Opposite trends of glycosides and alkaloids in *Dendrobium nobile* of different age based on UPLC-Q/TOF-MS combined with multivariate statistical analyses

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**Abstract**

**Introduction:** Alkaloids and glycosides are the active ingredients of the herb *Dendrobium nobile*, which is used in traditional Chinese medicine. The pharmacological effects of alkaloids include neuroprotective effects and regulatory effects on glucose and lipid metabolism, while glycosides improve the immune system. The pharmacological activities of the above chemical components are significantly different. In practice, the stems of 3-year-old *D. nobile* are usually used as the main source of *Dendrobii Caulis*. However, it has not been reported whether this harvesting time is appropriate.

**Objective:** The aim of this study was to compare the chemical characteristics of *D. nobile* in different growth years (1–3 years).

**Methods:** In this study, ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q/TOF-MS) was employed to analyze the constituents of *D. nobile*. The relative abundance of each constituent was analyzed with multivariate statistical analyses to screen the characteristic constituents that contributed to the characterization and classification of *D. nobile*. Dendrobine, a component of *D. nobile* that is used for quality control according to the Chinese Pharmacopoeia, was assayed by gas chromatography.

**Results:** As a result, 34 characteristic constituents (VIP > 2) were identified or tentatively identified as alkaloids and glycosides based on MS/MS data. Moreover, the content of alkaloids decreased over time, whereas the content of glycosides showed the opposite trend. The absolute quantification of dendrobine was consistent with the metabolomics results.

**Conclusion:** Our findings provide valuable information to optimize the harvest period and a reference for the clinical application of *D. nobile*.
1  |  INTRODUCTION

Dendrobium is the second largest genus of the plant family of Orchidaceae. Approximately 1,500 Dendrobium species are currently known. Dendrobii Caulis, Ganoderma, Cordyceps, and Ginseng Radix et Rhizoma are honored as “superior-grade” herbs in an ancient book named “Shen nong’s Classic of Materia Medica,” which is one of the Four Classics of traditional Chinese medicine. Moreover, Dendrobii Caulis has the effects of nourishing Yin and clearing heat, and it is applicable to situations such as yin injury and depletion of body fluid, dry mouth and polydipsia, less food and retching, deficiency and heat after illness, and dark eye.

In the Chinese Pharmacopeia, D. nobile is listed as the primary medicinal source of Dendrobii Caulis. Pharmacological research confirmed that D. nobile improves cognitive dysfunction, reduces gastric damage, has anti-tumor effects, and can be used to treat diabetes. Due to its beneficial effects, D. nobile is also used as a tonic and functional food, such as Dendrobii Liquor. Broad applications increased the demand for D. nobile and hence resulted in a sharp decline of wild D. nobile populations. In the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II, wild D. nobile is included.

To meet the demands, D. nobile was developed. The alkaloids and glycosides of D. nobile are considered as the principal biologically active components with extensive pharmacological effects. The alkaloids are used to protect neurons and regulate glucose and lipid metabolism, among others. Dendromoniliside A, isolated from the stem of Dendrobium moniliforme, improves immune activity by stimulating the proliferation of B cells and inhibiting the proliferation of T cells in vitro. Dendronobiloside A, isolated from the stem of D. nobile, was found to stimulate the proliferation of murine T and B lymphocytes in vitro. The above compounds have different pharmacological effects. In the main producing region, the stems of D. nobile are usually harvested in the third year. However, little evidence is available to support this practice. To find out the best time to harvest, understanding the differences in metabolite profile between stems grown for various years is necessary.

Fourteen alkaloids, including dendrobine, dendrobine-N-oxide, dibolionine, and dendroxine, were isolated from D. nobile in previous studies. Among them, dendrobine is used as a quality marker for the quality control (QC) of D. nobile according to the Chinese Pharmacopeia. However, our previous study indicated that the content of dendrobine in D. nobile decreases over time. Moreover, it is unknown whether other alkaloids in D. nobile follow the same trend as dendrobine. In addition to alkaloids, some non-alkaloid chemical constituents, such as polysaccharides, sesquiterpenes, phenanthrene, bibenzyl, and fluorenones, also exist in D. nobile. How the levels of these non-alkaloid chemical constituents in D. nobile change with age is also unclear. Therefore, it is unclear whether dendrobine should be used as a quality marker for the QC of D. nobile.

In the present study, ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q/TOF-MS) coupled with multivariate statistical analysis was employed to probe the characteristic components of D. nobile in different growth years to find accurate quality markers in D. nobile.

2  |  EXPERIMENTAL PROCEDURES

2.1  |  Chemicals and reagents

Dendrobine (purity > 99%) was purchased from the National Institutes for Food and Drug Control (Beijing, China). Naphthalene (purity > 99%, internal standard [IS]) was purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS-grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). LC-MS-grade formic acid was purchased from CNW Technologies GmbH (Dusseldorf, Germany). Formic acid and methanol (AR) were obtained from Chengdu Kelong Chemical Co., Ltd. (Chengdu, China).

