Water-soluble non-starch polysaccharides of wild-simulated *Dendrobium catenatum* Lindley plantings on rocks and bark of pear trees

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ABSTRACT

The total water-soluble polysaccharide (TP) of *Dendrobium catenatum* is composed of starch and active non-starch polysaccharides (NSPs) with glucomannan as the main structural type. Although the TP content has been used as a quality assessment indicator for many years, the NSPs content in samples from different environments and growth seasons have not been reported. In this study, we found that NSPs had stronger antioxidant activity than TP. The NSPs content was higher in wild-simulated environments including rocks and trees compared to plantings grown in greenhouse. The culture mode and growth period affected the ratio of NSPs and starch. Facility cultivation provided optimal growth conditions but produced more starch, whereas wild-simulated cultivation resulted in a higher ratio of NSPs, particularly in September. Therefore, cultivation by lithophytation and epiphytation may be preferable to facility plantings, which is expected to be enormously useful for the current production and quality control of *D. catenatum*.

1. Introduction

Dendrobium catenatum Lindley (synonym *D. officinale* Kimura et Migo) (Si, Zhang, Luo, Liu, & Liu, 2017), a lithophytic orchid found in subtropical and temperate regions, is one of the most valuable traditional Chinese medicinals, as well as an edible, flowering herbs. Its fresh stem can be chewed directly and the dried stem, called "Tiepi Fengdou" in Chinese, can be stewed in porridge, soups, and other dishes; as such, it is widely used in China and Southeast Asia for its extraordinary tonic efficacy (Si et al., 2017). Because the *D. catenatum* stem has many medicinal attributes, such as immunomodulating, anti-tumor, anti-fatigue, antidiabetic, and antioxidant properties, there is an increasing trend towards using it as a dietary supplement in functional beverages, health foods, and cosmetics (Chen et al., 2021a; Kanlayavattanakul, Lourith, & Chaikul, 2018).

Although wild *D. catenatum* has been listed as an endangered plant since the 1980s, tissue culture seeding and artificial cultivation techniques have been developed, allowing production to meet market needs over the past 20 years (Si, Wang, Liu, Liu, & Luo, 2017). Facility-aided cultivation in greenhouses is the most popular cultivation mode; cultivation in simulated natural environments, such as living tree epiphytic cultivation and rock lithophytic cultivation, has also advanced in recent years to further production efforts with ecological protection practices (Lin et al., 2017). However, little is known about the quality differences in *D. catenatum* under different cultivation conditions.

It is widely accepted that polysaccharides are the major bioactive component of the *D. catenatum* stem (Zhang et al., 2021). According to Chinese Pharmacopoeia (2020 Edition), total water-soluble polysaccharide (TP) has been commonly used as major quality assessment indicator of *D. catenatum*, and TP was acquired through hot water extraction and alcohol precipitation and quantified by phenol-sulfuric acid colorimetry (Liu, Liu, Zhang, & Si, 2021).The water-soluble carbohydrate polymers are ubiquitous in all known plants (Van Soest, Robertson, & Lewis, 1991). Starch, one of the most important water-soluble polysaccharides and major diet constituent, provides energy for human beings (Sumczynski, Bubelova, Sneyd, Erb-Weber, & Mlcek,

2015). Under the condition of adequate nutrition, however, excessive intake of starch will aggravate physiological diseases, such as type 2 diabetes and obesity(Panduro, Roman, Milán, Torres-Reyes, & Gonzalez-Aldaco, 2020). In a previous study (Li et al., 2019), we revealed the presence of starch in the stems of *D. catenatum* and hypothesized that the starch has a negative effect on the pharmacological activity of *D. catenatum*.

By contrast, water-soluble non-starch polysaccharides (NSPs) is considered an active ingredient of medicinal plants and functional foods known for their potential health-promoting effects, such as those related to alleviating oxidative stress and balancing intestinal microbiota and immune-system modulation (Chen et al., 2021b). Among NSPs, most of the research efforts have focused on mannan, glucan, and glucomannan (GM) (Korolenko, Bgatova, & Vetvicka, 2019). GMs have a main chain of β-(1,4)-linked D-glucopyranosyl and β-(1,4)-linked D-mannopyranosyl backbone sometimes with a α-(1,6)-linked D-galactose side chain (Korolenko et al., 2019). These NSPs have excellent immuno-enhancing, anti-tumor, hepatoprotective, cardioprotective, and antioxidant activity (Liu et al., 2022; Wang, Zhou, Wang, & Cao, 2022). Particularly, mannose, one of the degradation products of GMs, is taken up by the same transporter(s) as glucose but slows growth in several tumor types *in vitro* and *in vivo* (Gonzalez et al., 2018). Thus, there is an increasing demand for natural antioxidants and immunomodulators from polysaccharides of plants, due to their relatively low toxicity and relatively few side effects (Lin et al., 2019). *D. catenatum* is one of the most abundant natural plant resources with water-soluble polysaccharides, whose content of TP accounts for up to 50% of its dry matter content; its major structural type is GM in the stems (Yue, Zeng, & Ding, 2020). Recently, several studies e.g. (Deng et al., 2016) have assessed polysaccharides and their molecular weight distribution and monosaccharide composition in *Dendrobium* and *Panax* species, using saccharide mapping and chromatographic methods; however, the ability to apply such methods in the quantitative and qualitative determination of NSPs from different *D. catenatum* samples remains unknown.

It seems beyond dispute that the synthesis and accumulation of plant active ingredients largely depend on genes and the environment. Typically, cultivation conditions have a significant effect on the content and properties of polysaccharides from natural materials, including the growth stages (Détár et al., 2021), genetics (Pavloušek & Kumšta, 2011), and environmental conditions, such as light (Horrer et al., 2016), temperature (Wu et al., 2016), drought (Ahl et al., 2019), and precipitation (Walia et al., 2021). In previous studies, we examined the morphological and agronomic characteristics of wild-simulated *D. catenatum* plantings on rocks and the bark of pear trees and compared our findings to those obtained from facility-aided cultivation; our results revealed the complexity of the phytochemical contents and composition in wild simulated environments (Lin et al., 2017). In addition, the harvest time affects the polysaccharide content in the stems and leaves of *D. catenatum*; the TP content is highest before flowering, and the amount of glucose that constitutes TP decreases significantly after the flowering period (Liu et al., 2015). However, the content changes in NSPs, the core bioactive ingredients of *D. catenatum*, have not been clarified with respect to the cultivation conditions and growth stages.

In this study, we investigated the differences in starch and bioactive polysaccharide content in the stems of *D. catenatum* obtained from three cultivation modes (lithophyte on rocks, epiphyte on the bark of pear trees and facility cultivated on pots) and harvest times (May and September) by comparative analyses of the activity, structure, and contents. To the best of our knowledge, this is the first study to analyze the NSPs of *D. catenatum* in different cultivation modes. Consequently, our results are expected to provide useful information for current production and quality control practices related to *D. catenatum*.

2. Materials and methods

2.1. Plant materials

Wild *D. catenatum* is a scarce resource in nature. It requires a bit of luck for *D. catenatum* hunters to seek them. In recent years, it has been occasionally found in moderately damp mountains at an altitude of 1600 m in some provinces of China, such as Zhejiang, Guangdong, Fujian, Hunan, and Yunnan. Typically, the native environment where *D. catenatum* is living is the vertical rock wall of Danxia landform or the position close to the top of a tree trunk. Because of the scarcity of wild *D. catenatum,* its production is insufficient to meet market demand. As a result, the raw products of *D. catenatum* currently sold on the market are all come from artificial cultivation.

