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Structure characterization and immunomodulating effects of polysaccharides isolated from *Dendrobium officinale*

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1                   **Structure characterization and immunomodulating effects of**  
2                   **polysaccharides isolated from *Dendrobium officinale***

3  
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26

27 **Abstract**

28 A crude polysaccharide fraction (cDOP) has been determined to be the characteristic  
29 marker of *Dendrobium officinale*, an expensive tea material in Asia, but its chemistry and  
30 bioactivity have not been studied. In work reported here, cDOP was de-starched (DOP, 90%  
31 yield), and separated into two polysaccharides, DOP-1 and DOP-2, which were characterized  
32 by monosaccharide composition and methylation analyses, and spectral analyses (FT-IR and  
33  $^1\text{H}$  and  $^{13}\text{C}$  NMR). Both are composed of mannose and glucose, at similar ratios; and have a  
34 similar structure with a backbone of 1,4-linked  $\beta$ -D-mannopyranosyl and  $\beta$ -D-glucopyranosyl  
35 residues. Significant differences were observed only in their molecular weights. Bioassay  
36 using mouse macrophage cell line RAW 264.7 indicated that **DOP and its two sub-fractions**  
37 enhance cell proliferation, TNF- $\alpha$  secretion, and phagocytosis in a dose-dependent manner.  
38 They also induced the proliferation of lymphocytes alone and with mitogens. DOP-1 and  
39 DOP-2 are thus proven to be major, active polysaccharide markers of *D. officinale*.

40

41 **Keywords:** *Dendrobium officinale*, polysaccharides, chemical structure, RAW264.7 cells,  
42 lymphocytes

43

## 44 1. Introduction

45 *Dendrobium* is one of the largest genera in the plant family Orchidaceae. More than  
46 1100 species of *Dendrobium* have been identified, with a wide distribution throughout Asia,  
47 Europe and Australia (1). Totally, 74 species are found in China and more than 50 of them  
48 can be used as “*Shihu*”, a well-known and expensive China tea. *Dendrobii Officinalis Caulis*,  
49 called *Tiepi Shihu* in Chinese, which is derived from dried stems of *Dendrobium officinale*  
50 Kimura et Migo, is traditionally recognized as the best *Dendrobium* species for tonic  
51 purposes, such as benefiting the stomach, supplementing body fluids and strengthening  
52 immunity (2, 3). In addition, its green stem can be either chewed directly or stewed in  
53 porridge, soup and dishes as a high-quality food in diets (4). More and more companies tend  
54 to use *D. officinale* stem as dietary supplements to develop functional beverages (5).

55 For selling in herbal markets, the stems of *D. officinale* are always heated, twisted into a  
56 spiral or spring form, and finally dried; in this form, it is commonly known as *Tiepi Fengdou*.  
57 Nowadays, due to extremely scarce wild resources and increasing demand, *Tiepi Fengdou* has  
58 become one of the most expensive tea materials in herb markets worldwide, particularly in  
59 Southeast Asia. The high diversity of species in the *Dendrobium* genus, a lack of  
60 distinguishing macroscopic identifying characteristics, and high price of *Tiepi Fengdou* have  
61 led to the occurrence of adulterants, confused species, and counterfeits (6). Authentication of  
62 *Tiepi Fengdou* has been a challenge for a long time. A reliable, accurate means of identifying

63 true *Teipi Fengdou* would bring clarity to an uncertain market, allowing authorities to  
64 regulate and certify the authentic species, giving consumers confidence, and safeguarding the  
65 reputation of this expensive China tea.

66         Continuous efforts have been made for authentication of *D. officinale* (2). Different  
67 methods, like microscopic identification, DNA barcoding, fingerprinting small molecules,  
68 and fingerprinting monosaccharide composition, have been studied but all have significant  
69 weaknesses. Neither microscopic identification nor DNA barcoding is easy, and both need  
70 well-trained hands to operate the instruments. More importantly, neither of them can present  
71 quantitative analysis and therefore they will not work if the sample is true but its chemicals  
72 have been extracted. Fingerprinting of both small molecules and hydrolysis products of  
73 polysaccharide (monosaccharide composition) has also been studied (7-11); however, these  
74 methods cannot reliably distinguish *Teipi Shihu* from other *Dendrobium* species.

75         In our previous study, we collected authentic *Teipi Shihu* samples from 5 GAP farms  
76 (mainly in Yunnan Province), 10 other *Dendrobium* species, and 13 commercial samples  
77 from HK local herb stores; and compared their molecular size distribution patterns using  
78 HPLC coupled with HPGPC (high-performance gel permeation chromatography). Excitingly,  
79 the authentic samples from GAP farms displayed identical and unique HPGPC profiles with a  
80 huge peak (named cDOP) with molecular size in the range of 276-877 kDa. This peak was  
81 selected as the authentication marker, because it has not been found in other *Dendrobium*

82 species. This marker has been successfully used as a QC marker in qualitative and  
83 quantitative analysis of *Dendrobium* samples using HPGPC methods; however, little is  
84 known about its chemical structure and bioactivity.

85 In this study, we report the isolation and purification of two water soluble linear  
86 glucomannans (DOP-1 and DOP-2) from this QC marker. We have characterized their  
87 chemical structures using monosaccharide composition analysis, methylation analysis, and  
88 spectral analysis of their FT-IR, ESI-MS, GC-MS, and NMR spectra. Their effects on murine  
89 macrophage RAW 264.7 cells and murine lymphocytes were also assayed.

## 90 2. Materials and methods

### 91 2.1. Materials

92 The authentic *Dendrobium officinale* sample, also named *Tiepi Fengdou*, was kindly  
93 provided by certified production area in mainland China and authenticated by Dr. Chen  
94 Hubiao (12). A voucher specimen was deposited at the School of Chinese Medicine in Hong  
95 Kong Baptist University, Hong Kong.

96 Monosaccharide standards for D-mannose (Man) and D-glucose (Glc) were purchased  
97 from Merck Co. (Darmstadt, Germany). Thermostable  $\alpha$ -amylase (29,559 units/mL),  
98 iodomethane, sodium borohydride, sodium borodeuteride (98% atom% D), and dextran  
99 standards with different molecular weights ( $M_w$ :  $1.1 \times 10^6$ ,  $6.7 \times 10^5$ ,  $4.1 \times 10^5$ ,  $2.7 \times 10^5$ ,  $1.5 \times 10^5$ ,

100  $8.0 \times 10^4$ ,  $2.5 \times 10^4$ , and  $1.2 \times 10^4$  Da) were all purchased from Sigma-Aldrich (St. Louis, MO,  
101 USA). Deuterium oxide (99.9% D) was purchased from J&K Scientific Ltd (Beijing, China).  
102 A total starch assay kit (K-TSTA) was obtained from Megazyme (Wicklow, Ireland). All the  
103 other chemicals or solvents were of analytical grade. Ultrapure water was produced by  
104 Milli-Q system (Millipore, Billerica, USA).

105 LPS (from *Escherichia coli* 0111:B4), Griess reagent (modified), Concanavalin A (Con  
106 A), polymyxin B sulfate salt (poly B), and  
107 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from  
108 Sigma-Aldrich (St. Louis, MO, USA). Mouse IL-6 and TNF- $\alpha$  ELISA kits were purchased  
109 from eBioscience (San Diego, CA, USA). The kit (CellTiter 96<sup>®</sup> AQueous One Solution Cell  
110 Proliferation Assay) was purchased from Promega Inc. (Madison, WI, USA).