2.2  |  Sample collection

The fresh stems of 1- to 3-year-old D. nobile were harvested from Chishui city of Guizhou Province in China in October 2019. Samples were collected in triplicate for each feature. Details are listed in Table 1.

2.3  |  Sample preparation to profile the metabolome of D. nobile by UPLC-Q/TOF-MS

Clean fresh D. nobile stems were dried at 60°C in a drying oven. The dried stems were ground into fine powder (300 mesh), and then 75 mg was soaked in 1 mL of 70% (v/v) methanol, followed by ultrasonication (50 kHz, 400 W) for 30 min. Next, the liquid was centrifuged at 9705 g for 5 min, and the supernatant was used for UPLC-Q/TOF-MS analysis.

2.4  |  UPLC/Q-TOF/MS parameters

A 1290 Infinity II UPLC system (Agilent, MA, USA) coupled with a Synapt Q/TOF-MS System (Agilent, MA, USA) was used to profile the metabolome of D. nobile. Sample solution aliquots of
| Sample number | Cluster number | Stem number | Growth year | Collection time | Origin | Species of epiphytic stone |
|---------------|----------------|-------------|-------------|----------------|--------|---------------------------|
| 2019-10-1-1   | 2019-10-1      | 1           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-1-2   | 2019-10-1      | 2           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-1-3   | 2019-10-1      | 3           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-1-4   | 2019-10-1      | 4           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-1-5   | 2019-10-1      | 5           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-1-6   | 2019-10-1      | 6           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-1-7   | 2019-10-1      | 7           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-1-8   | 2019-10-1      | 8           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-1-9   | 2019-10-1      | 9           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-2-1   | 2019-10-2      | 1           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-2-2   | 2019-10-2      | 2           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-2-3   | 2019-10-2      | 3           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-2-4   | 2019-10-2      | 4           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-2-5   | 2019-10-2      | 5           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-2-6   | 2019-10-2      | 6           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-2-7   | 2019-10-2      | 7           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-2-8   | 2019-10-2      | 8           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-2-9   | 2019-10-2      | 9           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-3-1   | 2019-10-3      | 1           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-3-2   | 2019-10-3      | 2           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-3-3   | 2019-10-3      | 3           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-3-4   | 2019-10-3      | 4           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-3-5   | 2019-10-3      | 5           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-3-6   | 2019-10-3      | 6           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-3-7   | 2019-10-3      | 7           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-3-8   | 2019-10-3      | 8           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-4-1   | 2019-10-4      | 1           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-4-2   | 2019-10-4      | 2           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-4-3   | 2019-10-4      | 3           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-4-4   | 2019-10-4      | 4           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-4-5   | 2019-10-4      | 5           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-4-6   | 2019-10-4      | 6           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-4-7   | 2019-10-4      | 7           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-4-8   | 2019-10-4      | 8           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-4-9   | 2019-10-4      | 9           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-5-1   | 2019-10-5      | 1           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-5-2   | 2019-10-5      | 2           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-5-3   | 2019-10-5      | 3           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-5-4   | 2019-10-5      | 4           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-5-5   | 2019-10-5      | 5           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-5-6   | 2019-10-5      | 6           | 2 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-5-7   | 2019-10-5      | 7           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-5-8   | 2019-10-5      | 8           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-5-9   | 2019-10-5      | 9           | 3 years     | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-6-1   | 2019-10-6      | 1           | 1 year      | 2019-10-10     | Chishui city, Guizhou Province | Danxia Stone |