D. catenatum 'Jingpin Tianmushan', numbered Zhejiang SSVDC0112018, was cultivated in a standard planting base in Lin'an, Zhejiang Province, China. The precise location was 119°26′11″ E, 30°20′30″ N, at 280 m above sea level. Fig. 1 shows the three cultivation modes: lithophyte (plant epiphytes on vertical rock walls with no obstructions above, under natural light), pear tree epiphyte (shading of trees and leaves of 70–80%, with a light intensity of 3000–5000 lx), and greenhouse cultivation (with 70% net shade and an irrigation spray device). A common advantage of the first two modes is that the wild living environment of *Dendrobium* is restored as much as possible.

Polysaccharide content is higher during the growing season, with the highest content occurring before the flowering period in May and the lowest content coinciding with autumn and winter seasons (Yu, Guo, Si, Wu, & Wang, 2014). As such, we sampled the plantings at two time points for our analyses. The biennial stems from the three cultivation modes were collected on May 9, 2018, and September 6, 2018. Samples were freeze-dried to obtain a constant weight, crushed, passed through a 60-mesh sieve, and then stored for further use in a dryer. The botanical origin of plants was identified by Prof. Jinping Si. The voucher specimens were deposited in the School of Forestry and Biotechnology of Zhejiang A&F University.

2.2. Chemicals and reagents

Pullulan standards (molecular weight (Mw): 6.2, 10.0, 21.7, 48.8, 113.0, 200.0, 366.0, and 805.0 kDa) were purchased from American Polymer Standards Corporation (APSC) (Mentor, OH, USA). α-Amylase was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). D-mannose (Man), D-glucose (Glc), L-rhamnose (Rha), D-xylose (Xyl), D-galactose (Gal), D-galacturonic acid (GalA), and D-glucuronic acid (GlcA) were obtained from the National Institutes for Food and Drug Control (Beijing, China). Iodine, potassium iodide, and ABTS$^+$ were acquired from Aladdin Biochemical Technology Co, Ltd (Shanghai, China). 1-phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Macklin Biochemical Technology C., Ltd (Shanghai, China). Deionized water was prepared using a Millipore Milli-Q Plus system. Acetonitrile was chromatographic grade; all other reagents were of analytical grade.

An Agilent 1200 high performance liquid chromatography system (DAD Detector; RID Detector, Agilent Technologies, Santa Clara, CA, USA), a Nicolet Nexus FT-IR spectrometer (Thermo Nicolet Nexus, Waltham, MA, USA), a SpectraMax 190 light absorption microplate reader (Molecular Devices, San Jose, CA, USA), and an ALPHA 1–2 LD Plus lyophilizer (Christ, Germany) were used for the analyses.

2.3. Preparation of test sample solution

The reflux of powdered *D. catenatum* (0.15 g) was performed with distilled water (25 mL) for 2.5 h at 100 °C. After cooling, an aliquot of extracted solution was centrifuged at 6000× g for 30 min. The supernatant (5 mL) was precipitated for 1 h below 4 °C via ethanol addition to a final concentration of 80% (v/v). The resulting precipitate was collected by centrifugation and washed twice with 80% ethanol (20 mL).

A **B** **C**

Fig. 1. *D. catenatum* cultivated in 3 different environments: (A) Lithophyte on rocks. (B) Epiphyte on the bark of pear trees. (C) Facility cultivated on pots with the substrate of pine barks.

The residue was re-dissolved in hot water to 5 mL; then samples (2 mL) were freeze-dried. The crude polysaccharide solution was deemed TP.

In addition, 5 mL the supernatant was taken for α-amylase digestion (Xu et al., 2016). The supernatant was added to the same amount of 10 mM Tris-maleate buffer for 30 min. The sample was placed in a boiling water bath and boiled for 7 min to gelatinize the starch granules. After cooling and equilibrating the sample to 40 °C, α-amylase solution (15 U/ mL) was added to the sample and incubated for 1.5 h at 40 °C. To test if the starch had degraded completely, an iodine reagent test (0.5% iodine in 5% potassium iodide) was conducted. Then, four volumes of cold absolute ethanol were added, and precipitate polysaccharides were allowed to precipitate overnight at –20 °C. Next, the sample was centrifuged at 6000× g at room temperature for 1 h. The supernatant was removed and the samples were washed twice with cold 80% ethanol (20 mL). The residue was collected as NSPs and re-dissolved in hot water for high-performance gel permeation chromatography–refractive index detection (HPGPC-RID) analyses.

2.4. Starch-iodide reaction

The iodine-potassium iodide solution was prepared by dissolving 0.25 g iodine and 2.0 g potassium iodide in 25 mL distilled water. The starch–iodide reaction was carried out in a mixture of the chromogenic agent and 200 μL each sample (Juliano, 1971).

2.5. Scavenging activity of polysaccharide on ABTS$^+$ free radicals

Determination of antioxidant activity was carried on the basis of previous reports, with some modifications (Zhang et al., 2017b). Briefly, ABTS radical cations were produced by reacting 25 mL ABTS$^+$ solution (7 mM) with 0.44 mL potassium persulfate (2.45 mM); the mixture was allowed to stand in the dark at room temperature for 16 h. At the time of use, the ABTS$^+$ solution was diluted with PBS (0.1 M, pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. The mixture solution, containing 150 μLpolysaccharide of different concentrations (1, 2, 3, and 4 mg/mL) and 100 μL ABTS, was allowed to undergo reaction in dark conditions for 30 min, followed immediately by absorbance measurements recorded at 734 nm using a Spectra Max 190 system (Molecular Devices). VC (4 mg/ mL) was used as positive control set. The scavenging activity of polysaccharide on ABTS free radicals was calculated according to the following:

ABTS scavenging activity $(\%) = [1 - (A_s - A_b)/A_0] \times 100$

where A_s represents the absorbance of the sample reaction, A_b represents the absorbance of the sample reaction without adding the ABTS solution, and A_0 represents the absorbance of the sample reaction without adding the sample reaction.

2.6. Evaluation of polysaccharide content by phenol-sulfuric acid colorimetry

The phenol-sulfuric acid method was used for polysaccharide content analyses of TP and NSPs, according to the Chinese Pharmacopoeia (2020 Edition). Briefly, 1 mL 5% phenol was added to 1000 μL polysaccharide solution. Then the solution was mixed with 5 mL concentrated sulfuric acid via shaking. The reaction solution was placed in a boiling water bath for 20 min. After the reaction solution had cooled in an ice bath for 5 min, the absorbance was measured at 488 nm. Glucose standard solutions with a concentration range of 10 to 100 μg/mL were used to calculate a standard curve; the reaction solution to which 1000 μL distilled water was added was used as a blank control. Each sample was set as three replicates.