111 Animals: Inbred strain female (6 to 8 week-old) BALB/c mice were purchased from the  
112 Laboratory Animal Services Centre of The University of Hong Kong. The animals were  
113 provided with standard pellet diet and water ad libitum and maintained under controlled  
114 conditions of temperature and humidity, with a 12 h light/dark cycles. All experiments with  
115 animals were carried out in accordance with the Animals Ordinance, Department of Health,  
116 Hong Kong Special Administration Region, China for the care and use of experimental  
117 animals. All of the experimental protocols were first approved by the Committee on Use of  
118 Human and Animal Subjects in Teaching and Research of the Hong Kong Baptist University.



119           2.2. *Extraction and purification of polysaccharides from D. officinale*

120           The dried powder of *Dendrobium officinale* (200 g) was defatted with 95% ethanol for 2  
121 days at room temperature under stirring. After filtration, the residue was treated twice with 2  
122 L ultrapure water at 100 °C for 2 h and filtered. The combined extracts were centrifuged  
123 (8,000 rpm, 10 min), and concentrated in a rotary evaporator under reduced pressure at 55 °C.  
124 Then the gel-like solution was precipitated by adding 3 volumes of 95% ethanol under  
125 vigorous stirring. After standing overnight at 4 °C, the precipitate was obtained by  
126 centrifugation (8,000 rpm, 10 min), and further washed with absolute ethanol, acetone and  
127 ether. Finally, the precipitate was dissolved in water, dialyzed against ultrapure water for 3  
128 days, concentrated and lyophilized to yield the crude polysaccharide (cDOP).

129           Then, cDOP was de-starched according to Xing's method (13), and the obtained purified  
130 *Dendrobium officinale* polysaccharide was designated as DOP. A total of 100 mg of DOP was  
131 dissolved in 10 mL of ultrapure water and loaded onto a DEAE-650M anion-exchange  
132 chromatography column (5.0 i.d. × 30 cm). The column was eluted with ultrapure water first  
133 and then with 0.01, 0.03, 0.05, 0.10, and 0.30 M NaCl solutions at the flow rate of 1.5  
134 mL/min. Fractions (20 mL) were collected by a fraction collector and the carbohydrate  
135 content was analyzed by the phenol-sulphuric acid assay method (14). **Two fractions, namely**  
136 **DOP-1 (eluted by ultrapure water) and DOP-2 (eluted by 0.01 M NaCl), were successfully**  
137 **separated from DOP, dialyzed for 48 h, concentrated and lyophilized.**

138           2.3. *Structure analysis*

139           2.3.1. *Molecular weight determination*

140           Homogeneity and the molecular mass of DOP, DOP-1, and DOP-2 were determined  
141 using high-performance gel-permeation chromatography (HPGPC) according to the method  
142 of Xu (12). The analyses were performed on an Agilent 1100 series HPLC-DAD system  
143 (Agilent Technologies, Palo Alto, CA, USA) coupled with evaporative light scattering  
144 detector (ELSD, Grace, Deerfield, USA). Dextran standards with different molecular weights  
145 ( $M_w$ :  $1.1 \times 10^6$ ,  $6.7 \times 10^5$ ,  $4.1 \times 10^5$ ,  $2.7 \times 10^5$ ,  $1.5 \times 10^5$ ,  $8.0 \times 10^4$ ,  $2.5 \times 10^4$ , and  $1.2 \times 10^4$  Da) were  
146 used for the calibration curve.

147           2.3.2. *Chemical components and monosaccharide composition*

148           The total sugar content was measured by phenol-sulphuric acid spectrophotometric  
149 assay (15) and **D-mannose** was used as equivalents. The content of uronic acid was  
150 determined by the modified carbazole and sulphuric acid method (16) with **D-glucuronic acid**  
151 as the standard. Protein content was determined by spectrophotometric method (17) using  
152 bovine serum albumin (BSA) as the standard. For monosaccharide composition analysis,  
153 samples (5 mg) were hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 2  
154 h. Then, the resulting samples were analyzed using high-performance liquid chromatography  
155 (HPLC) coupled with ELSD detector according to the method of Yang (18) with some

156 modifications. Instead of the gradient elution, an isocratic mobile phase consisting of 22:78  
157 (v/v) mixtures of water and ACN was used.

### 158 *2.3.3. Infrared spectroscopic analysis of polysaccharides*

159 DOP and its two fractions (DOP-1 and DOP-2) were characterized by FT-IR  
160 spectroscopy on a Thermo Nicolet 5700 infrared spectrophotometer (Thermo Electron,  
161 Madison, WI, USA) at room temperature (25 °C) using KBr pellets. Samples were dried at 55  
162 °C in a vacuum drying oven for 24 h prior to analysis, and spectra were scanned between  
163 4000 cm<sup>-1</sup> to 450 cm<sup>-1</sup> with the resolution of 4 cm<sup>-1</sup>.

### 164 *2.3.4 Methylation and GC-MS analysis*

165 The methylation analysis of DOP, DOP-1, and DOP-2 were carried out according to the  
166 method of Carre (19) with some modifications (20, 21). Briefly, the dried samples were  
167 dissolved in anhydrous DMSO with constant stirring at 50 °C for 20 h to obtain a well  
168 dissolved solution. Then, dry NaOH powder (20 mg) was added, and the mixture was stirred  
169 at room temperature for 4 h. After that, methyl iodide (1.0 mL) was added slowly, with the  
170 mixture in an ice bath, and another 0.5 mL methyl iodide was added 30 min later. The  
171 solution was stirred for 2.5 h. The methylated polysaccharides were extracted with CH<sub>2</sub>Cl<sub>2</sub>,  
172 and then washed with distilled water three times. In order to remove water, the extract was  
173 passed through an **anhydrous** NaSO<sub>4</sub> column and the eluted solution was evaporated by a

174 stream of nitrogen. The dried methylated samples were detected by FT-IR spectroscopy to  
175 ensure that the peak of –O-H in the region 3500-3100 cm<sup>-1</sup> had been eliminated. Then the  
176 per-methylated samples were hydrolyzed, reduced and acetylated as partially methylated  
177 alditol acetates (PMAA) which were further analyzed by GC-MS for linkage analysis.  
178 Aliquots of the PMAA were separated on a SP-2330 capillary column (30 m×0.25 mm I.D.,  
179 0.2 μm film thickness, Supelco, Bellefonte, PA, USA). The oven temperature was set initially  
180 at 160 °C, increased at the rate of 2 °C/min to 210 °C, and then increased at 5 °C/min to 240  
181 °C.

### 182 *2.3.5 NMR spectrum*

183 The polysaccharides (30 mg) were deuterium-exchanged three times and finally  
184 dissolved in 0.5 mL of D<sub>2</sub>O (99.9% D). The <sup>1</sup>H and <sup>13</sup>C NMR spectra were measured on a  
185 Bruker Advance 400 MHz NMR spectrometer (Bruker, Rheinstetten, Germany) at 298 K.  
186 Acetone was used here as the internal standard ( $\delta_{\text{H}}$  2.225 ppm,  $\delta_{\text{C}}$  31.45 ppm), and Bruker  
187 TopSpin program was used to acquire and process the NMR data.

## 188 *2.4. Immunomodulating effects determination*

### 189 *2.4.1. Cell cultures*

190 The murine macrophage cell line RAW264.7 was obtained from American Type Culture

191 Collection (Manassas, VA) and was propagated in DMEM high glucose medium (Invitrogen  
192 Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Gibco,  
193 Carlsbad, CA, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Carlsbad,  
194 CA, USA).