(Continues)
| Sample number | Cluster number | Stem number | Growth year | Collection time | Origin | Species of epiphytic stone |
|---------------|----------------|-------------|-------------|-----------------|--------|----------------------------|
| 2019-10-10-6-2 | 2019-10-10-6  | 2           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-6-3 | 2019-10-10-6  | 3           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-6-4 | 2019-10-10-6  | 4           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-6-5 | 2019-10-10-6  | 5           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-6-6 | 2019-10-10-6  | 6           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-6-7 | 2019-10-10-6  | 7           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-6-8 | 2019-10-10-6  | 8           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-6-9 | 2019-10-10-6  | 9           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-1 | 2019-10-10-7  | 1           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-2 | 2019-10-10-7  | 2           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-3 | 2019-10-10-7  | 3           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-4 | 2019-10-10-7  | 4           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-5 | 2019-10-10-7  | 5           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-6 | 2019-10-10-7  | 6           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-7 | 2019-10-10-7  | 7           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-8 | 2019-10-10-7  | 8           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-9 | 2019-10-10-7  | 9           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-7-10| 2019-10-10-7  | 10          | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-8-1 | 2019-10-10-8  | 1           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-8-2 | 2019-10-10-8  | 2           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-8-3 | 2019-10-10-8  | 3           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-8-4 | 2019-10-10-8  | 4           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-8-5 | 2019-10-10-8  | 5           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-8-6 | 2019-10-10-8  | 6           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-8-7 | 2019-10-10-8  | 7           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-8-8 | 2019-10-10-8  | 8           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-8-9 | 2019-10-10-8  | 9           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-9-1 | 2019-10-10-9  | 1           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-9-2 | 2019-10-10-9  | 2           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-9-3 | 2019-10-10-9  | 3           | 1 year      | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-9-4 | 2019-10-10-9  | 4           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-9-5 | 2019-10-10-9  | 5           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-9-6 | 2019-10-10-9  | 6           | 2 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-9-7 | 2019-10-10-9  | 7           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-9-8 | 2019-10-10-9  | 8           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
| 2019-10-10-9-9 | 2019-10-10-9  | 9           | 3 years     | 2019-10-10      | Chishui city, Guizhou Province | Danxia Stone |
0.1 μL were separated on a Waters CORTECS UPLC C18 column (100 mm × 2.1 mm, 1.6 μm) at 40°C with a flow rate of 0.4 mL/min. The mobile phases consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile, v/v). The elution gradients were as follows: 0–0.5 min, 5% B; 0.5–4 min, 5%–40% B; 4–5 min, 40%–75% B; 5–5.1 min, 75%–95% B; 5.1–6.5 min, 95% B; 6.5–6.6 min, 95%–5% B; 6.6–10 min, 5% B. Electronic spray ionization (ESI) was used in the TOF-MS system. The ion source temperature was 350°C, the nebulizer pressure was 44 psi, the drying gas flow was 10 L/min, the sheath gas flow temperature was 350°C, the sheath gas flow was 11 L/min, the Vcap voltage was 4,000 V, and the nozzle voltage was 1,000 V. In the mass analyzer, the scan mode was auto MS/MS, and the ions were scanned from m/z 50 to 1,200. [Correction added on 20 April 2022, after first online publication: In the first sentence under ‘2.4 UPLC/Q-TOF/MS parameters’, the text “Waters Corp., Milford, MA, USA” has been changed to “Agilent, MA, USA”; and the elution gradients have been changed to “0–0.5 min, 5% B; 0.5–4 min, 5%–40% B; 4–5 min, 40%–75% B; 5–5.1 min, 75%–95% B; 5.1–6.5 min, 95% B; 6.5–6.6 min, 95%–5% B; 6.6–10 min, 5% B.”]

2.5 | Dendrobine quantification in D. nobile by GC

Dendrobine in D. nobile was quantified following Part I of the Chinese Pharmacopeia, edition 2015. Chromatography was performed on an Agilent gas chromatograph (Agilent Technologies 7820, USA) equipped with a DB-1 column (30 m × 0.25 mm, 0.25 μm, Agilent Technologies). The linearity, precision, reproducibility, and stability were validated for dendrobine quantification.

2.6 | Establishment of the in-house library for D. nobile

To ensure the efficiency and accuracy of UPLC-HRMS chemical profiling, an in-house chemical library was constructed by retrieving several databases, including SciFinder (https://scifinder.cas.org/), Web of Science (http://www.webofscience.com), PubMed (https://pubmed.ncbi.nlm.nih.gov/), ChemSpider (http://www.chemspider.com/), and PubChem (https://pubchem.ncbi.nlm.nih.gov/). More than 700 references about Dendrobium Sw. were utilized to build the library including 430 compounds, which involved as much prior knowledge and available chemical information as possible, such as Chinese and English names, detailed molecular formulae, molecular weight, and CAS numbers in a Microsoft Office Excel document, as well as chemical structures in MOL files. Through use of the “Find” function of Excel, those compounds which matched the determined molecular formula were found as potential candidates. Only those metabolite data which further matched the experimental UPLC-Q/TOF-MS/MS data were finally selected for establishing the identity of an unknown constituent.

2.7 | Data processing

The raw data were processed by peak matching, peak alignment, ion fusion, and deconvolution using Agilent Mass Hunter Profinder software (version 10.0). Principle component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed in SIMCA-P software (version 14.0). Other visualizations were performed in R (version 4.0.2). The statistical analysis of multiple comparisons was performed using one-way ANOVA in SPSS 18.0 (IBM, Chicago, USA). Data in histograms and box plots are expressed as mean ± SD. P-values of less than 0.05 were considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Profiling of the metabolome in D. nobile stems with different ages

In the present study, the metabolites in D. nobile stems were separated by the UPLC system and detected in both positive and negative modes of Q/TOF-MS. The total ion chromatograms are shown as the basis of the peak chromatograms (BPCs) for typical D. nobile samples cultivated for 1, 2, and 3 years. In the BPCs of both positive and negative modes, chromatographic peaks mainly appeared at the retention time range from 1 to 5 min (Figure 1A, C). In the BPC of the positive mode, a couple of peaks showed decreasing trends along with the increase of growth years (Figure 1B). Oppositely, in the BPC of the negative mode, another set of peak responses increased along with the growth years of the plants (Figure 1D).

Moreover, in total 509 and 368 ions with various m/z values were extracted from the BPCs in positive and negative modes, respectively. The peak areas of each ion in the extracted ion chromatograms were used to represent the quantities of the corresponding constituents while the comparisons were made between samples. In score plots of PCA, using all ions detected in both positive and negative modes, the general profiles of D. nobile grown for 1, 2, and 3 years were observed, except a couple of outliers (Figure 1E). The locations of the three clusters representing D. nobile samples of different ages were distributed along with component 1, indicating that the constituents with larger loading values in component 1 were more affected by age. Thus, based on the chromatograms and the PCA results, the metabolome of D. nobile stems was altered in an age-dependent manner.

