factors	TAC	TPC	TFC	TTC	TAAC
Longitude	0.424	0.228	-0.236	-0.489	0.271
Latitude	0.759*	0.300	-0.451	-0.927**	0.534
Altitude	-0.092	0.464	-0.232	0.436	-0.466

TAC, total alkaloid content; TPC, total polysaccharide content; TFC, total flavonoid content; TTC, total tannin content; TAAC, total amino acid content; *P < 0.05; **P < 0.01

PLSR analysis

PLSR was carried out to analyze the association between phytochemical contents of *D. nobile* and 10 climatic factors. As shown in *Figure 5*, the score scatter plot indicated that the 6 regions were scattered within the 95% confidence interval (Hotelling's T-squared ellipse), and values of R2 > 0.5, implying selected samples meet the modeling requirements and the quality of samples could be guaranteed.



a **b**

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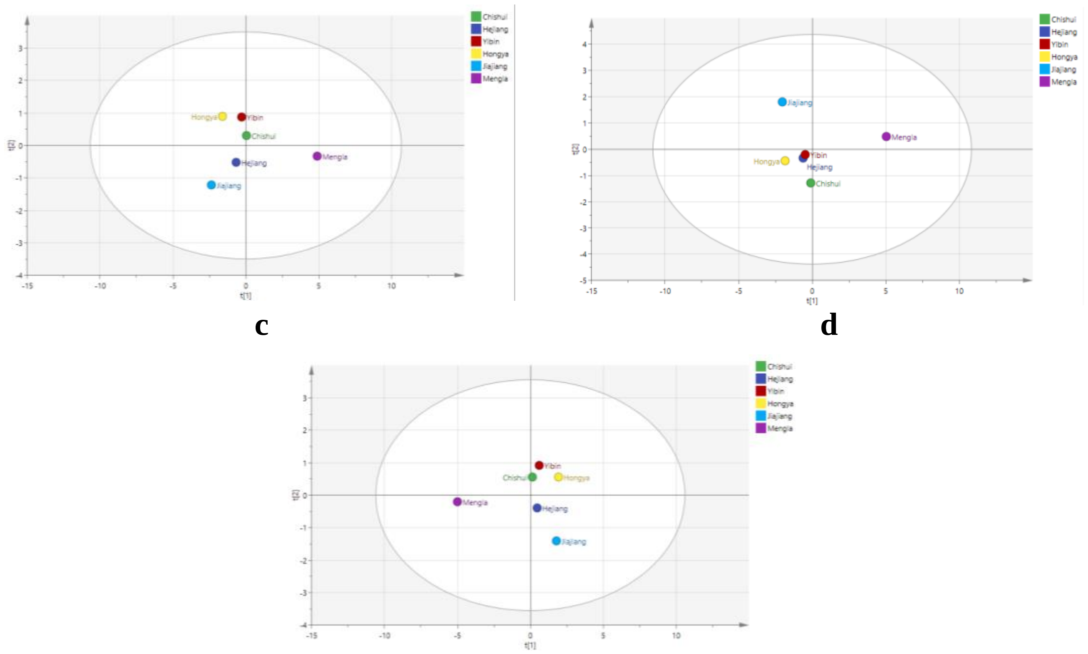

c d

e

Figure 5. *Score scatter plot of the 6 regions. (a) Total alkaloid content; (b) Total polysaccharide content; (c) Total flavonoid content; (d) Total tannin content; (e) Total amino acid content*

