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Comparison of antitumor activities of different polysaccharide fractions from the stems of *Dendrobium nobile* Lindl

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ABSTRACT

The antitumor activities of extracted polysaccharide fractions from the stems of *Dendrobium nobile* Lindl were investigated. Polysaccharides were sequentially extracted from the stems of *D. nobile* to obtain three fractions, i.e. water extract fraction (DNP-W), 5% NaOH extract fraction (DNP-OH) and 5% HCl extract fraction (DNP-H). Further the DNP-W was isolated to give six sub-fractions (DNP-W1, DNP-W2, DNP-W3, DNP-W4, DNP-W5 and DNP-W6) by anion-exchange chromatography. The monosaccharide profile, protein content, uronic acid content, total carbohydrate content, viscosity and molecular weight of nine polysaccharide fractions were analyzed. Both the in vivo and in vitro antitumor activities of nine polysaccharide fractions were evaluated and compared. Results indicated that DNP-W1 and DNP-W3 exhibited high antitumor activities against Sarcoma 180 in vivo and HL-60 in vitro. The results suggested that DNP-W1 and DNP-W3 could be considered as an effective natural antitumor source.

1. Introduction

The dried or fresh stems of several *Dendrobium* species (Orchidaceae) are widely used in traditional Chinese and folk medicine to nourish the stomach and promote the production of body fluid (Jiangsu New Medical College, 1986). *Dendrobium nobile* Lindl (Chinese name "Jin-Chai-Shi-Hu") is one of the most famous *Dendrobium* species and has been recorded in the Chinese Pharmacopoeia (2005 Edition). To elucidate the pharmacological mechanism of *D. nobile*, much research has been carried out on the low molecular compounds (Chen & Chen, 1935; Hedman & Leander, 1972; Li, Xu, Wu, Hirata, & Niwa, 1991; Morita, Fujiwara, Yoshida, & Kobayashi, 2000; Veerraju, Rao, Rao, Rao, & Rao, 1989; Ye, Qin, & Zhao, 2002; Zhang et al., 2008). And several compounds have been found to possess antitumor and anti-mutagenic activity (Miyazawa, Shimamura, Nakamura, & Kameoka, 1997; Ye et al., 2002; Zhang et al., 2008). In contrast, the polysaccharides from *D. nobile* have been little reported, even though some polysaccharides from different *Dendrobium* species have been demonstrated to possess curing-cataract, immuno-stimulating and anti-mutagenic activities (Chen & Guo, 2000; Hua, Zhang, Fu, Chen, & Chan, 2004; Luo, Deng, & Zha, 2008; Zha, Luo, Luo, & Jiang, 2007). In this paper, water, alkali and acid extracts polysaccharide were isolated from the stems of *D. nobile*, and their characteristics and antitumor activities in vivo/vitro were investigated.

2. Materials and methods

2.1. Material and reagents

The wild stems of *D. nobile* were collected from Sichuan province of China in May, 2007. The stems were crushed into a powder after being dried in an oven (40 °C, 7 days). Voucher specimens were deposited in the herbarium of the School of Biotechnology and Food Engineering, Hefei University of Technology (No. DNP0002). [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) and DEAE-cellulose were purchased from Sigma–Aldrich, St. Louis, USA. Trifluoroacetic acid (TFA) and Pei-cellulose were purchased from E. Merck, Darmstadt, German. Dextrans were purchased from Fluka Co., St. Louis, USA. All reagents used in this study were analytical grade.

Human acute promyelocytic leukemia HL-60 (ATCC CCL-240) and human hepatocellular carcinoma HepG2 (ATCC HB-8065) were purchased from the American Type Culture Collection (Rockville, MD, USA).

2.2. Extraction and isolation of polysaccharides

The powdered materials were pre-extracted for 48 h in a Soxhlet system with acetone and subsequently for another 48 h with methanol. The extracts were discarded and the residue was extracted three times with hot distilled water (90 °C, 2 h) and the

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water solutions were condensed and precipitated with 4 vols EtOH to give water extract polysaccharide (DNP-W). The residue divided into two groups, then the first was extracted with 5% NaOH at 4 °C for 10 h. The pH of solution was adjusted to 7 with 1 M HCl and centrifuged. The supernatant was precipitated with 4 vols EtOH to give alkali extract polysaccharide (DNP-OH). The second group was extracted with 5% HCl at 4 °C for 10 h to give acid extract polysaccharide (DNP-H). All the polysaccharides were treated with Sevag reagent (Staub, 1965) to remove protein, extensively dialyzed (molecular weight cut of 3500 Da).

The water extract polysaccharide (DNP-W) was fractionated on DEAE-cellulose column (1.6 cm × 60 cm), eluted with water and stepwise by 0.05, 0.1, 0.2, 0.3 and 0.5 M NaCl solutions to give six sub-fractions (DNP-W1, DNP-W2, DNP-W3, DNP-W4, DNP-W5 and DNP-W6).

The carbohydrate content was determined spectrophotometrically by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein content was determined by the Kjeldahl Nitrogen Determination method (Kjeldahl, 1883). The uronic acid content was determined by the *m*-hydroxylbiphenyl method (Blumenkrantz & Asboe-Hansen, 1973).