3.2 | Discovery of the constituents in D. nobile stems of various ages

To screen the constituents that can be used to distinguish between samples of various ages, the outliers observed in the score plots of PCA were removed from the data, and a PLS-DA was performed and passed the permutation tests. In the score plot of PLS-DA (Figure 2A),
points representing samples from different planting years were located away from each other, while points representing samples with the same ages were clustered together. In addition, the PLS-DA model also had a good predictive ability and credibility: $R^2_Y = 0.709$; $Q^2_{(cum)} = 0.789$. To find out which contents were significantly different among *D. nobile* stems aged 1, 2, and 3 years, a variable importance in projection (VIP) plot was generated (Figure 2B). In the VIP plot, in total 34 constituents with VIP scores of more than 2 were selected (Table 2). Most constituents detected in positive mode were distributed on the right side of the plots, which meant that their levels may be high in 1-year-old *D. nobile* stems. In contrast, most points located on the left side of the VIP plots, representing highly expressed contents in 3-year-old *D. nobile* stems, were detected by MS in negative mode. To confirm this finding, a one-way hierarchical clustering analysis heatmap was generated by using data of 34 constituents with PLS-DA VIP scores of more than 2. Based on the expression pattern along the planting years, the constituents were generally clustered into two groups (Figure 2C). The contents of 16 out of 34 constituents (cluster 1) decreased over time (Figure 2D), while the contents of 18 out of 34 constituents (cluster 2) increased over time (Figure 2E). Most constituents in cluster 1 were observed by MS in positive mode, whereas most constituents in cluster 2 were detected by MS in negative mode, confirming our PCA results.