2.7. Analyses of polysaccharides by high-performance gel permeation chromatography with a differential refractive index detector (HPGPC-RID)

The content and molecular weight of specific polysaccharides from *D. catenatum* were measured via HPGPC-RID according to a previous study (Balli et al., 2020), with minor modifications. Briefly, two columns (length: 300 mm; internal diameter: 7.8 mm; Welch, Shanghai, China) with particles 5 μm in diameter were used in XtimateTM *SEC*-1000 and XtimateTM *SEC*-300 analyses. An Agilent 1200 high-performance liquid chromatography (HPLC) system was coupled to a RID system for measurements conducted at 35 °C. The mobile phase used double-distilled water at a flow rate of 1.0 mL/min. The average weight-averaged molecular weight (Mw) values of the polysaccharides were determined based on a standard curve produced by Pullulan standards (Sigma-Aldrich). The polysaccharide solution was filtered through a 0.22 μm nylon membrane for analyses.

2.8. Monosaccharide composition analyses

The monosaccharide compositions of sample polysaccharides were determined using a PMP-labeling procedure. Polysaccharide solution (1 mL) was hydrolyzed with 500 μL 3 M hydrochloric acid at 110 °C for 1 h in a sealed test tube. Subsequently, excess hydrochloric acid was removed by neutralization with the addition of 500 μL 3 M NaOH. Next, the polysaccharide hydrolysis solution (400 μL) was labeled with PMP by adding 400 μL 0.3 M NaOH and 400 μL 0.5 M PMP methanol solution. The mixture was incubated at 70 °C for 100 min. The reaction product was neutralized with 400 μL 0.3 M HCl solution and extracted three times with 2 mL chloroform. The aqueous phase was filtered through a 0.22 μm nylon membrane for HPLC analyses. The HPLC analyses were coupled to a DAD detector and a Venusil MP C$_{18}$ column (250 mm × 4.6 mm) maintained at 35 °C. The column (250 mm × 4.6 mm) was eluted with a mobile phase consisting of 0.02 M ammonium acetate solution and acetonitrile (83:17, v/v) at 1 mL/min. Standard monosaccharides

solution mixed with 0.10 mg/mL Man, 0.10 mg/mL Glc, 0.10 mg/mL Rha, 0.12 mg/mL Xyl, 0.09 mg/mL Gal, 0.12 mg/mL GalA, and 0.10 mg/mL GlcA was determined using the same procedure. The monosaccharide content was calculated according to the calibration curve (peak area and concentration) of each monosaccharide standard.

2.9. Fourier transform infrared spectroscopy analyses

The IR spectra of *D. catenatum* polysaccharides were recorded using a Fourier transform infrared (FT-IR) spectrophotometer. The sample (2 mg) was ground into powder with spectroscopic-grade potassium bromide (KBr) powder and pressed into a disk for FT-IR measurement in the frequency range of 4000–400 cm^{-1}.

2.10. Data processing and statistical analyses

All experiments were repeated at least three times. The results were generated using EXCEL software (version 2007, Microsoft Corp., Redmond, WA, USA) and are expressed as the mean ± standard deviation. SPSS 19.0 software (IBM SPSS, Chicago, IL, USA) was used for the statistical analyses. Statistical significance was determined using one-way analysis of variance with GraphPad Prism 6.0 software (San Diego, CA, USA). A p-value < 0.05 was considered statistically significant. HPLC data were first processed by Agilent ChemStation and exported as AIA files to professional software, the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (version 2004A), for calibration of the retention time of peaks. Principal component analyses were carried out using SIMCA 13.0 software (Umetrics, Malmö, Sweden).

3. Results and discussion

3.1. Extraction of NSPs from D. Catenatum

TP was extracted according to a classic method according to the Chinese Pharmacopoeia (2020 edition) as well as our previous study (Zhang et al., 2017a). After extraction with hot water and precipitation in 80% alcohol, the TP of *D. catenatum* was obtained. Fig. 2A shows the blue color of the starch–iodide reaction product indicating that TP from *D. catenatum* contained starch. Then the starch in the obtained TP was digested using α-amylase (final concentration: 15 U/mL) into small sugar molecules, and removed using alcohol (Li et al., 2019). As expected, after α-amylase digestion, the positive blue color response of polysaccharides on iodine and the potassium iodide solution had disappeared (Fig. 2A). Thus, the NSPs solution was obtained without starch. Since the main component of NSPs in *D. catenatum* is glucomannan, which contains β-(1,4)-linked D-glucopyranosyl and β-(1,4)-

linked D-mannopyranosyl bonds and cannot be hydrolyzed by α-amylase, the loss of NSPs was only 1.3% in the previous study so the loss of this part could be neglected (Y. Li et al., 2019).

3.2. In vitro antioxidant activity of polysaccharides

As shown in Fig. 2B, the *in vitro* antioxidant activity of NSPs was significantly stronger than that of the TP. In contrast to the well-known antioxidant standard vitamin C (Vc), the 4 mg/mL NSPs solution showed similar antioxidant activity. The radical scavenging rate of the sample from lithophyte cultivation was significantly higher than those of other tested samples. The IC$_{50}$ of ABTS$^+$ scavenging rate was calculated for the concentrations of 1, 2, 3, and 4 mg/mL each NSPs sample; the average IC$_{50}$ value was 1.89 mg/mL. Similar with the NSPs from *D. devonianum*, the *D. catenatum* NSPs also showed very strong scavenging abilities for ABTS$^+$ radicals (Fan, Lin, & Luo, 2022). The increased antioxidant activity of the NSPs is likely due to starch removal, which is consistent with results showing that the starch content in wheat is negatively correlated with antioxidant activity (Sumczynski et al., 2015). However, it is not clear as to why there are differences in NSPs activity with respect to the cultivation method, which requires further study.

3.3. Phenol-sulfuric acid colorimetry analyses

As shown in Fig. 3, in the flowering period of May, the TP contents of lithophytic, tree epiphytic, and facility cultivation of *D. catenatum* were 512.2, 473.3, and 462.8 mg/g, respectively; after pretreatment with α-amylase digestion, the contents decreased by 14.0%, 20.9%, and 24.3% respectively, which means that the proportion of starch in TP can be as high as one-fifth, particularly in facility cultivation. Consistently, in September, corresponding to the period after flowering, the TP contents were 418.2, 385.9, and 431.4 mg/g accordingly for lithophytic, tree epiphytic, and facility cultivation, respectively, and α-amylase pretreatment reduced polysaccharide content by 6.0%, 7.7%, and 19.5%. Therefore, TP and NSPs content and the ratio between NSPs and TP were influenced by the cultivation environment and the development stage.