#### 195 *2.4.2. Cell viability assay*

196 The viability of cells was measured using MTT assay. Briefly, RAW264.7 cells ( $5 \times 10^3$   
197 cells/well) were plated in 96-well microplates overnight and then treated with serial  
198 concentrations of DOP, DOP-1 and DOP-2 for 24 h, respectively. Equal volume of medium  
199 was used as vehicle control. After treatment, cells were stained with MTT at a final  
200 concentration of 0.5 mg/mL in PBS (pH 7.4) for another 4 h in dark and then the medium was  
201 discarded. The formazan crystals present in cells were dissolved by dimethyl sulfoxide. The  
202 absorbance was read at 570 nm in a Benchmark Plus microplate reader (Bio-Rad, Richmond,  
203 CA). The results were expressed as ratio of absorbance values of treatment to vehicle control  
204 cells.

#### 205 *2.4.3. ELISA for quantitative analysis of cytokines*

206 RAW264.7 cells ( $1 \times 10^4$  cells/well) were seeded in 96-well plates and incubated  
207 overnight. Then, **DOP and its two sub-fractions** at 125, 250, and 500 µg/mL, respectively  
208 (plus 10 mg/mL of polymyxin B for eliminating the functions of LPS) were added and the

209 cells were incubated for another 24 h. The cell supernatants were collected by centrifugation  
210 at 1,000×g for 10 min. The amounts (pg/mL) of IL-6 and of TNF-α secretion in culture  
211 supernatants were determined in duplicate by using an ELISA kit according to the  
212 manufacturer's instructions. LPS (100 ng/mL) and LPS plus polymyxin B were used here as  
213 controls.

#### 214 *2.4.4. Phagocytic assay*

215 Dextran ( $M_w$  12 kDa), which is characterized by low immunogenicity and high  
216 resistance to cleavage, was fluorescein isothiocyanate-conjugated and prepared for the  
217 phagocytic assay according to the published method (22, 23). RAW264.7 cells ( $4 \times 10^5$   
218 cells/well) were seeded in 12-well plates overnight and then were exposed to cell culture  
219 medium, serial concentrations of DOP, DOP-1 and DOP-2, and LPS for 24 h. Then the cells  
220 were treated with FITC-dextran (0.1 mg/mL) and incubated for 1 h. After incubation, cells  
221 were washed four times in cold PBS and were analyzed by flow cytometry (BD Biosciences,  
222 USA).

#### 223 *2.4.5. Lymphocyte proliferation assays*

224 Spleens were collected from 6-8 week-old BALB/c mice after killing them by cervical  
225 dislocation. Single cell suspension of splenocytes was prepared according to the method

226 described by Matsuzaki (24). Briefly, the spleens were cut into several pieces and pressed  
227 through 70  $\mu\text{m}$  cell strainer (BD falcon, BD Biosciences, San Jose, CA) into culture medium  
228 using a syringe plunger. After centrifugation at  $100\times g$  for 10 min, cell pellets were washed  
229 twice with Hank's balanced salt solution (HBSS, Gibco, Carlsbad, CA, USA). Spleen cells  
230 were re-suspended in red cell lysis buffer in order to lyse red cells and incubated at room  
231 temperature for 5 min. The lymphocytes were collected and washed twice with RPMI-1640  
232 without FBS. Proliferation of mouse spleen cells was measured using the lymphocyte  
233 transformation assay as previously described (25, 26). Briefly, the resulting pellet was  
234 re-suspended and diluted to  $5\times 10^6$  cells/mL with RPMI-1640 after the cell viability was  
235 assessed by trypan blue exclusion. The cell suspension was incubated in 96-well culture  
236 plates, 100  $\mu\text{L}$  per well. The lymphocyte proliferation of *Dendrobium officinale*  
237 polysaccharides was tested by incubating the mouse splenocytes in the absence or presence of  
238 DOP, DOP-1, and DOP-2 at various concentrations for 48 h. It is known that LPS and Con A  
239 stimulate B cells and T cells, respectively. Thus, we used the optimal concentration of LPS  
240 (20  $\mu\text{g}/\text{mL}$ ) and Con A (1.25  $\mu\text{g}/\text{mL}$ ) as positive control compounds. After that, 20  $\mu\text{L}$  of  
241 CellTiter 96® AQueous One Solution Cell Proliferation reagent was added into each well at 4  
242 h before the end of incubation. The absorbance of cells in each well was measured by  
243 Benchmark Plus microplate reader (Bio-Rad, Richmond, CA) at a wave length of 490 nm,  
244 and the results of OD values were represented as mean  $\pm$  SD.

245 To detect co-stimulatory activity of *Dendrobium officinale* polysaccharides and  
246 mitogenic stimulators (Con A and LPS), the mouse splenocytes were cultured with different  
247 concentrations of DOP, DOP-1, and DOP-2 (125, 250, and 500  $\mu\text{g}/\text{mL}$ ) and two different  
248 mitogens (Con A and LPS) at their predetermined optimal concentrations (Con A: 1.25  
249  $\mu\text{g}/\text{mL}$ ; LPS: 20  $\mu\text{g}/\text{mL}$ ) for 48 h. Subsequent treatment and determinations were performed  
250 as described previously.

## 251 2.5. Data analysis

252 All data were expressed as mean  $\pm$  SD. Statistical analysis was performed using one way  
253 ANOVA, and statistical significance was determined at \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ,  
254 \*\*\*\*  $p < 0.0001$ .

## 255 3. Results

### 256 3.1. Fractions and molecular characterization

257 As shown in Figure 1A, DOP was purified by ion-exchange chromatography on  
258 DEAE-650M. Two main fractions DOP-1 (eluted with ultrapure water) and DOP-2 (eluted  
259 with 0.01 M NaCl) were obtained, accounting for 66.4% and 23.6% of total polysaccharides  
260 by weight, respectively. They both exhibited high homogeneity in HPGPC examination  
261 (Figure 1B). The weight-average molecular weight ( $M_w$ ) of the DOP and its two fractions



262 (DOP-1 and DOP-2) were close, which were found to be  $7.3 \times 10^5$ ,  $8.1 \times 10^5$ , and  $6.7 \times 10^5$  Da,  
263 respectively, which illustrated that DOP could be successfully separated into DOP-1 and  
264 DOP-2 by ion-exchange chromatography.

### 265 3.2. Chemical components and monosaccharide compositions

266 The chemical compositions of cDOP, DOP, DOP-1, and DOP-2 are summarized in Table  
267 1. The average starch content of cDOP was 10.0%, while after de-starching with thermostable  
268  $\alpha$ -amylase, starch was not found in DOP, DOP-1, and DOP-2. A higher percentage of neutral  
269 sugar and lower percentages of protein and uronic acid were found in DOP, DOP-1, and  
270 DOP-2, as compared to cDOP.

271 DOP, DOP-1, and DOP-2 showed similar monosaccharide composition (Figure 1C).  
272 Only two monosaccharides were found, namely mannose (Man) and glucose (Glc). The  
273 mannose percentage, glucose percentage, and mannose/glucose ratios (M/G) are shown in  
274 Table 2. The mannose/glucose ratios for DOP, DOP-1, and DOP-2 were  $5.8 \pm 0.1$ ,  $5.6 \pm 0.1$ , and  
275  $5.9 \pm 0.1$ , respectively.