### 3.3 Assignment of the general structures of constituents showing age-dependent expression in *D. nobile* stems

To identify the structures or structural features of the 34 constituents with age-dependent expression in *D. nobile* stems, information including the exact molecular weight, fragment ions in MS, chromatographic retention time, reference literature, and reference compounds was integrated. Before the structure identification, as much information of
compounds reported in *D. nobile* was collected as possible. In the positive mode of MS, by matching the exact \( m/z \) values of quasi-molecular ions with the exact molecular weights of the reported compounds in *D. nobile*, in total 17 structures were screened out. Most of those structures were potentially dendrobine-type alkaloids. Furthermore, the chromatographic retention time of constituent #232-p was identical to that of the reference compound dendrobine (Figure 3A). Constituents #339-p, #419-p, #278-p, and #207-p were identified as dendroxine, \( N \)-isopentenyl-dendrobinium, nobilonine, and \( N \)-methyl-dendrobinium by searching an in-house library and comparing their formulae and fragmentation information with the literature.\(^{11}\) Constituents #273-p and #418-p were identified as the isomers
| ID   | VIP | Rt (min) | Add ion     | m/z actual | m/z theoretical | Molecular formula | Identification                                      | MS/MS                                                                 |
|------|-----|----------|-------------|------------|-----------------|-------------------|---------------------------------------------------|-----------------------------------------------------------------------|
| 232-p| 9.93| 3.09     | [M+H]^+     | 264.1969   | 264.1958        | C_{14}H_{23}NO_2  | Dendrobine                                        | 218.1910; 176.1441; 105.0699; 70.0655; 145.1014;                      |
| 273-p| 7.90| 3.32     | [M+H]^+     | 280.1917   | 280.1907        | C_{14}H_{25}NO_3  | 2-Hydroxydendrobine or 6-hydroxydendrobine        | 263.1884; 220.1326; 136.1118;                                       |
| 44-n | 6.41| 0.60     | [M–H]^-     | 191.0567   | 191.0561        | C_{9}H_{12}O_{6}  | Quinic acid                                       | 127.0399; 93.0343; 85.0295;                                        |
| 339-p| 4.22| 3.69     | [M+H]^+     | 292.1902   | 292.1907        | C_{13}H_{25}NO_3  | Dendroxine                                        | 248.2001; 205.1445; 178.1210; 93.069; 56.0490;                     |
| 418-p| 4.15| 2.29     | [M+H]^+     | 264.1954   | 264.1958        | C_{14}H_{25}NO_2  | Isomer of dendrobine                              | 176.1441; 122.0970;                                                |
| 121-n| 3.88| 2.75     | [M–H]^-     | 445.2073   | 445.2079        | C_{21}H_{34}O_{10}| Dendromoniliside D or isomer                      | 321.1341; 265.1450; 161.0461; 101.0239; 71.0134;                   |
| 153-p| 3.48| 2.82     | [M+H]^+     | 262.1799   | 262.1802        | C_{14}H_{23}NO_2  | Unknown                                          | 95.0854;                                                            |
| 419-p| 3.40| 4.22     | M^+         | 332.2597   | 332.2584        | C_{21}H_{34}NO_2  | N-isopentenyl-dendrobinium                       | 264.1973; 176.1435; 69.0704;                                      |
| 230-n| 3.36| 3.51     | [M–H]^-     | 559.2750   | 559.2760        | C_{27}H_{44}O_{12}| Dendronobiloside C/D or isomer                   | 517.0897; 298.0759; 179.0552; 119.0338; 89.0239; 71.0142;         |
| 41-n | 3.23| 0.62     | [M–H]^-     | 341.1085   | 341.1085        | C_{13}H_{22}O_{11}| Sucrose                                           | 89.0240; 71.0135; 59.0135;                                        |
| 438-p| 3.17| 4.53     | M^+         | 292.1919   | 292.1907        | C_{14}H_{26}NO_3  | Fragment of N-isopentenyl-dendrobinium           | NA                                                                  |
| 251-n| 3.11| 3.51     | [M–H]^-     | 559.2750   | 559.2760        | C_{27}H_{44}O_{12}| Dendronobiloside C/D or isomer                   | 517.0897; 298.0759; 179.0552; 119.0338; 89.0239; 71.0142;         |
| 130-n| 3.02| 2.83     | [M–H]^-     | 445.2070   | 445.2079        | C_{21}H_{34}O_{10}| Dendromoniliside D or isomer                      | 413.2164; 360.1420; 283.1530; 221.1526; 161.0424; 101.0245;      |
| 34-p  | 2.88| 0.68     | [M+H]^+     | 262.1286   | 262.1285        | C_{11}H_{19}NO_6  | Isomer of succinylcarnitine                       | 244.1183; 216.1239; 198.1128; 72.0807;                            |
| 171-n| 2.85| 3.09     | [M–H]^-     | 577.1565   | 577.1563        | C_{27}H_{30}O_{14}| Violanthin                                        | 533.1282; 503.1175; 457.1128; 383.0769; 353.0652; 191.0315;       |
| 282-p| 2.80| 3.42     | [M+H]^+     | 306.2073   | 306.2064        | C_{16}H_{27}NO_3  | 1H-Cyclopent[cd]indole-5-carboxylic acid, decahydro-1,7b-dimethyl-7-methylene-6-(1-methylethyl)-2-oxo-, methyl ester, (2a,4a,5b,6a,7aa,7ba) - (9CI), malonyl-L-carnitine or isomer | 292.1860; 263.1867; 233.0591; 175.1476; 133.1012; 107.0852;       |
| 20-p  | 2.75| 0.60     | [M+H]^+     | 248.1132   | 248.1129        | C_{10}H_{17}NO_6  | Isomer of malonyl-L-carnitine                     | 230.1028; 182.0819; 116.0710; 98.0597; 87.0439;                   |
| 163-n| 2.73| 3.02     | [M–H]^-     | 445.2073   | 445.2079        | C_{21}H_{34}O_{10}| Dendromoniliside D or isomer                      | 413.2148; 281.1389; 221.1548; 162.0436; 89.0240; 59.0133;         |
| 53-p  | 2.62| 0.93     | [M+H]^+     | 276.1447   | 276.1442        | C_{13}H_{21}NO_6  | Unknown                                          | 258.1355; 230.1391; 86.0962;                                     |
| 172-n| 2.60| 3.14     | [M–H]^-     | 577.2012   | 577.2079        | C_{23}H_{34}O_{10}| Unknown                                          | NA                                                                  |
| 267-n| 2.58| 3.77     | [M–H]^-     | 559.2750   | 559.2760        | C_{22}H_{44}O_{12}| Dendronobiloside C/D or isomer                   | 536.0630; 457.1714; 179.0559; 119.0347; 89.0244; 59.0135;         |
unknown means the compound is not found in *Dendrobium*.

2-hydroxydendrobine or 6-hydroxydendrobine, isomer of dendrobine, by searching our in-house library. However, the exact structure could not be confirmed due to a lack of reference standard or literature. Constituents #438-p was deduced as in-source fragments of N-isopentenyl-dendrobinium by comparison of the retention time and fragmentation information with their corresponding precursor ions. Constituents (1) #34-p, (2) #282-p, (3) #20-p, and (4) #57-p showed not only extremely similar exact molecular weights, but also similar fragmentation mechanisms as (1) succinylcarnitine, (2) 1H-cyclopent|cd|indole-5-carboxylic acid, decahydro-1,7b-dimethyl-7-methylene-6-(1-methylethyl)-2-oxo-, methyl ester, (2aa,4aa,5b,6a,7aa,7ba) - (9CI), malonyl-L-carnitine, (3) malonyl-L-carnitine, and (4) 2-methyl-1H-indol-7-yl-β-D-mannopyranoside or isomer 2-methyl-1H-indol-7-yl-β-D-mannopyranoside, respectively. The exact molecular weights of constituents #153-p, #53-p, #58-p, #47-p, and #147-p matched nothing in the in-house library; however, based on the predicted molecular formulae, they could be identified as alkaloids although their exact structures remain unknown. As their expression followed the positive age-dependent patterns as observed for dendrobine, dendroxine, N-isopentenyl-dendrobinium, nobilnine, and N-methyl-dendrobium, we also assumed they are dendrobine-type alkaloids.