The cultivation conditions significantly affected polysaccharide content. The stems of *D. catenatum* collected from the lithophytic mode contained the highest NSPs content, whereas facility cultivation produced the most starch. It is interesting to note that *D. catenatum* cultivated in the greenhouse had the highest TP content in September, but contained the lowest active polysaccharide NSPs as a result of the highest content of starch among the three cultivation modes. As the NSPs content was greater in lithophytic samples than epiphytic ones, and even higher than facility-cultivated samples from both May and September, it can be deduced that the simulated natural cultivation
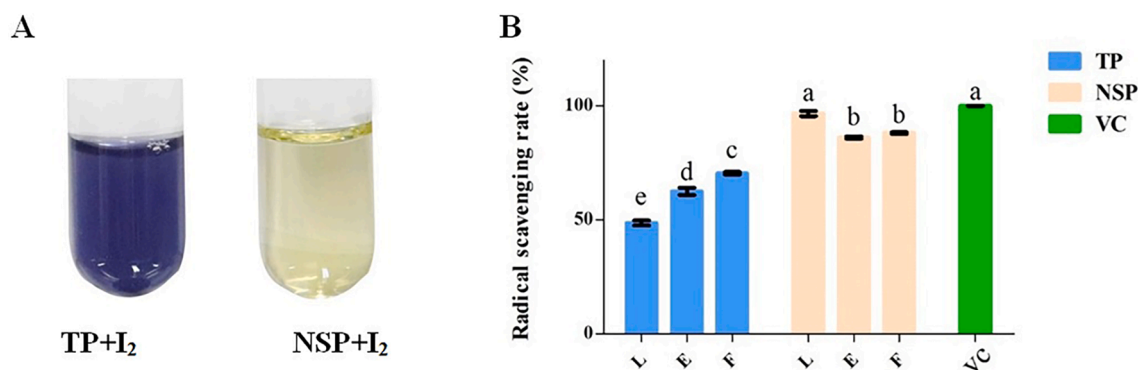

Fig. 2. Polysaccharides and their *in vitro* antioxidant activity of *D. catenatum*. (A) Starch-iodide reaction of total polysaccharide (TP) and non-starch polysaccharides (NSPs). (B) ABTS$^+$ scavenging rate of TP and NSPs (4 mg/mL) from *D. catenatum* cultivated in different patterns. **L**: lithophyte on rocks; **E**: epiphyte on the bark of pear tree; **F**: facility cultivated on pots. The data are presented as means ± SD from three independent experiments. Different lowercases within each bar mean a statistical difference at *P* < 0.05.
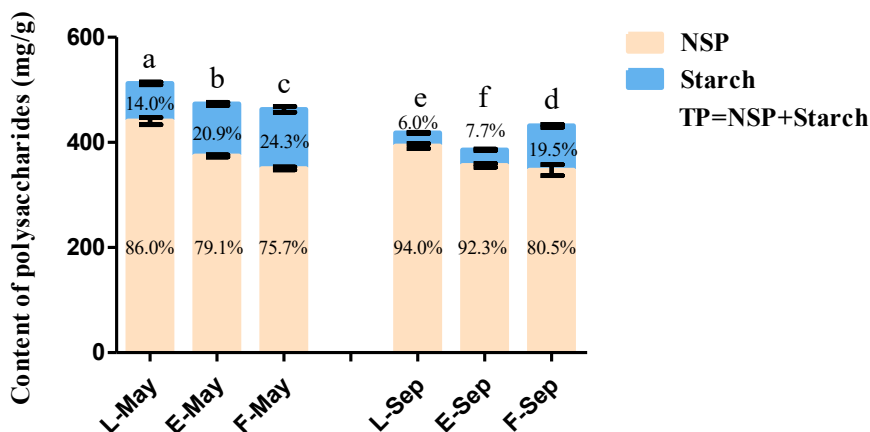
Fig. 3. NSPs and starch content (mg/g) of *D. catenatum* in different cultivation patterns and harvest times. The unit of mg/g was calculated as milligrams of polysaccharides per gram of *D. catenatum* powder dry weight. The data are presented as means ± SD from three independent experiments. Different lowercases above each columns show significant difference in the TP content (*P* < 0.05)The relative percentages of each NSPs and Starch in TP are also marked on the figure.

pattern is conducive to the synthesis and accumulation of active polysaccharide components, whereas *D. catenatum* grown in a greenhouse, the best growth mode for maximum yield, showed relatively poor quality due to starch (inactive polysaccharides) accumulation.

The harvest time also had a significant effect on polysaccharide content. In the same environment, both TP and NSPs contents were higher in May than in September. May is the time during which *D. catenatum* begins to bloom in Zhejiang Province, China. Blooming consumes a significant amount of nutrient starch (Liu et al., 2014). Thus, the stems of *D. catenatum* synthesize and accumulate more starch before blooming in May. As a result, there was a significant decrease in TP content. The content of NSPs, accounting for 75.5% to 94.0% of the TP,

decreased after flowering to a varying degree, thus suggesting that GM and other NSPs may also act as energy storage substances and may be consumed during the flowering period.

3.4. Content of specific polysaccharides in D. Catenatum

Although it is difficult to discriminate the starch and NSPs using a direct one-step on-line HPGPC-RID method due to the complexity of the components, the method combining two-step HPGPC-RID analyses with α-amylase digestion has proven to be very efficient for quantitative determination of polysaccharide content including TP and NSPs. The polysaccharide contents of the three cultivation modes were analyzed;
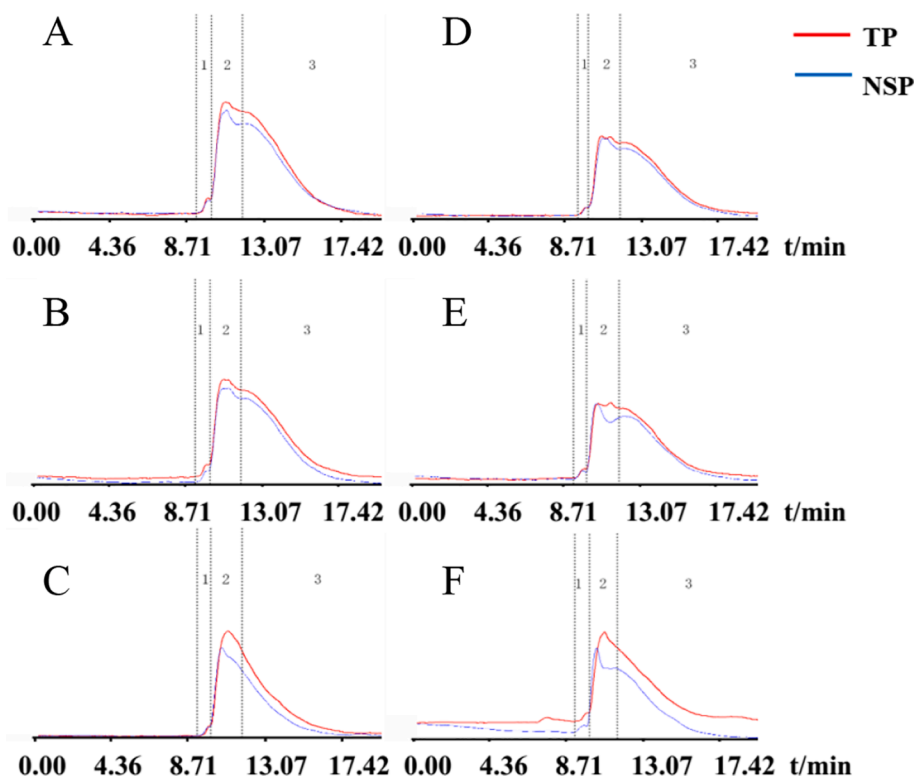

Fig. 4. HPSEC chromatograms of total polysaccharides (red line) and non-starch polysaccharides (blue line) from *D. catenatum* samples. (**A, B, C**) samples collected from rock (L), pear tree (E) and greenhouse (F) in May; (**D, E, F**) samples collected from rock (L), pear tree (E) and greenhouse (F) in September. Elution time for three different molecular weight fractions are shown (vertical dash line). 1, 2, 3, means the different HPLC fractions of polysaccharides.

the results are shown in Fig. 4. The standard curve and regression equation were established as follows: $\log M_w = 9.6971 - 0.3076\,t$, with R = 0.9969 (in which Mw is the weight-average molecular weight and t is the retention time). The polysaccharides from *D. catenatum* were divided into three fractions according to the chromatogram peak shapes and their relative molecular mass: fraction 1 (4.3×10^6–6.6×10^6 Da), fraction 2 (1.5×10^6–4.3×10^6 Da), and fraction 3 (0.0085×10^6–1.5×10^6 Da). The standard curve for calculating the polysaccharide content of each fraction was $Y = 79949X + 1596.4$, with $R^2 = 0.9991$ (where Y is the peak area of each polysaccharide fraction and X is the content of polysaccharide fractions calculated using the Pullulan standard with M_w of 8.05×10^5 Da). Our results revealed a linear relationship from 0.01 to 2.00 mg/mLof polysaccharide concentration. The established HPGPC-RID method was applied to quantify TP (including starch and specific polysaccharides), NSPs (amylase-treated polysaccharides), and their three fractions in *D. catenatum* samples. Table S1 summarizes the contents of the investigated polysaccharides.