The typical outputs of Variable-Importance-in-the-Projection (VIP) and regression coefficients in each PLSR model were presented in *Figure 6*. A VIP score above 1.0 indicated a significant contribution of variables to the relationship between them and compound contents (Carrascal et al., 2009). Combining the VIP value with the regression coefficient, annual extreme minimum temperature (1.226), annual mean minimum temperature (1.146), annual mean temperature (1.133), and active accumulated temperature ≥ 10°C (1.129) were determined as key factors affecting content of total alkaloid and negatively associated between them. Annual precipitation (1.856), annual sunshine hour (1.056), and annual extreme minimum temperature (1.049) were considered as important factors affecting the content of total polysaccharides, among them, annual precipitation, annual sunshine hour showed positive associated with that, while annual extreme minimum temperature showed negative associated with that. Annual frost-free day (1.487), annual extreme minimum temperature (1.213) and annual mean minimum temperature (1.036) showed strong contribution to the enrichment of flavonoid, all of them showed positive relationship. Active accumulated temperature ≥10°C (1.218), annual mean temperature (1.214), annual extreme minimum temperature (1.204), annual mean minimum temperature (1.203), annual mean maximum temperature (1.203), and annual sunshine hour (1.158) were considered as main factors influencing the content of tannin, moreover, all of these variables showed positive correlated with the total tannin content. Annual frost-free day (1.446), annual sunshine hour (1.163), annual mean maximum temperature (1.134), annual mean temperature (1.062), active accumulated temperature ≥10°C (1.052) were considered as important factors affecting the synthesis of amino acid, among which,

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annual frost-free day showed positive correlation with the total amino acid content, while the four remain variables showed negative correlation with that.