In the negative mode of MS, 17 constituents with PLS-DA VIP scores of more than 2 were screened out. In the same way as above, using the exact molecular weight values, ion fragmentation mechanisms, and the information of reference compounds, constituents #44-n, #41-n, #171-n, and #47-n were uniquely identified as quinic acid, succrose, violanthin, and protocatechualdehyde, respectively. By matching the exact molecular weights with members of our in-house library, constituents #121-n, #230-n, #251-n, #130-n, #163-n, #267-n, #280-n, and #150-n were identified as dendronobilosides or dendromonilisides (Figure 4B). Their MS/MS fragment ions were also linked to parts of the predicted structures. Among those constituents, #280-n was uniquely identified as dendronobiloside A based on their formulae and fragmentation information obtained from the literature (Figure 4A). Others were only identified as isomers of their corresponding predicted structures because of the lack of

### Table 2 (Continued)

| ID   | VIP  | Rt (min) | Add ion   | m/z actual | m/z theoretical | Molecular formula | Identification                  | MS/MS                           |
|------|------|----------|-----------|------------|-----------------|------------------|---------------------------------|---------------------------------|
| 278-p| 2.55 | 3.43     | [M+H]+    | 294.2068   | 294.2064        | C_{14}H_{27}NO_{3} | Nobilnine                      | 249.1491; 203.1425; 175.1484; 95.085; |
| 280-n| 2.54 | 3.91     | [M−H]−    | 563.3060   | 563.3073        | C_{20}H_{46}O_{12} | Dendronobiloside A              | 401.2472; 113.0229; 101.0231; 71.0131; 59.0131; |
| 58-p | 2.53 | 1.33     | M−        | 103.0540   | 103.0542        | C_{6}H_{17}+      | Unknown                         | 91.0540; 77.0386; 51.0228; |
| 249-n| 2.52 | 3.71     | [M−H]−    | 561.2871   | 561.2917        | C_{20}H_{46}O_{12} | Unknown                         | 399.2333; 161.0429; 101.0232; 59.0129; |
| 150-n| 2.44 | 2.98     | [M−H]−    | 413.2173   | 413.2181        | C_{21}H_{34}O_{8} | Dendronobiloside E/Dendromoniliside A | 350.1051; 293.1501; 265.1447; 216.1370; 101.0241; 59.0132; |
| 207-p| 2.35 | 3.00     | M−        | 278.2118   | 278.2115        | C_{13}H_{20}NO_{2}^{+} | N-Methyl-dendrobinium          | 250.2194; 220.1355; 187.0016; 133.1005; 105.0689; 84.0807; |
| 120-n| 2.31 | 2.81     | [M−H]−    | 445.1319   | 445.1351        | C_{16}H_{28}O_{12} | Unknown                         | NA                             |
| 47-p | 2.26 | 0.88     | [M+H]+    | 300.1441   | 300.1442        | C_{14}H_{21}NO_{4} | Unknown                         | NA                             |
| 147-p| 2.18 | 2.82     | [M+H]+    | 469.1355   | 469.1341        | C_{20}H_{34}O_{12} | Unknown                         | NA                             |
| 47-n | 2.15 | 0.64     | [M−H]−    | 137.0238   | 137.0244        | C_{6}H_{12}O_{3}  | Protocatechualdehyde            | NA                             |
| 36-n | 2.12 | 0.61     | [M−H]−    | 357.1026   | 357.1038        | C_{12}H_{22}O_{12} | Lactobionic acid or isomer      | 173.0448; 89.0242; |
| 57-p | 2.10 | 1.30     | [M+H]+    | 310.1283   | 310.1285        | C_{13}H_{18}NO_{4} | 2-Methyl-1H-indol-7-yl-β-D-mannopyranoside or isomer | 292.1179; 264.1227; 120.0809; |
| 131-n| 2.07 | 2.88     | [M−H]−    | 479.1686   | 479.1685        | C_{23}H_{34}N_{6}O_{6} | Unknown                        | NA                             |

Unknown means the compound is not found in *Dendrobium*. 

17 constituents with PLS-DA VIP scores of more than 2 were screened out. In the same way as above, using the exact molecular weight values, ion fragmentation mechanisms, and the information of reference compounds, constituents #44-n, #41-n, #171-n, and #47-n were uniquely identified as quinic acid, succrose, violanthin, and protocatechualdehyde, respectively. By matching the exact molecular weights with members of our in-house library, constituents #121-n, #230-n, #251-n, #130-n, #163-n, #267-n, #280-n, and #150-n were identified as dendronobilosides or dendromonilisides (Figure 4B). Their MS/MS fragment ions were also linked to parts of the predicted structures. Among those constituents, #280-n was uniquely identified as dendronobiloside A based on their formulae and fragmentation information obtained from the literature (Figure 4A). Others were only identified as isomers of their corresponding predicted structures because of the lack of
corresponding reference compounds. Constituent #36-n was tentatively identified as one of the isomers of lactobionic acid. Regarding constituents #172-n, #249-n, #120-n, and #131-n, the exact molecular weight values matched nothing in our in-house library or any reference standards. Therefore, their structures remain unknown.