The proportions of the three fractions in polysaccharides (TP and NSPs) were similar among the samples with respect to the different harvest times and cultivation modes. Fraction 1 accounted for 1.73–3.21% of polysaccharides, fraction 2 accounted for 26.88–34.31%, and fraction 3 accounted for 63.68–70.80%. Although the method of HPGPC-RID method using the Pullulan standard with Mw of 8.05×10^5 Da was different from the polysaccharide content measured by phenol-sulfuric acid method, the sum of the contents of the three fractions was consistent with the results of TP and NSPs content determined using glucose as the standard of the phenol-sulfuric acid method. With respect to the TP content for the three cultivation modes, the order from high to
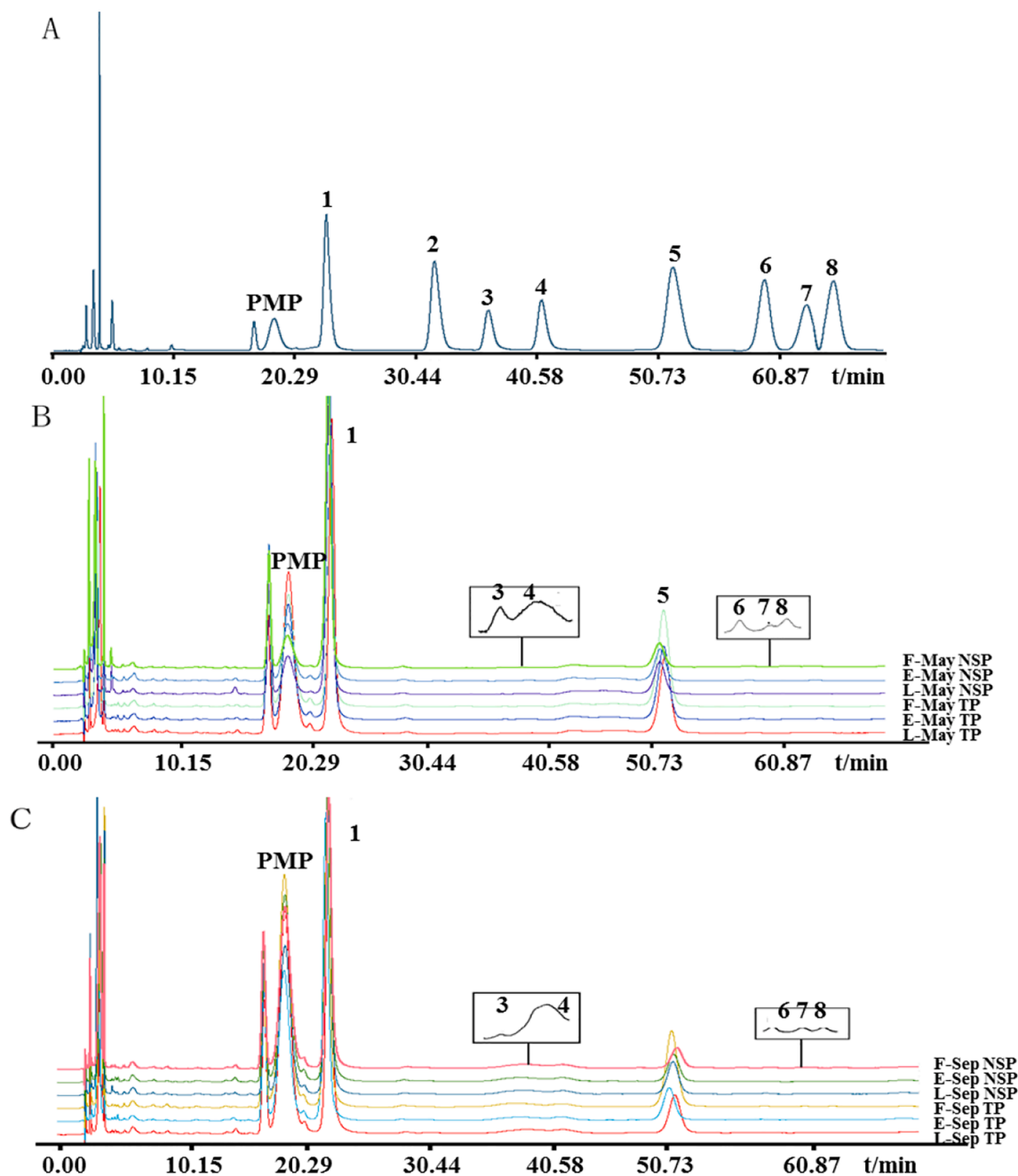

Fig. 5. HPLC chromatograms of monosaccharides. (**A**) Mixed standards, including 1. mannose (man), 2. rhamnose (rha), 3. glucuronic acid (glcA), 4. galacturonic acid (galA), 5. glucose (glc), 6.galactose (gal), 7. xylose (xyl), 8. arabinose (ara). (**B**) The composition of polysaccharides from *D. catenatum* cultivated in different environment and collected in May. (**C**) Samples collected in September.

low was as follows: lithophytic > epiphytic > facility-aided cultivation. The harvest time affected the TP and NSPs content, in which the total content was higher in May than in September.

Comparing the curves of TP and NSPs, the peak height was considerably lower after starch removal in NSPs (Fig. 3). The starch was relatively more distributed in fraction 3 when samples were collected in May than in September. For example, the *D. catenatum* collected from pear trees in May contained 2.19, 10.88, and 44.49 mg/g starch in the three fractions (1–3, respectively), compared to the results from September showing amounts of 0.64, 17.21, and 7.69 mg/g. These results indicate that starch in *D. catenatum* had no fixed molecular weight, consistent with studies on rice and buckwheat (Deng, Yang, Li, Zeng, Li, Zhong, & Ren, 2021; Wang, Wang, Wang, Qiu, & Li, 2021). In addition, flowering consumed more starch with related lower molecular weight fractions. By contrast, from May to September, *D. catenatum* of lithophyte consumed 49.95 mg/g fraction 3 of NSPs, the samples from pear tree epiphyte consumed 12.48 mg/g, whereas the greenhouse cultured ones increased 5.56 mg/g. This may be because facility-aided cultivated *D. catenatum* has sufficient starch to provide energy for flowering, as opposed to simulated naturally cultivated ones, particularly cliff-cultured plants, that cannot provide enough starch and consequently consume NSPs to support flowering.