### 276 3.3. Infrared spectroscopy

277 The FT-IR spectra of cDOP, DOP, DOP-1, and DOP-2 (Figure 1D) were similar. The  
278 broad and strong absorbance bands at  $3436 \text{ cm}^{-1}$  between the region  $3500\text{-}3100 \text{ cm}^{-1}$  can be

279 attributed to the vibration of hydroxyl groups of polysaccharides and water involved in  
280 hydrogen bonding (27). The absorption at around  $2891\text{ cm}^{-1}$  ( $3000\text{-}2800\text{ cm}^{-1}$ ) was assigned  
281 to the C-H stretching vibration of the methyl group (28, 29), and bonds approximately  $1736$   
282  $\text{cm}^{-1}$  were assigned to the valence vibration of C=O (13). Furthermore, peaks at around  $1379$   
283  $\text{cm}^{-1}$  and  $1250\text{ cm}^{-1}$  were due to symmetric C-H bending vibration of the methyl group and  
284 the C-O vibration of O-acetyl groups, respectively (30). The bonds at  $878\text{ cm}^{-1}$  and  $813\text{ cm}^{-1}$   
285 revealed the presence of  $\beta\text{-D}$ -mannopyranose units and  $\alpha\text{-D}$ -galactopyranose units,  
286 respectively (31). The assignment of the absorption peaks are given in Table 3, and are  
287 consistent with the results of monosaccharide composition analysis (Table 2).

#### 288 *3.4. Methylation and GC-MS analysis*

289 The major sugar residues and their positions at which they were substituted in the  
290 polysaccharides were determined by methylation and GC-MS analysis. The individual  
291 residues were qualitatively determined according to the retention times of their derivatives in  
292 GC and by comparison of mass spectra with literature data (20, 32). The linkage patterns of  
293 **DOP and its two fractions** are summarized in Table 4. They showed similar linkage modes,  
294 mainly 1,4-linked mannosyl and 1,4-linked glucosyl units. It is suggested that **DOP and its**  
295 **two sub-fractions** mainly comprise linear glucomannan. Meanwhile, a small number of  
296 terminal groups (T-Man<sub>p</sub>) and branched residues 1,2,4-linked-Man<sub>p</sub>, 1,3,4-linked-Man<sub>p</sub>, and

297 1,4,6-linked-Manp were also found in DOP, DOP-1, and DOP-2, inferring that some terminal  
298 mannosyl groups were attached to the *O*-2, *O*-4 and *O*-6 positions of 1,4-linked Manp.

### 299 3.5. NMR spectroscopy

300 The 1D <sup>1</sup>H and <sup>13</sup>C NMR spectra of DOP-1 and DOP-2 at 298 K are shown in Figure 2.  
301 They look identical. Based on data in the literature (13, 33, 34), the signals at around δ<sub>H</sub> 2.08  
302 ppm and δ<sub>C</sub> 21.54-21.81 ppm were assigned to the protons and methyl carbons in acetyl  
303 groups. And the <sup>13</sup>C resonances at δ<sub>C</sub> 173.79-173.83 ppm were assigned to carbonyl carbons  
304 in *O*-acetyl groups. There were two peaks at around δ<sub>C</sub> 103.75 and 101.40 ppm in the  
305 anomeric region of <sup>13</sup>C NMR spectra of both DOP-1 and DOP-2 (Figure 2B); these belong to  
306 β-1,4-linked Glcp and β-1,4-linked Manp, respectively. The results above indicate that both  
307 DOP-1 and DOP-2 have a backbone of 1,4-linked β-D-mannopyranosyl and  
308 β-D-glucopyranosyl residues, which is consistent with the results of methylation analysis.

309 In summary, we conclude that the structures of DOP-1 and DOP-2 are similar to each  
310 other. The backbones of the two polysaccharides consist of units of  
311 β-D-Manp-(1→4)-β-D-Glcp-(1→.

### 312 3.6. Effects of DOP and its two sub-fractions on proliferation of RAW264.7 cells

313 The cell proliferation effects of DOP and its two fractions on RAW264.7 cells are shown

314 in Figure 3A. When administered at varied concentrations of 3.75, 7.5, 15, 30, 62, 125, 250,  
315 500, and 1000  $\mu\text{g}/\text{mL}$  together with polymyxin B (10  $\mu\text{g}/\text{mL}$ ), **DOP and its two fractions**  
316 induced cell proliferation but polymyxin B did not inhibit it. However, the effect of LPS was  
317 excluded by polymyxin B. DOP had significant proliferation effects at different  
318 concentrations from 3.75 to 1000  $\mu\text{g}/\text{mL}$ . The proliferation rate increased along with the  
319 concentration from 3.75  $\mu\text{g}/\text{mL}$  to 125  $\mu\text{g}/\text{mL}$  ( $p < 0.0001$ ), but declined to the control value at  
320 the high concentration of 1000  $\mu\text{g}/\text{mL}$ . Similar results were also observed for DOP-1 and  
321 DOP-2. In summary, none of DOP, DOP-1, and DOP-2 showed cytotoxicity on RAW264.7  
322 cells. Therefore, the three concentrations of 125, 250, and 500  $\mu\text{g}/\text{mL}$  were chosen for further  
323 study.

### 324 *3.7. Effects of DOP and its two sub-fractions on cytokine production of RAW 264.7 cells*

325 As shown in Figure 3B, DOP, DOP-1, and DOP-2 exhibited similar effects on the  
326 TNF- $\alpha$  production of RAW 264.7 cells with the positive control LPS. The effect of LPS was  
327 suppressed by polymyxin B, but polymyxin B did not inhibit TNF- $\alpha$  production of **DOP and**  
328 **its two fractions**. The effect on TNF- $\alpha$  production of DOP, DOP-1, and DOP-2 was  
329 dose-dependent at the concentrations of 125, 250, and 500  $\mu\text{g}/\text{mL}$ . All of DOP, DOP-1, and  
330 DOP-2 had little effect on NO and IL-6 production (data not shown).

### 331 *3.8. Phagocytic activities of DOP and its two sub-fractions*

332 As shown in Figure 3C, the positive control LPS significantly increased the  
333 phagocytosis rate of RAW264.7 cells at the concentrations of 2 µg/mL, while polymyxin B  
334 inhibited this effect. DOP, DOP-1, and DOP-2 all exhibited similar inducing effects in a  
335 dose-dependent manner at the concentrations of 125, 250, and 500 µg/mL, but polymyxin B  
336 did not suppress their effects. DOP significantly increased phagocytosis rates from  
337  $18.3 \pm 0.42\%$  (125 µg/mL,  $p < 0.001$ ) to  $26.6 \pm 1.70\%$  (500 µg/mL,  $p < 0.0001$ ), and comparable  
338 activities were also observed in groups of DOP-1 and DOP-2.

### 339 *3.9. Proliferation of mouse spleen lymphocytes after stimulation with DOP and its two* 340 *sub-fractions*

341 As shown in Figure 4A, Con A and LPS significantly triggered T lymphocyte and B  
342 lymphocyte proliferation, respectively, and these results were consistent with previous studies  
343 (35, 36). *DOP and its two sub-fractions* also stimulated lymphocyte proliferation *in vitro*. The  
344 OD490 values of DOP, DOP-1, and DOP-2 groups were significantly higher than that of the  
345 control group at concentrations of 125, 250, and 500 µg/mL.

### 346 *3.10. Changes of T and B lymphocyte proliferation in synergistical stimulation by DOP* 347 *and its two sub-fractions with mitogen*

348 As shown in Figure 4B, the OD490 values of DOP *and its two sub-fractions* groups were

349 significantly higher than that of the cell control group, and rose in a dose-dependent manner  
350 at the concentrations of 125, 250, and 500  $\mu\text{g}/\text{mL}$ . Compared to the value of positive control  
351 LPS group, DOP, DOP-1, and DOP-2 co-cultured with LPS groups also significantly  
352 increased OD490 values at the concentrations of 125 250, and 500  $\mu\text{g}/\text{mL}$ . DOP-2 displayed  
353 significant mitogenic activity on mouse lymphocytes, yet it did not have significant  
354 proliferation effect at the concentration of 125  $\mu\text{g}/\text{mL}$  as compared to the LPS group. When  
355 co-cultured with *D. officinale* polysaccharides, the lympho-proliferative response induced by  
356 optimal concentration of Con A (1.25  $\mu\text{g}/\text{mL}$ ) was significantly enhanced (Figure 4C)  
357 compared with the Con A-stimulating group and control group in a dose-dependent manner.