3.4 Statistics of the structure-confirmed constituents with age-dependent expression and validation with dendrobine quantification

To find a marker compound to determine the age of *D. nobile*, the identification of one or multiple structure-confirmed constituents with an age-dependent expression pattern is important. Among all constituents with age-dependent expression patterns, #232-p, #339-p, #419-p, #278-p, and #207-p, which were detected in positive mode, were uniquely identified as dendrobine, dendroxine, N-isopentenyl-dendrobinium, nobiline, and N-methyl-dendrobinium, respectively; #44-n, #41-n, #171-n, #280-n, and #47-n, which were detected in negative mode, were uniquely identified as quinic acid, sucrose, violanthin, dendronobiloside A, and protocatechualdehyde, respectively. The statistical significance of the age-dependent expression of these structure-confirmed constituents was analyzed by one-way ANOVA followed by Tukey’s test (Figure 5). Constituents #339-p and #207-p passed the test between year 1 and year 2 and between year 1 and year 3; however, they failed to pass the test between year
 Constituent #278-p merely passed the test between year 1 and year 3. Moreover, constituent #171-n failed to pass the test between any two years. Constituents #232-p, #419-p, #44-n, #41-n, #280-n, and #47-n passed the test between any two years, thus showing an age-dependent expression pattern not only based on the visualized data but also at a statistically significant level. Thus, the above constituents can be used as markers to distinguish between D. nobile of different age.

The absolute quantification of dendrobine was used to validate the statistical analysis results using peak areas. This quantification method was established by other institutions and was validated in our group. As shown in Figure 6A, dendrobine and naphthalene did not interfere with each other under this detection condition. The retention time of dendrobine in the sample solution is the same as that of the reference standard solution, and there is no background interference. The separation coefficient of dendrobine from adjacent peaks was greater than 1.5, indicating that the method was specific. The correlation coefficient of the standard curve was 0.9997, indicating that dendrobine has a good linear relationship in the linear range of 0.30-50.00 μg/mL (Figure 6B). The recovery rate (95.81–95.91%), relative standard deviation (RSD) between injections (0.48%), RSD between samples (1.50%), and RSD between samples stored for various
durations (0.37%) showed that the method was accurate, precise, stable, and reliable (Figure 6C). In agreement with the MS data, the GC data confirmed that dendrobine content in *D. nobile* decreased with age. The difference of dendrobine content between two groups was statistically significant (*P* < 0.05, Figure 6D), confirming the MS results.
The reason behind the trends of glycosides and alkaloids in D. nobile of different age

In most plants, the levels of glycosides of naturally existing molecules increase as plants get older. The most well-known example is the saponin family. Saponins are glycosides of triterpenes with four rings. This family is biosynthesized in various well-known medicinal plants and saponins are expressed in an age-dependent manner in Panax ginseng, Panax notoginseng, and Polygala tenuifolia. Another famous glycoside family is formed by the flavonoid glycosides, which also exhibit a positively age-dependent expression pattern. Even in some regular food sources, small molecules with very simple structures, like isothiocyanates in curly kale, follow the same pattern. Thus, it is not surprising that positively age-dependent expression patterns of glycosides were observed in the present study.

However, for alkaloids, completely opposite observations were made in D. nobile. Alkaloids are also expressed in a broad range of plants. Generally, alkaloid contents increase while plants grow. Examples include cinchonine, cinchonidine, quinidine, and quinine in Cinchona ledgeriana Moens, as well as galanthamine, lycorine, and lycoramine in Lycoris chinensis. However, in the case of dendrobine-type alkaloids in D. nobile, with increasing age, the contents decreased. This may be linked to the plant defense system. Dendrobium spp. possess aerial roots and require microorganisms to support nutrition. Thus, while utilizing microorganisms, there is a need to prevent attacks by phytopathogenic microbes during plant development. This hypothesis is partially supported by another study regarding dendrobine in a microbe named Trichoderma longibrachiatum. In addition to D. nobile, dendrobine was also detected in this microorganism. Dendrobine-containing T. longibrachiatum showed much stronger anti-bacterial activity than other similar
microbes without dendrobine. Possibly, dendrobine protects young *D. nobile* against phytopathogenic microbes, while this might not be necessary in older *D. nobile*, which have a mature immune system. This speculation remains to be confirmed by future experiments.