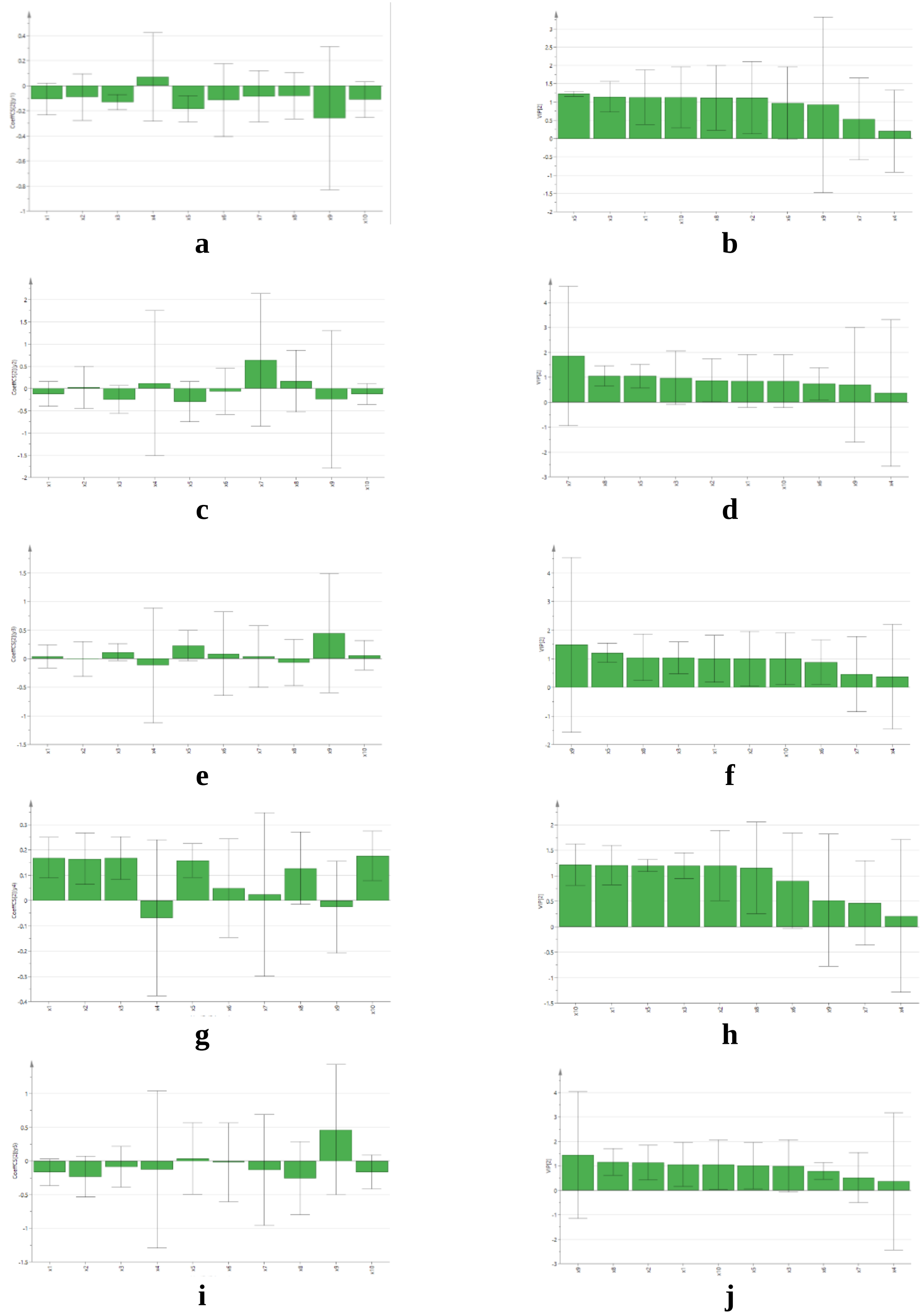

Figure 6. *Regression coefficients (a, c, e, g, i) and VIP values (b, d, f, h, j) based on PLSR analysis in D. nobile. (a, b) Total alkaloid content; (c, d) Total polysaccharide content; (e, f) Total flavonoid content; (g, h) Total tannin content; (i, j) Total amino acid content; Lateral axis: x1: Annual mean temperature; x2: Annual mean maximum temperature; x3: Annual mean minimum temperature; x4: Annual extreme maximum temperature; x5: Annual extreme minimum temperature; x6: Annual mean relative humidity; x7: Annual precipitation; x8: Annual sunshine hour; x9: Frost-free day; x10: Active accumulated temperature ≥10°C*

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Discussion

Phytochemical diversity

In this study, the results of ANOVA suggested that the content of total alkaloid, total polysaccharide, total flavonoid, total tannin and total amino acid in *D. nobile* showed significant different among the 10 populations ($P < 0.01$), and the mean value of CV among them was 15.12%, which more than 10%. Both *D* and *H′* also revealed the diversity in the 10 populations using the 5 phytochemical contents. At present, the content of total alkaloid was only considered as the marker ingredient to evaluate the quality of *D. nobile* by the Chinese Pharmacopeia (National Pharmacopoeia Committee, 2020). The findings suggested that the content of total alkaloid in *D. nobile* cultivated in Sichuan and Guizhou provinces than that in Yunnan province. It provided a theoretical basis for better quality of *D. nobile* in Sichuan and Guizhou. Additionally, *D. nobile* from different population could be utilized as special germplasm resources based on its higher content of total polysaccharide, total flavonoid, total tannin and total amino acid respectively. On the whole, phytochemical content heterogeneity in different populations means the species is likely to have more chance to adapt to various environment conditions, thus increasing probability of surviving in the context of climate change (Visser, 2008). In practice, the spatial heterogeneity of phytochemical content is conducive to the maintenance and preservation of biodiversity, provides rich raw materials for the cultivation of new varieties of *D. nobile.*

Comparison of morphological and phytochemical diversity

In our series of research work, we analyzed the variation at the morphological, phytochemical and genetic levels for the same samples of *D. nobile* respectively. In former study, the phenotypic cluster of the 10 populations based on 18 morphological traits was constructed (He et al., 2021). In the present study, the cluster of the same 10 populations using 5 phytochemical contents was analyzed. The findings showed the similarity and difference between the two clusters. The similarity lied in ML population far away in southern Yunnan formed a group alone, and the other 9 populations which were distributed in Sichuan and Guizhou respectively gathered together. It indicated both morphological and quality traits of *D. nobile* cultivated in Sichuan and Guizhou were closer. On the other hand, the difference was that LJ and XM population which was located in Hongya and in Jiajiang county respectively composed of another group in morphological cluster, while in phytochemical cluster, the LJ population was first clusterd with the FM and FB population located in Hejiang county, and XM population was first clustered with the FX population located in Chishui city, then these populations, together with the WL population formed a sub-subgroup. Similar results had been reported that there were completely inconsistencies between morphological and phytochemical clustering in *D. officinale*, another species of the genus *Dendrobium* (Liu et al., 2016). This result implied that environmental factors might influence on the formation of morphology and synthesis of secondary metabolism by different ways.