#### 358 **4. Discussion**

359 *Teipi Shihu* has a complicated chemical profile. In addition to bibenzyls, phenanthrenes,  
360 sesquiterpenoids, and other small compounds, polysaccharides are the dominant component,  
361 with content up to 50% of the total dry weight (37). In our previous study (12), we  
362 demonstrated the molecular size distribution pattern of the water extract of *Teipi Shihu*, and  
363 found DOP to be the unique polysaccharide marker with its content being close to 30% of the  
364 total dry weight. There are also some other polysaccharides, in addition to DOP.

365 Five polysaccharide fractions have been obtained from *D. officinale* through a sequential  
366 ethanol precipitation method (38). Another study using anion-exchange chromatography on

367 DEAE-cellulose (NH<sub>2</sub>) separated six fractions from a crude *D. officinale* polysaccharide (39,  
368 40). Despite the differences in molecular weights, mannose-to-glucose ratios, and number of  
369 acetyl groups, all these polysaccharides are types of acetyl-glucomannan. In our study, DOP  
370 and its two sub-fractions also showed the identical general features: acetyl-glucomannan  
371 having a high degree of polymerization and a limited degree of branching.

372 Up to now, only two studies have reported the structural details of de-starched and  
373 HPGPC-homogenous 2-*O*-acetyl-glucomannan isolated from *D. officinale* (13, 39).  
374 Comparison with these literature data reveals some similarities and differences between these  
375 reported polysaccharides and the *Dendrobium officinale* polysaccharides in our study.  
376 Consistent with data in the literature (13, 39), our data also indicates that the backbone of  
377 DOP-1 and DOP-2 has (1→4)-linked β-D-mannopyranosyl residues and β-D-glucopyranosyl  
378 residues. Both the literature and our studies show identical 1D NMR spectra. But, in our  
379 results, the *M<sub>w</sub>* of DOP-1 and DOP-2 ( $8.1 \times 10^5$  and  $6.7 \times 10^5$  Da, respectively) was a bit larger  
380 than the data in the literature (13, 39, 40). The reported polysaccharides also contained traces  
381 of arabinose (39) or galactose and arabinose (13, 40). However, we found no  
382 monosaccharides other than mannose and glucose in DOP-1 and DOP-2.

383 The chemical structures of these polysaccharides have not been directly confirmed by  
384 high-quality 2D NMR spectra. In order to obtain a high-quality NMR spectrum, the  
385 polysaccharide sample needs to be dissolved in D<sub>2</sub>O or DMSO to form a homogenous

386 solution at the concentrations of approximate 3–4% (w/v). But *Dendrobium* polysaccharides  
387 including DOP-1 and DOP-2 in the present study always have high viscosity and relatively  
388 low solubility in D<sub>2</sub>O. Therefore it is hard to get their high-quality 2D NMR spectra. A  
389 previous study also encountered this problem (13). In that case, the authors had to degrade  
390 their polymer sample using endo-β-mannanase, and tried to characterize the chemical  
391 structure by analyzing the major enzyme-hydrolysis products for which 1D/2D NMR spectra  
392 are satisfactory.

393 Natural polysaccharides isolated from *D. officinale* have been shown to possess various  
394 bioactivities, especially the effect to modulate immune function (3, 40-43). Our results  
395 regarding the immunomodulatory activity of DOP and its two fractions indicate that *D.*  
396 *officinale* polysaccharides exert significant immunomodulatory effects on innate immune  
397 responses mediated by spleen lymphocytes and macrophages. They significantly stimulated  
398 the proliferation of splenocytes which is related to immunity improvement of T-lymphocytes  
399 and/or B-lymphocytes (Marciani et al., 2000). Therefore, DOP-1 and DOP-2 can be  
400 considered pharmaceutically active components of DOP extracted from *D. officinale*.

401 Known to be one of the target cells of polysaccharides, macrophages represent the first  
402 line of host defense (44) and are the most important antigen-presenting cells. Macrophages  
403 possess many immune functions, such as phagocytosis, antigen processing, and cytokine  
404 production (45). Macrophages are in a state of dormancy and their functions can be activated



405 by immunoenhancers (46). One of the most useful indicators of macrophage activation would  
406 be an enhancement of phagocytic activity, which represents a vital step in the immunological  
407 defense system. Consistent with previous studies, our results demonstrate that all  
408 polysaccharides in *D. officinale* increase macrophages' phagocytic activity significantly (42).  
409 Administration of DOP may result in the initiation of an immune reaction against foreign  
410 pathogens and tumors. Apart from the phagocytosis, activated macrophages accompanied by  
411 **TNF- $\alpha$**  production also play an important role in anti-tumor activity (47, 48). In this study, *D.*  
412 *officinale* polysaccharides induced production of TNF- $\alpha$  in macrophages in a dose-dependent  
413 manner. This result is in agreement with the previous study (40, 42).

414 Some reports in the literature have showed that polysaccharides in *D. officinale* induce  
415 NO production (40, 42) and IL-6 production (42); however, increase in NO and IL-6  
416 production was not detected in our studies. The immunomodulating activities of  
417 polysaccharides are associated with their chemical composition, molecular weight,  
418 conformation, glycosidic linkage, degree of branching and so on (49, 50). From these aspects,  
419 our polysaccharides should contain some differences from those of the previous studies. In  
420 this study, **DOP and its two sub-fractions** were found to contain the same monosaccharide  
421 composition, identical linkage pattern, and similar degree of branching. This might explain  
422 why they showed similar effects on RAW264.7 cells and spleen lymphocytes.

423 In conclusion, the unique polysaccharide marker DOP of *D. officinale* was separated

424 into two purified polysaccharides DOP-1 and DOP-2. **DOP and its two sub-fractions** showed  
425 identical chemical structures with slightly different molecular weights. Furthermore, they all  
426 have significant immunomodulatory activity *in vitro*, including activation of macrophages  
427 and lymphocytes. DOP-1 and DOP-2 are thus proven to be major, active polysaccharide QC  
428 markers of *D. officinale*.

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### 433 **Conflict of Interest**

434 The authors declare no conflict of interest.

435

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578

579

## 580 **Figure Captions**

581

582 **Figure 1.** (A) Chromatogram of the polysaccharides from *Dendrobium officinale* on  
583 ion-exchange column (DEAE-650M). (B) HPGPC chromatogram of cDOP (crude DOP),  
584 DOP (non-starch DOP), DOP-1 (non-starch DOP-1), and DOP-2 (non-starch DOP-2) (c=2.0  
585 mg/mL). (C) HPLC-ELSD chromatogram of standard monosaccharide mixture and  
586 hydrolysis products of mixed standards, DOP, DOP-1, and DOP-2. Black line: Standard  
587 mixture of mannose (Man, c=1.6 mg/mL) and glucose (Glc, c=1.6 mg/mL). (D) FT-IR  
588 spectra of cDOP, DOP, DOP-1, and DOP-2.

589 **Figure 2.** <sup>1</sup>H (A) and <sup>13</sup>C (B) NMR spectra of DOP-1 and DOP-2, obtained at 298 K.