3.5. Monosaccharide composition

HPLC with pre-column derivatization has been widely employed for qualitative and quantitative analyses of compositional monosaccharides in polysaccharides from plants(Li et al., 2013). The results in Fig. 5 and Table S2 show that the HPLC profiles of TP and NSPs from *D. catenatum* were similar; the main compositional monosaccharides were Man and Glc with the concentration from 20.05 mg/g to 125.54 mg/g, as well as minor GlcA, Gal, Xyl and Ara with the concentration less than 5.23 mg/g. However, the monosaccharide content fluctuated with a change in harvest time, cultivation mode, and enzyme pretreatment. The glucose content showed a remarkable decrease after α-amylase treatment (up to 58.11 mg/g in the samples collected from greenhouse in May), which confirmed that the polysaccharides of *D. catenatum* contained starch.

The glucose content of greenhouse samples was twice that of lithophyte and 2.4 times that of pear epiphyte (Table S2). But the contents sharply decreased in facility-grown samples after enzyme digestion, whereas lithophyte and epiphyte samples experienced a slight decrease. In the samples collected in May, the glucose content decreased 38.41 mg/g, 47.71 mg/g and 58.11 mg/g from rock lithophyte, pear tree epiphyte, and facility cultivation patterns respectively. Meanwhile, the index of glucose content decreased by 0.88 mg/g, 7.56 mg/g and 56.21 mg/g in September. This result indicated that samples cultivated in the greenhouse accumulated much more starch than that in wild-simulated cultivation patterns in September, when the trend of the samples was similar to that in May. Moreover, there was no siginificant difference in the content of glucose contents for the three cultivation modes in May. But four months after, the glucose content for these three cultivation modes showed significant changes. Furthermore, glucose decreased predominantly from May to September, as a possible result of flowering that consumed glucose for energy.

In addition, there were no significant differences in the mannose or glucose content of NSPs. However, the molar ratio of mannose to glucose changed dramatically for the three cultivation modes and two growth stages. Particularly, in the three samples collected in May and the greenhouse samples in September, the ratio was less than 2, which was caused by the high content of starch composed of glucose. Turning to NSPs, the ratio of Man:Glc was ranged from 3.33 to 5.64. It suggested that the high proportion of mannose in NSPs is closely related to its enhanced biological activity (Huang et al., 2015).

3.6. Structure analyses by FT-IR spectroscopy

The main observed modes in the *D. catenatum* polysaccharides appeared at 3406, 2928, 2890, 1735, 1652, 1637, 1377, 1249, 1151, 1092, 1065, 1024, 958, 896, 878, and 809 cm^{-1} (Fig. 6). The mode at 3406 cm^{-1} is the characteristic stretching vibration of the –OH groups coupled with hydrogen bonding. In addition, the mode at 2928 and 2890 cm^{-1} represents C—H stretching and arises from methyl units. Ester carbonyl groups (C═O), mainly sourced from the acetyl group of GM appeared at 1735 cm^{-1}. The modes at 1652 and 1637 cm^{-1} are associated with the bending vibration of O—H. In addition, stretching vibration of the C—O fragment was observed at 1249, 1151, 1092, 1065, 1024, and 958 cm^{-1}. The existence of β-1,4 glycosidic and β-1,4 mannosidic linkages characteristic of GM were assigned to C—O—C stretching at 1024 and 1249 cm^{-1}. The peaks at 896 cm^{-1} were of β-anomeric carbon, indicating that the two fractions mainly contained β-type glycosidic linkages (Y. Y. Deng et al., 2014). In addition, C—H bending appeared at 878 and 809 cm^{-1} and is attributed to the β-pyranose form of glucose and mannose, respectively (Huang et al., 2016).

The difference between the TP and the NSPs is the obvious reduction in the intensity of the entire IR spectrum as a result of starch digestion. However, the absorption peak intensity at 958 cm^{-1} and the uronic acid content in September were higher than in May, when the absorption intensity of the rock wall and pear tree at 1735 and 958 cm^{-1} in different habitat samples became greater than that observed for greenhouse samples. In September, the absorption intensity of the rock wall and pear tree at 956 cm^{-1} in different habitat samples appeared higher than that in the greenhouse. In May and September, the absorption intensity of the rock wall and pear tree at 809 cm^{-1} appeared to be greater than that in the greenhouse, and the proportion of artificially wild cultivated mannose was larger. After amylase treatment, the absorption intensity of characteristic peaks at 809 cm^{-1} increase on the samples from rock wall, pear tree and greenhouse, and the absorption intensity of characteristic peaks at 1735 and 958 cm^{-1} on the pear tree and the greenhouse also increase. In September, the absorption intensity of characteristic peaks at 1735 and 809 cm^{-1} of the rock wall and pear tree increased slightly, and that at 956 and 810 cm^{-1} of the greenhouse increased. Thus, the culture environment and harvest season affected the structure of polysaccharides and starch-free polysaccharides. After α-amylase treatment, the proportion of uronic acid and mannose in each sample increased; thus, the potential pharmacological activity may be enhanced.

4. Conclusion

The present study revealed that the changes in NSPs content in *D. catenatum* differed from that of TP, due to the degree of starch accumulated in different environments and harvesting periods. Wild-simulated cultivation, including lithophytation and epiphytation, was beneficial for obtaining polysaccharides with low starch and high NSPs content, particularly in September. As NSPs possess enhanced antioxidant activity, wild-simulated cultivation is recommended as a means of obtaining higher-quality stems of *D. catenatum*. In addition, epiphytation on the bark of pear trees might be a more recommended cultivation model than lithophytation because of the higher biomass during the same growing time. Furthermore, NSPs analyses methods are not only capable of ensuring *D. catenatum* quality but also could play a significant role in the quality control of their functional food products. Our results are expected to be useful regarding the quality content of NSPs and the selection of culture mode and harvest time. Our findings also provide a reference for developing quality evaluation methods for NSPs in other herbal medicines.

Declaration of Competing Interest

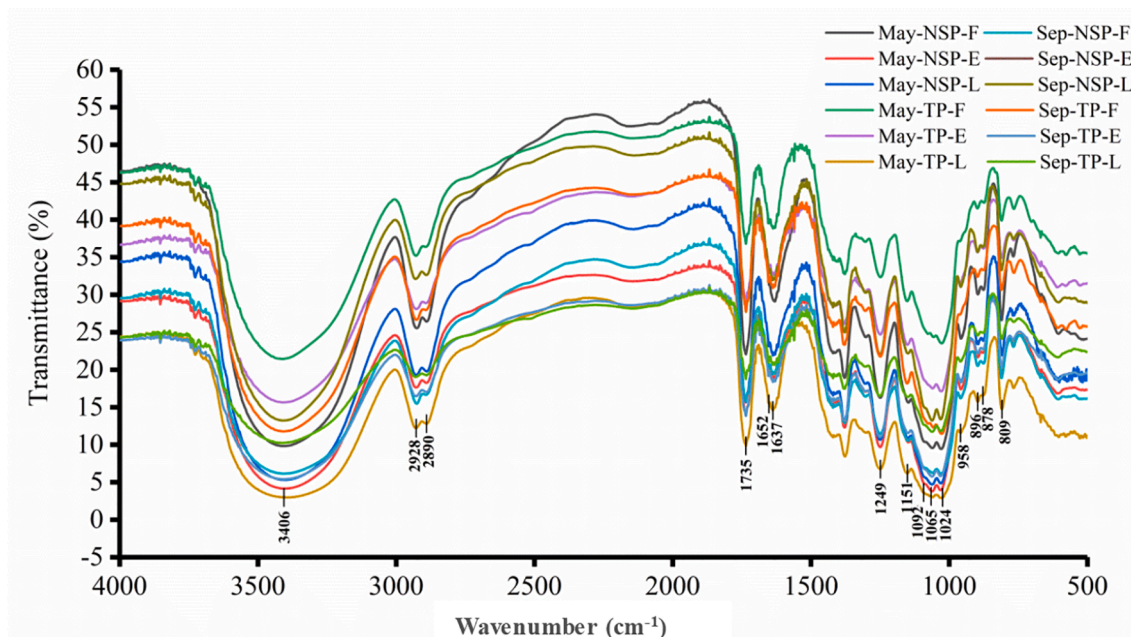The authors declare the following financial interests/personal