Factors affecting phytochemical diversity

According to the existing biological knowledge, phenotype is the result of the interaction of genetics and environment. Genetic differences can lead to phenotypic diversity, including chemical diversity. In previous study, the genetic diversity and

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population structure was evaluated in the 10 same populations of *D. nobile* based on genotype-by-sequencing (GBS), genetic distinctiveness was existed within and among populations. Abundant genetic variation was the basis of phenotypic variation (He et al., 2022). Production of active ingredients in medicinal plant is also strongly influenced by environmental factors besides genotypes (Valls et al., 2007; Gairola et al., 2010). There was a view that genetic factors were the main role for the differences of phenotypic traits if samples distributed in narrow range (Du et al., 2018). In other words, environmental factors had a greater impact on the phenotype when samples collected distributed in wider range. The 10 sampled populations distributed in different regions, including Sichuan, Guizhou and Yunnan in Southwest China with wide range of longitude and latitude. Geographical differences also lead to different levels of genetic diversity to adapt to environment. Especially for medicinal plant, environmental conditions (climate, geology and ecology) play an important role in the formation of quality.

This study revealed that the variation of content of total alkaloid, polysaccharide, flavonoid, tannin and total amino acid among populations of *D. nobile* could be attributed to various climatic variables including temperature, humidity, sunshine etc. In particular, extreme temperature and accumulated temperature were the key factors for the synthesis of the five active ingredients of *D. nobile* respectively. Owing to different kinds of active ingredient, the most important environmental factors contributing to the content were also different. Low temperature and less active accumulated temperature were beneficial to the accumulation of alkaloid. Precipitation and sunshine were beneficial to synthesize of polysaccharide. Frost-free day and extreme temperature were the key climatic factors influencing synthesis of flavonoid. Temperature, sunshine and accumulated temperature promote tannin enrichment. Variables such as frost-free day, temperature and sunshine played leading role in accumulation of amino acid. It had been reported that temperature, relative humidity and sunshine showed significant correlation with the content of total polysaccharide, alkaloid and flavonoid in *D. officinale* under different cultivation modes (Yuan et al., 2020). Similar results were reported that the variation in total flavonoid content among populations could be attributed to various environmental factors including climatic conditions (Gobbo-Neto and Lopes, 2007) in other plant like *Valeriana jatamansi* (Jugran et al., 2016).

At the same time, it should be also noted that the content of total alkaloid and tannin among the studied populations of *D. nobile* were influenced by latitude. With the increase of latitude, the content of total alkaloid increased, while the content of total tannin decreased. The effect of geographical factors and climatic factors on the content of total alkaloid and tannin were mutually confirmed. The reason for this was that the temperature would decrease as the latitude increases.

Environmental stress

It is well known that secondary metabolites in plant could be synthesized under environmental stress contribute to the defense mechanism (Hall et al., 1982; Tang et al., 1994; Gharibi et al., 2016). In medicinal plant, studies also point out that low temperature was beneficial to the accumulation of active ingredient in *Panax ginseng* (Nam et al., 2003), and *Astragalus membranaceus* (Pan et al., 2007). In *Atractylodes lancea*, the synthesis of volatile oil was the result of stress of potassium deficiency and high temperature (Guo et al., 2007). Moderate environmental stresses such as temperature, water and light could promote the accumulation of active component in

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Scutellaria baicalensis (Yuan et al., 2016). Appropriate drought stress was meaningful for increasing the content of iridoid in *Gentiana rigescens* (Liu et al., 2020). Interestingly, in this study, it was observed that some of the active ingredient (polysaccharide, flavonoid, tannin) were enriched under normal growth and development conditions, while other chemical compounds were accumulated as a result of environmental stress. According to correlation analysis, it was found that environmental stress such as low temperature, short sunshine hour and less accumulative temperature were favorable for biosynthesis of alkaloid and amino acid in *D. nobile* respectively.