590 **Figure 3.** Effects of DOP, DOP-1, and DOP-2 on the cell viability (A), TNF-α production (B),  
591 and phagocytosis activity (C) of RAW264.7 cells.

592 Mouse macrophages RAW264.7 cells were treated with different concentrations of DOP,  
593 DOP-1, and DOP-2, positive control LPS, LPS plus polymyxin B (10 μg/mL) or vehicle for  
594 24 h. MTT method, ELISA assay, and FACScan flow cytometry were used for analyzing cell  
595 viability, TNF-α production, and phagocytosis rate, respectively. The data are presented as the  
596 mean±SD. \*p<0.05, \*\*p<0.01, \*\*\*p<0.0001, \*\*\*\*p<0.0001 compared with the control  
597 group.

598 **Figure 4.** Proliferation of lymphocytes after stimulation by DOP, DOP-1, and DOP-2 at  
599 different concentrations and/or mitogen (LPS or Con A). (A) Lymphocytes (5×10<sup>5</sup> cells/well)  
600 in 96-well-plate were incubated with DOP, DOP-1, and DOP-2 at different concentrations for  
601 48 h. Two positive controls were used: LPS (20 μg/mL), a lipopolysaccharide which is a  
602 mitogen of B cells, and Con A (1.25 μg/mL), a phytohemagglutinin which is a mitogen of T  
603 cells. (B and C) Lymphocytes (5×10<sup>5</sup> cells/well) were treated by LPS/Con A with or without  
604 DOP, DOP-1, and DOP-2 at the indicated concentrations for 48 h. The cell viability was  
605 measured with tetrazolium compound (MTS) method. The different DOP and mitogens were  
606 dissolved and applied in medium. Control cells were treated with medium. The data are  
607 presented as the mean±SD. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\* p<0.0001 compared with

608 the control group.

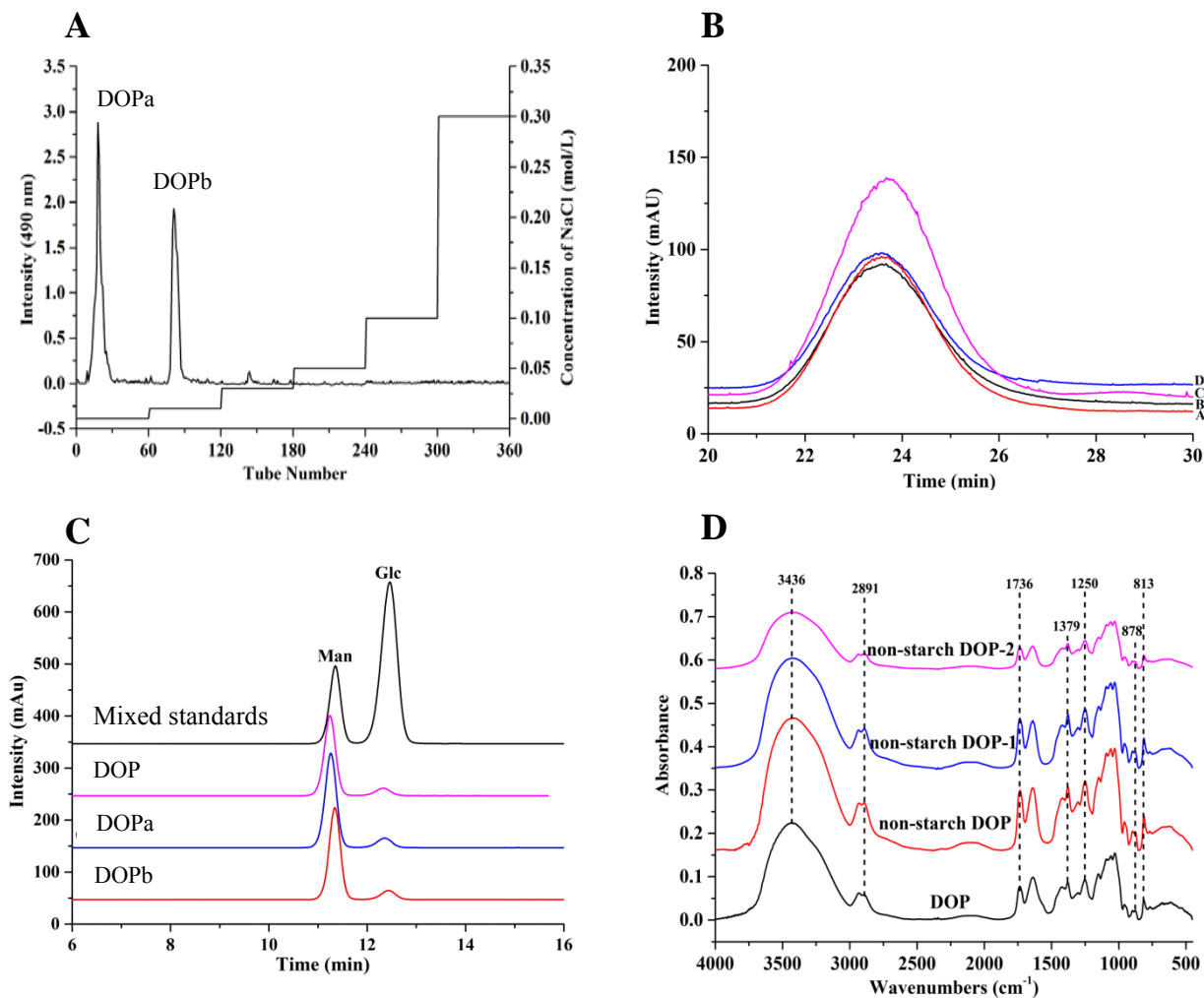
609 **Table 1** Chemical compositions of CDOP, DOP, DOP-1, and DOP-2.

610 **Table 2** Monosaccharide analysis of DOP, DOP-1, and DOP-2.