Fig. 6. The representative FT-IR spectra of polysaccharide solution in *D. catenatum*.

relationships which may be considered as potential competing interests: Jinping Si reports administrative support was provided by Ministry of Science and Technology of the People's Republic of China. Jingjing Liu reports financial support was provided by National Natural Science Foundation of China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100309.

References

Ahl, L. I., Mravec, J., Jørgensen, B., Rudall, P. J., Rønsted, N., & Grace, O. M. (2019). Polysaccharide composition of folded cell walls in drought:tressed succulent *Aloe* species. *Plant Cell and Environment, 42*, 2458–2471. https://doi.org/10.1111/pce.13560

Balli, D., Cecchi, L., Khatib, M., Bellumori, M., Cairone, F., Carradori, S., … Mulinacci, N. (2020). Characterization of Arils Juice and Peel Decoction of Fifteen Varieties of Punica granatum L.: A Focus on Anthocyanins. *Ellagitannins and Polysaccharides. Antioxidants (Basel), 9*(3), 238. https://doi.org/10.3390/antiox9030238

Chen, X., Wang, Y., Shen, M., Yu, Q., Chen, Y., Huang, L., & Xie, J. (2021b). The water-soluble non-starch polysaccharides from natural resources against excessive oxidative stress: A potential health-promoting effect and its mechanisms. *International Journal of Biological Macromolecules, 171*(1), 320–330. https://doi.org/10.1016/j.ijbiomac.2021.01.022

Chen, W.-H., Wu, J.-J., Li, X.-F., Lu, J.-M., Wu, W., Sun, Y.-q., … Qin, L.-P. (2021a). Isolation, structural properties, bioactivities of polysaccharides from Dendrobium officinale Kimura et. Migo: A review. *International Journal of Biological Macromolecules, 184*, 1000–1013. https://doi.org/10.1016/j.ijbiomac.2021.06.156

Deng, Y., Chen, L.-X., Han, B.-X., Wu, D.-T., Cheong, K.-L., Chen, N.-F., … Li, S.-P. (2016). Qualitative and quantitative analysis of specific polysaccharides in *Dendrobium huoshanense* by using saccharide mapping and chromatographic methods. *Journal of Pharmaceutical and Biomedical Analysis, 129*, 163–171. https://doi.org/10.1016/j.jpba.2016.06.051

Deng, F., Yang, F., Li, Q. P., Zeng, Y. L., Li, B., Zhong, X. Y., . . . Ren, W. J. (2021). Differences in starch structural and physicochemical properties and texture characteristics of cooked rice between the main crop and ratoon rice. *Food Hydrocolloids, 116*, 106643. doi: ARTN 106643 10.1016/j.foodhyd.2021.106643.

Deng, Y.-Y., Yi, Y., Zhang, L.-F., Zhang, R.-F., Zhang, Y., Wei, Z.-C., … Zhang, M.-W. (2014). Immunomodulatory activity and partial characterisation of polysaccharides from Momordica charantia. *Molecules, 19*(9), 13432–13447. https://doi.org/10.3390/molecules190913432

Détár, E., Zámbori-Németh, É., Gosztola, B., Harmath, A., Ladányi, M., & Pluhár, Z.s. (2021). Ontogenesis and harvest time are crucial for high quality lavender – Role of the flower development in essential oil properties. *Industrial Crops and Products, 163*, 113334. https://doi.org/10.1016/j.indcrop.2021.113334

Fan, Y., Lin, M., & Luo, A. (2022). Extraction, characterization and antioxidant activities of an acidic polysaccharide from *Dendrobium devonianum*. *Journal of Food Measurement and Characterization, 16*(1), 867–879. https://doi.org/10.1007/s11694-021-01211-2

Gonzalez, P. S., O'Prey, J., Cardaci, S., Barthet, V. J. A., Sakamaki, J.-I., Beaumatin, F., … Ryan, K. M. (2018). Mannose impairs tumour growth and enhances chemotherapy. *Nature, 563*(7733), 719–723. https://doi.org/10.1038/s41586-018-0729-3

Horrer, D., Flütsch, S., Pazmino, D., Matthews, J. A., Thalmann, M., Nigro, A., … Santelia, D. (2016). Blue Light Induces a Distinct Starch Degradation Pathway in Guard Cells for Stomatal Opening. *Current Biology, 26*(3), 362–370. https://doi.org/10.1016/j.cub.2015.12.036

Huang, K., Li, Y., Tao, S., Wei, G., Huang, Y., Chen, D., & Wu, C. (2016). Purification, Characterization and Biological Activity of Polysaccharides from Dendrobium officinale. *Molecules, 21*(6), 701–717. https://doi.org/10.3390/molecules21060701

Huang, X. J., Nie, S. P., Cai, H. L., Zhang, G. Y., Cui, S. W., Xie, M. Y., & Phillips, G. O. (2015). Study on *Dendrobium officinale* O-acetyl-glucomannan (Dendronan): Part IV. Immunomodulatory activity *in vivo*. *Journal of Functional Foods, 15*, 525–532. https://doi.org/10.1016/j.jff.2015.03.054

Juliano, B. O. (1971). A simplified assay for milled-rice amylose. *Cereal Science Today, 16* (10), 334–340.

Kanlayavattanakul, M., Lourith, N., & Chaikul, P. (2018). Biological activity and phytochemical profiles of *Dendrobium*: A new source for specialty cosmetic materials. *Industrial Crops and Products, 120*(120), 61–70. https://doi.org/10.1016/j.indcrop.2018.04.059

Korolenko, T. A., Bgatova, N. P., & Vetvicka, V. (2019). Glucan and mannan -two peas in a pod. *International Journal of Molecular Sciences, 20*(13), 3189. https://doi.org/10.3390/ijms20133189

Li, Y., Liu, J. J., Zhang, X. F., Wu, S. H., Li, S. P., & Si, J. P. (2019). Study on quantification method of non-starch polysaccharides in *Dendrobium catenatum*. *China Journal of Chinese Materia Medica, 44*(15), 3221–3225. https://doi.org/10.19540/j.cnki.cjcmm.20190527.104

Li, H., Long, C., Zhou, J., Liu, J., Wu, X., & Long, M. (2013). Rapid analysis of mono-saccharides and oligo-saccharides in hydrolysates of lignocellulosic biomass by HPLC. *Biotechnology Letters, 35*(9), 1405–1409. https://doi.org/10.1007/s10529-013-1224-4