2.3. Monosaccharide analysis of polysaccharides

Polysaccharides were hydrolyzed with 2 M TFA (4 ml) at 120 °C for 4 h in a sealed tube. After removal of TFA, the hydrolyzates were dissolved in H_2O (2 ml) and reduced with $NaBH_4$ (40 mg) at room temperature for 2 h. The resulting alditols were acetylated with Ac_2O (2 ml) at 100 °C for 1 h and analyzed by GC. The alditol acetates were identified by their relative retention times on GC. GC was performed on a Shimadzu GC-9A instrument equipped with a hydrogen flame ionization detector, using a 3% OV-225 column (0.25 mm × 28 m i.d.) at a temperature program of 170 °C (1 min) followed by 1 °C/min to 180 °C (1 min) and then 4 °C/min to 250 °C. The hydrogen flow rate was 20 ml/min and the ion-source temperature was 150 °C.

2.4. Intrinsic viscosity [η]

The viscosity [η] of polysaccharides was measured in 0.2 M NaCl at 25 °C by an Ubbelohde capillary viscometer (internal diameter size 0.8 mm). The flow time of the solvent was always higher that 120 s, the kinetic energy correction was negligible. Huggins and Kraemer equations were used to estimate intrinsic viscosity [η] (Huggins, 1942; Kraemer, 1938).

2.5. Molecular weight (Mw)

The molecular weight (Mw) of polysaccharides was determined with a multi-angle laser light scattering instrument equipped with a He–Ne laser at the angles from 26 to 142 at 25 °C. Astra software was utilized for data acquisition and analysis.

2.6. In vivo antitumor test

Sarcoma 180 tumor cells (1×10^5 cells/mouse) were inoculated into 8-week-old BALB/c mice (20 ± 1 g). The polysaccharides were dissolved in PBS (pH 7.2) and injected intraperitoneally once daily for 10 days at 24 h after tumor inoculation (dose: 40 mg kg^{-1} body). The same volume of PBS was injected into the control mice. The tumors were allowed to grow on the mice for 7 days before they were removed from the animals and weighed. The antitumor activities of the tested polysaccharide samples were expressed as an inhibition ratio calculated as $[(A - B)/A] \times 100\%$, where A and B are the average tumor weight of the control and treated groups, respectively.

2.7. In vitro proliferation assays

2.7.1. The antiproliferation of suspended HL-60 leukemic cells

The HL-60 leukemic cells (1×10^5 cells/ml) containing polysaccharides at concentration of 25, 50, 100 and 200 μg/ml in PBS solution were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% bovine serum under an atmosphere of 5% carbon dioxide at 37 °C for 72 h. The survival rate of the mammalian cells was assayed by a hemacytometer to count living cells that excluded the Trypan Blue dye.

2.7.2. The antiproliferation of adherent mammalian HepG2 cells

The mammalian HepG2 cells (1×10^5 cells/ml) were incubated with the polysaccharides at concentration of 25, 50, 100 and 200 g/ml and allowed to grow under the same conditions as the HL-60 cells mentioned before. The numbers of living HepG2 cells at the end of the 72 h incubation period were determined by MTT method (Mosmann, 1983). In these two assays, the tested polysaccharide samples were compared with control samples.

All in vitro results were expressed as the ratio of inhibition of tumor cell proliferation calculated as $[(A - B)/A] \times 100\%$, where A and B are the average number of viable tumor cells of the control and samples, respectively.

2.8. Statistics

Statistical evaluations in all experiments were performed by a Student's *t*-test. A P value of less than 0.05 was considered significant.

3. Results and discussion

3.1. Isolation

The yields of polysaccharides from the dried stems of *D. nobile* were 3.1%, 0.49% and 0.41% for DNP-W, DNP-OH and DNP-H, respectively. DNP-W was further fractionated on DEAE-cellulose column to give six sub-fractions, i.e. DNP-W1 (yield: 75% of the loaded polysaccharides), DNP-W2 (yield: 2% of the loaded polysaccharides), DNP-W3 (yield: 5% of the loaded polysaccharides), DNP-W4 (yield: 10% of the loaded polysaccharides), DNP-W5 (yield: 5% of the loaded polysaccharides) and DNP-W6 (yield: 3% of the loaded polysaccharides) (Fig. 1). In general, the content of polysaccharides from *D. nobile* is related to extraction conditions, plant sources and parts (Chen & Guo, 2000). The obtained data indicated that DNP-W is main constituent of polysaccharides from *D. nobile* and the content of which ranges from 1.32% to 7.51% in the dried materials (Chen & Guo, 2000; Zhu et al., 2007). Moreover, Fig. 1 shows that water eluate fraction DNP-W1 (75%) is main constituent in DNP-W and the similar results were also reported in *Dendrobium officinale* (Hua et al., 2004), *Dendrobium huoshanense* (Zha et al., 2007) and *Dendrobium moniliforme* (Chen, He, Hua, & Zhang, 2003).

3.2. Chemical analysis and molecular weight (Mw)

Monosaccharides composition, uronic acid, protein and total carbohydrate in different extracted polysaccharides were shown in Table 1. The results indicated that proteins were detected in all the samples except DNP-W1 and DNP-W2. DNP-W has the highest protein content 7.1%. Except for DNP-W1, there was a very low level of uronic acid in all polysaccharides. Monosaccharide analysis showed that all the polysaccharides were determined to be heteropolysaccharides containing mannose, galactose and glucose as major sugar and traces of other monosaccharide. However,