With increasing age, the increase of *Dendrobium*-specific glycosides and the decrease of dendrobine-type alkaloids in *D. nobile* may be related. Dendrobine is one of the typical structures of sesquiterpene alkaloids. Dendrobine has only been isolated from *D. nobile*, while dendrobine-type alkaloids have been detected in various *Dendrobium* species. The structures of dendrobine-type alkaloids are complex; thus, multiple biosynthesis pathways may exist. A study published in 2017 proposed a biosynthesis scheme of dendrobine. In this scheme, an oxide metabolite of copacamphane was considered as one of the mandatory intermediate compounds for dendrobine bioynthesis in *D. nobile*. The glycone of constituent #150-n (dendronobiloside A) in the present study is just the hydroxylated product of this intermediate. Similarly, in the aforementioned scheme, one of the intermediates, the mono-oxidative metabolite of the dehydrated picrotoxane, is just the dehydrogenated product of the glycone of dendronobiloside A that was assigned to constituent #280-n in the present study. Other than constituents #150-n, #280-n, constituents #121-n, #230-n, #251-n, #130-n, #163-n, and 267-n were also identified as dendronobilosides or dendromonilisides. All these aforementioned constituents share similar glycone structures that are also similar to intermediates in the biosynthesis pathway of dendrobine, while also being negatively correlated with dendrobine-type alkaloid accumulation in *D. nobile*. Thus, we hypothesize that glycosylation of intermediates in dendrobine-type alkaloid biosynthesis pathways may play a competitive role in the generation of dendrobine-type alkaloids in *D. nobile*.

The opposite accumulation trends seem to be observed just between dendrobine-type alkaloids and *Dendrobium*-specific glycosides, including dendronobilosides and dendromonilisides. A flavonoid glycoside named violanthin, assigned to constituent #171-n, was not influenced by the age of *D. nobile*, maybe due to the low degree of crosstalk between biosynthesis pathways of flavonoids and dendrobine-type alkaloids. Constituent #57-p is a special glycoside which was detected by MS in positive mode. This constituent was tentatively identified as the glycoside of a derivative of indole alkaloids and showed even a reversed expression pattern in contrast to dendronobilosides and dendromonilisides. A study published in 2020 proposed an alternative biosynthesis pathway of dendrobine, in which indole alkaloid derivatives play an important role. The shikimate pathway is upstream of the indole alkaloid pathway. Meanwhile, shikimate is biosynthesized from 3-dehydroquinic acid, which is a major metabolite of quinic acid. In the present study, we observed a decreasing trend of quinic acid during *D. nobile* growth. This age-dependent decreasing expression pattern of quinic acid was not only observed in the present study but also in several other plant species. Thus, taken together, the substrate-competitive effect from the generation of dendronobilosides and dendromonilisides and the substrate-reductive effect from the decreased synthesis of quinic acid and indole alkaloids may work together to contribute to the decreasing trend of dendrobine during *D. nobile* growth.

Dendrobine-type alkaloids share a sesquiterpene backbone that was verified to be synthesized mainly via the mevalonate pathway and the methyl-erythritol 4-phosphate pathway. In the two pathways, 16 key enzymes are involved. Among 16 key enzymes involved in terpenoid backbone biosynthesis, the expression levels of the genes encoding acetyl-CoA C-acetyltransferase (AACT), 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS), geranyl diphosphate synthase (GPPS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) were different between plants in different stages, including ginger rhizome, *Achillea millefolium*, and *Solanum tuberosum* L. The expression levels of AACT, DXS, and HDS in young ginger rhizome were higher than in mature ones. Moreover, the RT-PCR results show that the expression levels of genes encoding DXR and GPPS in leaves of *A. millefolium* at different developmental stages were also different; young leaves exhibit higher expression levels of the above genes than fully expanded leaves. HMGR enzyme activity showed the same trend. The above results are in agreement with the observation that the content of alkaloids in 1-year-old *D. nobile* stems is higher than that in 2- and 3-year-old stems. However, over time, sesquiterpenes are also glycosylated. Glycosylation of plant secondary metabolites, including terpenoids, flavonoids, and other small molecules, is mainly catalyzed by UDP-glucuronosyltransferases (UGTs). Previous studies have shown that the relative mRNA expression levels of UGTs are also different between different growth stages of plants. Additionally, the relative expression levels of UGTs, including UGTs, UGTs, UGTs (UGT71A34, UGT71A35, UGT71A34, UGT71W2, and UGT77T1), were confirmed to be higher in the mature stage than in the young stage in *Stevia rebaudiana*, litchi, and strawberry. Therefore, we deduced that the above enzymes, which are involved in the synthesis of alkaloids and glycosides, are among the major causes of the opposite trend observed for the two main compounds in *D. nobile*.

### 4 CONCLUSION

In the present work, the metabolites in *D. nobile* stems were analyzed using UPLC-Q/TOF-MS with multivariate statistical analysis. The results reveal that trends of alkaloids and sesquiterpene glycosides in the stems of *D. nobile* in different growth years were the opposite. The contents of alkaloids in stems were found to decrease with age, whereas the content of sesquiterpene glycosides follows an opposite trend. Furthermore, the absolute quantification of dendrobine confirmed the increasing trend of alkaloids. Combining our results with the pharmacological effects of the above chemical components, we recommend to use 1-year-old *D. nobile* for clinical treatment of neurological diseases, while 3-year-old *D. nobile* could be used to improve immunity. Finally, sesquiterpene glycosides might be quality-related constituents that could be used as quality markers in *D. nobile*. 
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DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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