Lin, S., Li, H. Y., Wang, Z. Y., Liu, X., Yang, Y., Cao, Z. W., … Qin, W. (2019). Analysis of methanolic extracts and crude polysaccharides from the leaves of *Chuanminshen violaceum* and their antioxidant activities. *Antioxidants (Basel), 8*(8), 266. https://doi.org/10.3390/antiox8080266

Lin, Y. K., Zhu, Y. Q., Si, J. P., Qin, L., Zhu, Y., Wu, L. S., & Liu, J. J. (2017). Effects of cultivation environments on *Dendrobium catenatum*. *China Journal of Chinese Materia Medica, 42*(16), 3084–3089. https://doi.org/10.19540/j.cnki.cjcmm.20170714.018

Liu, J. J., Liu, Z. P., Zhang, X. F., & Si, J. P. (2021). Effects of various processing methods on the metabolic profile and antioxidant activity of *Dendrobium catenatum* Lindley leaves. *Metabolites, 11*(6), 351. https://doi.org/10.3390/metabo11060351

Liu, Z. P., Xu, C. X., Liu, J. J., Si, J. P., Zhang, X. F., & Wu, L. S. (2015). Study on accumulation of polysaccharides and alcohol-soluble extracts contents of Dendrobium officinale leaves. *China Journal of Chinese Materia Medica, 40*(12), 2314–2317. https://doi.org/10.4268/cjcmm20151210

Liu, J., Yu, L., Wang, C., Zhang, Y., Xi, H., Si, J., … Yan, J. (2022). Preparation, structural features and *in vitro* immunostimulatory activity of a glucomannan from fresh *Dendrobium officinale* stems. *Frontiers in nutrition, 8*. https://doi.org/10.3389/fnut.2021.82380310.3389/fnut.2021.823803.s001

Liu, J., Zhang, H., Cheng, Y., Wang, J., Zhao, Y., & Geng, W. (2014). Comparison of ultrastructure, pollen tube growth pattern and starch content in developing and abortive ovaries during the progamic phase in hazel. *Frontiers in Plant Science, 5*, 528. https://doi.org/10.3389/fpls.2014.00528

Panduro, A., Roman, S., Milán, R., Torres-Reyes, L. A., & Gonzalez-Aldaco, K. (2020). *Chapter 10:Personalized nutrition to treat and prevent obesity and diabetes.*

Pavloušek, P., & Kumšta, M. (2011). Profiling of primary metabolites in grapes of interspecific grapevine varieties: Sugars and organic acids. *Czech Journal of Food Sciences, 29*(4), 361–372. https://doi.org/10.5344/ajev.2011.10037

Si, J. P., Wang, Q., Liu, Z. J., Liu, J. J., & Luo, Y. B. (2017). Breakthrough in key science and technologies in Dendrobium catenatum industry. *China Journal of Chinese Materia Medica, 42*(12), 2223–2227. https://doi.org/10.19540/j.cnki.cjcmm.2017.0102

Si, J. P., Zhang, Y., Luo, Y. B., Liu, J. J., & Liu, Z. J. (2017). Herbal textual research on relationship between Chinese medicine"Shihu"(*Dendrobium* spp.) and "Tiepi Shihu"(*D. catenatum*). *China Journal of Chinese Materia Medica, 42*(10), 2001–2005. https://doi.org/10.19540/j.cnki.cjcmm.20170415.002

Sumcznski, D., Bubelova, Z., Sneyd, J., Erb-Weber, S., & Mlcek, J. (2015). Total phenolics, flavonoids, antioxidant activity, crude fibre and digestibility in non-traditional wheat flakes and muesli. *Food Chemistry, 174*, 319–325. https://doi.org/10.1016/j.foodchem.2014.11.065

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science, 74*(10), 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Walia, M. K., Zanetti, F., Gesch, R. W., Krzyżaniak, M., Eynck, C., Puttick, D., … Monti, A. (2021). Winter camelina seed quality in different growing environments across

Northern America and Europe. *Industrial Crops and Products, 169*, 113639. https://doi.org/10.1016/j.indcrop.2021.113639

Wang, L. J., Wang, L. B., Wang, A. L., Qiu, J., & Li, Z. G. (2021). Effects of superheated steam on starch structure and physicochemical properties of buckwheat flour during storage. *Journal of Cereal Science, 99*, 103221. doi: ARTN 10322110.1016/j.jcs.2021.103221.uns.

Wang, X., Zhou, X., Wang, K., & Cao, X. (2022). Structural characterisation and bioactivity of polysaccharides isolated from fermented *Dendrobium officinale*. *J Sci Food Agric, 102*(1), 280–290. https://doi.org/10.1002/jsfa.v102.110.1002/jsfa.11356

Wu, Z. G., Jiang, W., Chen, S. L., Mantri, N., Tao, Z. M., & Jiang, C. X. (2016). Insights from the Cold Transcriptome and Metabolome of Dendrobium officinale: Global Reprogramming of Metabolic and Gene Regulation Networks during Cold Acclimation. *Frontiers in Plant Science, 7*(e0121658), 1653. https://doi.org/10.3389/fpls.2016.01653

Xu, Z., Wang, H., Wang, B., Fu, L., Yuan, M., Liu, J., … Ding, C. (2016). Characterization and antioxidant activities of polysaccharides from the leaves of Lilium lancifolium Thunb. *International Journal of Biological Macromolecules, 92*, 148–155. https://doi.org/10.1016/j.ijbiomac.2016.07.028

Yu, Q. X., Guo, Y. Y., Si, J. P., Wu, L. S., & Wang, L. H. (2014). Variation of polysaccharides and alcohol-soluble extracts content of *Dendrobium officinale*. *China Journal of Chinese Materia Medica, 39*(24), 4769–4772. https://doi.org/10.4268/cjcmm20142414

Yue, H., Zeng, H., & Ding, K. (2020). A review of isolation methods, structure features and bioactivities of polysaccharides from *Dendrobium* species. *Chinese Journal of Natural Medicines, 18*(1), 1–27. https://doi.org/10.1016/S1875-5364(20)30001-7

Zhang, J. Y., Guo, Y., Si, J. P., Sun, X. B., Sun, G. B., & Liu, J. J. (2017a). A polysaccharide of *Dendrobium officinale* ameliorates H₂O₂-induced apoptosis in H9c2 cardiomyocytes *via* PI3K/AKT and MAPK pathways. *International Journal of Biological Macromolecules, 104*(Pt A), 1–10. https://doi.org/10.1016/j.ijbiomac.2017.05.169

Zhang, Y., Zhang, L. H., Liu, J. J., Liang, J. L., Si, J. P., & Wu, S. H. (2017b). *Dendrobium officinale* leaves as a new antioxidant source. *Journal of Functional Foods, 37*, 400–415. https://doi.org/10.1016/j.jff.2017.08.006

Zhang, K.e., Zhou, X., Wang, J., Zhou, Y., Qi, W., Chen, H., … Xie, M. (2021). Dendrobium officinale polysaccharide triggers mitochondrial disorder to induce colon cancer cell death via ROS-AMPK-autophagy pathway. *Carbohydrate Polymers, 264*, 118018. https://doi.org/10.1016/j.carbpol.2021.118018