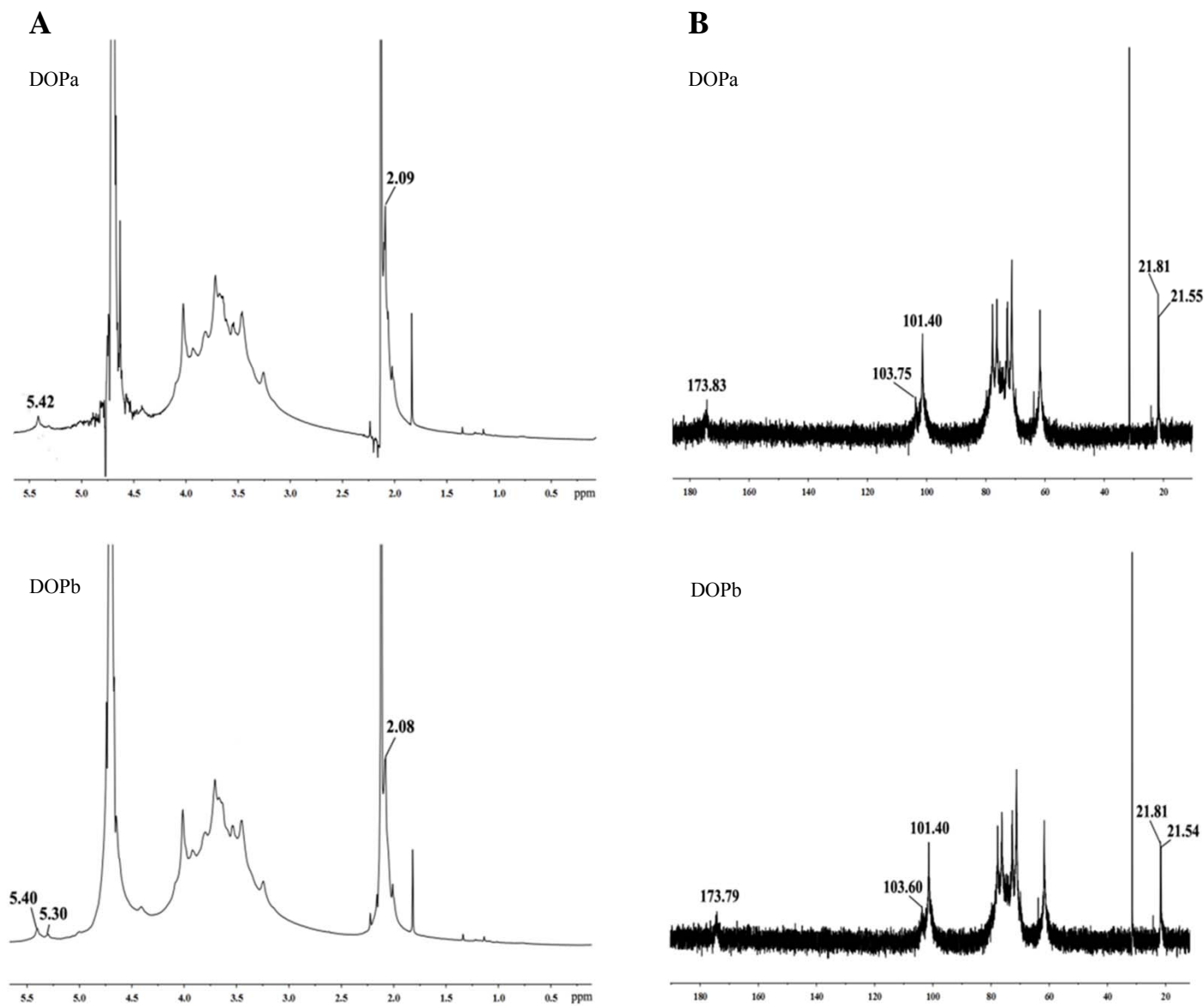
611 **Table 3** FT-IR spectra of DOP, DOP-1, and DOP-2: wave numbers and intensities of  
612 functional groups.

613 **Table 4** Glycosyl-linkage analysis of DOP, DOP-1, and DOP-2.

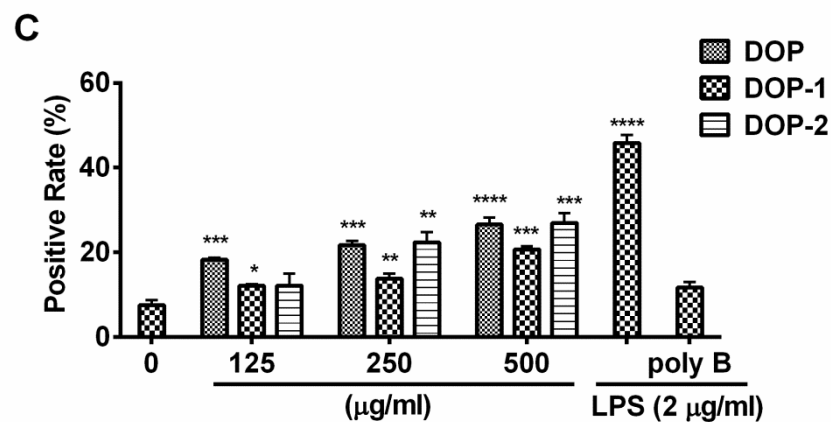
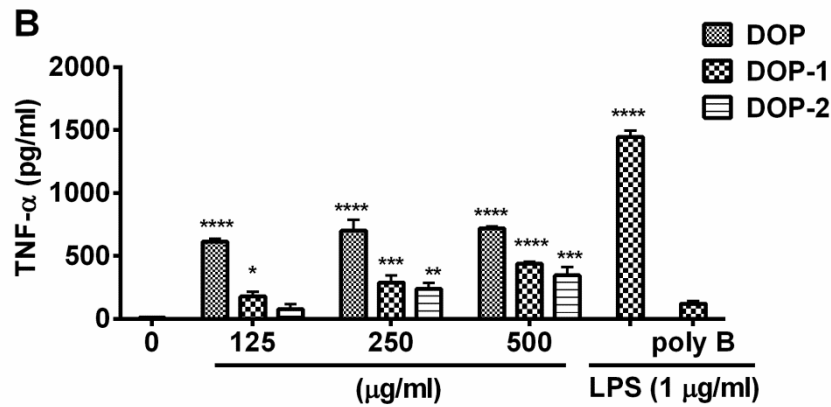
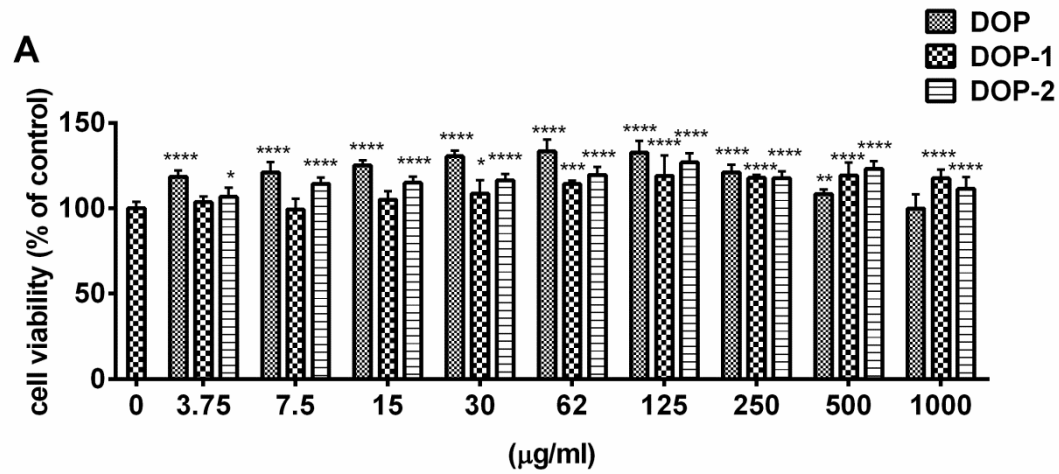
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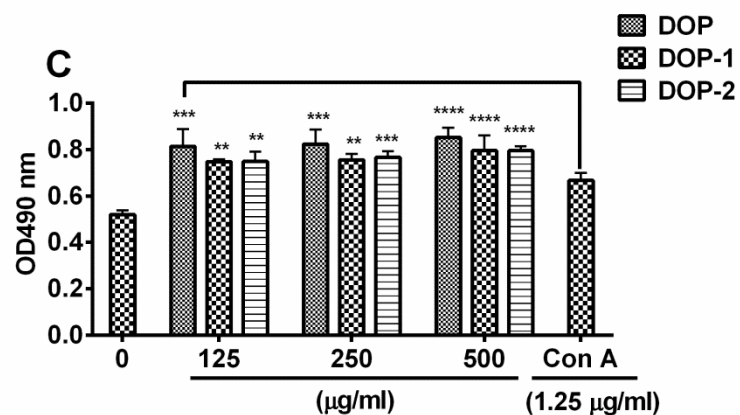
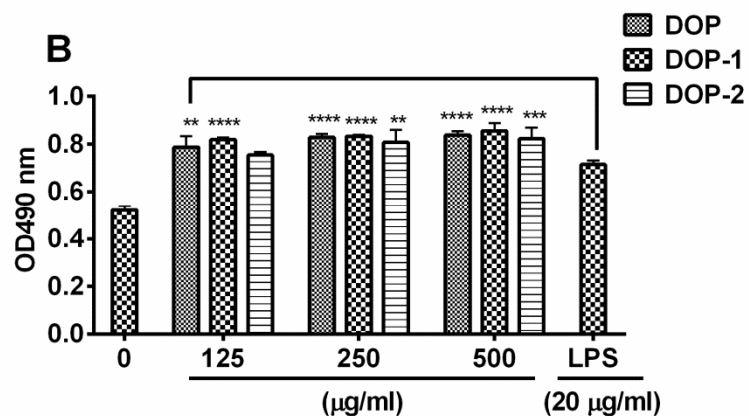
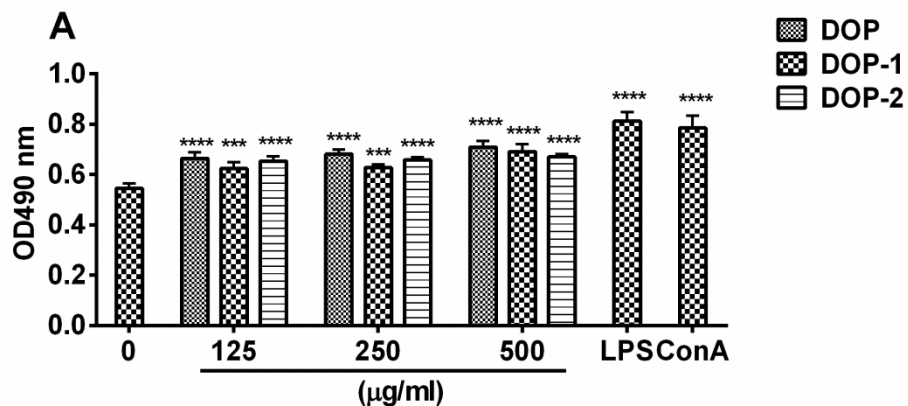
**Figure 1.** (A) Chromatogram of the polysaccharides from *Dendrobium officinale* on ion-exchange column (DEAE-650M). (B) HPGPC chromatogram of DOP, DOPa, and DOPb (C=2.0 mg/ml). (C) HPLC-ELSD chromatogram of standard monosaccharide mixture and hydrolysis products of DOP, DOPa, and DOPb. : Standard mixture of mannose (Man, c=1.6 mg/ml) and glucose (Glc, c=1.6 mg/ml); : DOPb; : DOPa; : DOP (D) FT-IR spectra of CDOP, DOP, DOPa, and DOPb.