Various hypotheses had been proposed to explain the impact of environmental stress on secondary metabolites, which were growth/differentiation balance (GDB) (Frisehknecht et al., 2001), carbon/nutrition balance (CNB) (Hamilton et al., 2001), optimum defense (OD) (Barto et al., 2005) and resource availability (RA) hypothesis (Byers, 2000) respectively. A common conclusion was put forward that the mounts of plant secondary metabolites would increase under environmental stress from different perspectives (Huang et al., 2007). The stress effects in alkaloid and amino acid synthesis in *D. nobile* in this study could be explained by the hypothesis of RA. This hypothesis hold that plant grown under severe natural conditions had the characteristics of slow growth and more amounts of secondary metabolites, while plant grown under good natural conditions had the characteristics of fast growth and less amounts of secondary metabolites because of natural selection. The present results also suggested that *D. nobile* grown in Yunnan were characteristics of taller and stronger in morphology than that in Sichuan and Guizhou due to the climate conditions in Yunnan were conducive to the growth and development, while the content of alkaloid in Yunnan was lower than that in Sichuan and Guizhou where temperature was less and sunshine hour shorter. Thus, the species of *D. nobile* showed slow growth and more amounts of secondary metabolites respectively. The quality of *D. nobile* grown in Sichuan and Guizhou was better. The results also provide data support for the suitability of medicinal use of *D. nobile* grown in Sichuan and Guizhou, where are the traditional authentic areas of the species. It lays a foundation for further study of the metabolic mechanism of the stress resistance and the influence of stress effect on the quality of *D. nobile* at the molecular level.

Conclusion

In conclusion, the content of total alkaloid, total polysaccharide, total flavonoid, total tannin, and total amino acid in cultivated *D. nobile* showed highly significant differences ($p < 0.01$) among the 10 sampled populations in Southwestern China, and higher diversity at phytochemical level. The findings suggested that *D. nobile* had higher quality in Sichuan and Guizhou than that in Yunnan. Phytochemical content heterogeneity in the different populations means the species is likely to have more chance to adapt to various environment conditions, thus increasing their probability of surviving in the context of climate change. The 10 populations were classified into two groups, two subgroups and three sub-subgroups. it was found that the content of total alkaloid showed significant positive correlation with latitude ($p < 0.05$), while that of total tannin showed strong significant negative correlation with latitude ($p < 0.01$). These findings suggested that temperature (including extreme temperature and accumulated temperature), precipitation, sunshine and frost-free day were considered as key factors influencing synthesis of the 5 active ingredients respectively. Low

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temperature and less accumulated temperature were favorable for enrichment of alkaloid and amino acid, which showing environmental stress.

Overall, this study represents a first step to understanding phytochemical heterogeneity and the association between environmental conditions and phytochemical diversity of cultivated *D. nobile* growing in Southwest China. The results of this study are expected to be useful to enrich the gene pool, to provide guidance for the introduction and the formulation of the regional national strategic plan for artificial cultivation. In addition, climatic and geographical variables selected in this study were not the only factors affecting the phytochemical content of *D. nobile*. It could be suggested that the number of sampled populations and other environmental factors should be increased for future research in order to better understand the phytochemical variation and the association of them with environmental variables, and more research about genotype × environment interaction should be investigated.

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Conflict of interests. The authors declare no conflicts of interests.

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