**Figure 2.**  $^1\text{H}$  (A) and  $^{13}\text{C}$  (B) NMR spectra of DOPa and DOPb, obtained at 298 K.



**Figure 3.** Effects of DOP, DOPa, and DOPb on the cell viability (**A**), TNF- $\alpha$  production (**B**), and phagocytosis activity (**C**) of RAW264.7 cells. Mouse macrophages RAW264.7 cells were treated with different concentrations of DOP, DOPa, and DOPb, positive control LPS, LPS plus polymyxin B (10  $\mu\text{g/ml}$ ) or vehicle for 24 h. MTT method, ELISA assay, and FACScan flow cytometry were used for analyzing cell viability, TNF- $\alpha$  production, and phagocytosis rate, respectively. The data are presented as the mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ , \*\*\*\* $p < 0.0001$  compared with the control group.



**Figure 4.** Proliferation of lymphocytes after stimulation by DOP, DOPa, and DOPb at different concentrations and/or mitogen (LPS or Con A). **(A)** Lymphocytes ( $5 \times 10^5$  cells/well) in 96-well-plate were incubated with DOP, DOPa, and DOPb at different concentrations for 48 h. Two positive controls were used: LPS (20 mg/ml), a lipopolysaccharide which is a mitogen of B cells, and Con A (1.25 mg/ml), a phytohemagglutinin which is a mitogen of T cells. **(B and C)** Lymphocytes ( $5 \times 10^5$  cells/well) were treated by LPS/Con A with or without DOP, DOPa, and DOPb at the indicated concentrations for 48 h. The cell viability were measured with tetrazolium compound (MTS) method. The different DOP and mitogens were dissolved and applied in medium. Control cells were treated with medium. The data are presented as the mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  compared with the control group.



**Table 1** Chemical compositions of CDOP, DOP, DOPa, and DOPb

	CDOP	DOP	DOPa	DOPb
Total starch (%) <sup>a</sup>	10.0 ± 0.60 <sup>b</sup>	ND <sup>c</sup>	ND	ND
Netural Sugar (%)	76.5 ± 6.9	92.5 ± 4.1	93.4 ± 8.0	92.1 ± 2.0
Protein (%)	4.79 ± 0.52	1.49 ± 0.50	1.39 ± 0.33	1.66 ± 0.12
Total uronic acid (%)	2.31 ± 0.01	2.15 ± 0.24	2.04 ± 0.10	1.93 ± 0.20

<sup>a</sup> %: molar percentage

<sup>b</sup> Data was shown as mean ± SD (n=3)

<sup>c</sup> ND: not detectable or lower than limits of quantification

**Table 2** Monosaccharide analysis of DOP, DOPa, and DOPb

Sample	Mannose (% w/w) <sup>a</sup>	Glucose (% w/w)	Mannose/Glucose
DOP	85.2 ± 0.54	14.8 ± 0.24	5.78 ± 0.11
DOPa	84.8 ± 0.16	15.2 ± 0.06	5.59 ± 0.03
DOPb	85.5 ± 0.62	14.5 ± 0.02	5.92 ± 0.01

<sup>a</sup>: Data were shown as mean ± SD, (n=3)

**Table 3** FT-IR spectra of DOP, DOPa, and DOPb: wave numbers and intensities of functional groups.

Wavenumbers (cm <sup>-1</sup> )	Functional groups
3436	Vibration of O-H
2891	C-H stretching vibration of CH <sub>3</sub>
1736	Valence vibration of C=O
1379	C-H bending vibration of CH <sub>3</sub>
1250	C-O vibration of O-acetyl groups
878	Mannose pyranosyl ring
813	

**Table 4** Glycosyl-linkage analysis of DOP, DOPa, and DOPb.

Peak number	Retention time (min)	Corresponding derivatives	Residue linkage	Peak area percentage (%) <sup>a</sup>		
				DOP	DOPa	DOPb
1	12.45	2,3,4,6-Me <sub>4</sub> -Manp	T-Manp	5.55	5.26	6.34
2	18.85	2,3,6-Me <sub>3</sub> -Manp	1,4-linked-Manp	73.60	76.25	74.20
3	20.51	2,3,6-Me <sub>3</sub> -GlcP	1,4-linked-GlcP	12.43	11.82	12.40
4	22.26	2,6-Me <sub>2</sub> -Manp	1,3,4-linked-Manp	2.46	2.33	2.07
5	24.45	3,6-Me <sub>2</sub> -Manp	1,2,4-linked-Manp	2.00	1.77	2.29
6	25.14	2,3-Me <sub>2</sub> -Manp	1,4,6-linked-Manp	3.79	2.57	2.70

<sup>a</sup> Calculated as a relative percentage of all derivatives present, based on the peak area.

<sup>b</sup> DOP, DOPa, and DOPb were the samples of water-extracted polysaccharide from *Dendrobium officinale*, its two fractions DOPa (eluted with ultrapure water) and DOPb (eluted with 0.01 M NaCl), respectively.