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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,

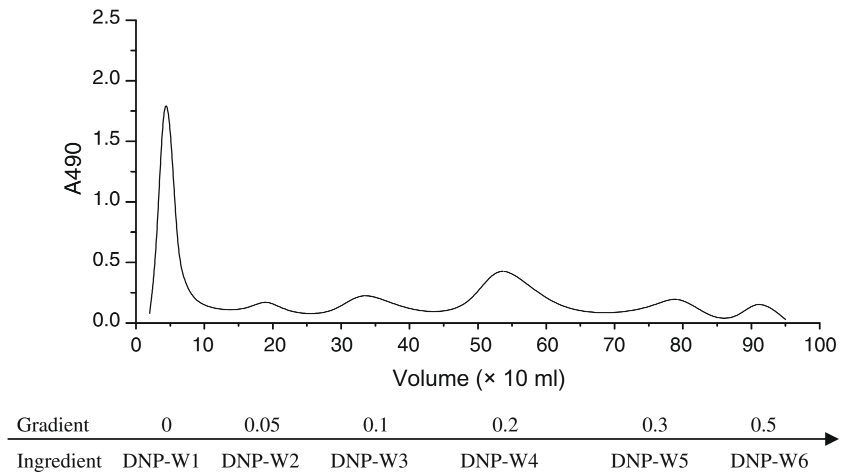

Fig. 1. DEAE-cellulose column chromatogram of DNP-W in condition of gradient elution.

Table 1
Monosaccharide composition, uronic acid content, protein content and total carbohydrate content of the polysaccharides from the stems of *D. nobile*.

Sample	Monosaccharide content in polysaccharides (%)						Ua (%)	Protein (%)	TA (%)
	Man	Glc	Gal	Rha	Ara	Xyl	n	n	n
DNP-W	42.2	31.1	13.6	2.8	7.4	2.9	2.1	7.1	90.3
DNP-OH	16.1	23.3	51.2	3.4	1.8	4.2	2.6	3.3	89.6
DNP-H	9.1	30.6	53.3	3.7	1.3	2.0	2.1	3.0	87.1
DNP-W1	64.2	28.1	6.6	n	1.1	n	n	n	100
DNP-W2	21.1	65.3	11.2	T	1.9	T	0.1	n	100
DNP-W3	21.2	52.6	14.3	5.9	4.6	1.1	1.4	3.9	98.3
DNP-W4	12.8	54.9	23.6	4.6	3.9	T	2.5	1.7	96.2
DNP-W5	7.5	52.5	29.3	4.7	2.1	3.9	4.5	1.2	96
DNP-W6	10.6	41.2	42.3	3.1	1.2	1.6	4.0	1.1	96.5

n, not detected; T, trace amount; Man, mannose; Glc, glucose; Gal, galactose; Rha, rhamnose; Ara, arabinose; Xyl, xylose; Ua, uronic acid; TA, total carbohydrate content.

the ratios of monosaccharide were difference between polysaccharide fractions. Compared with DNP-OH and DNP-H, DNP-W had high content of mannose (42.2%) and low content of galactose (13.6%). Among the six sub-fractions from DNP-W, DNP-W1 had less protein and more mannose than other fractions and the galactose content rapidly increased to become the predominant monosaccharide during the sequential fractions.

The viscosity $[\eta]$ and molecular mass (Mw) of polysaccharide fractions are showed in Table 2. As shown in Table 2, DNP-W has high $[\eta]$ 27.1 $cm^3\,g^{-1}$ and Mw 41.3×10^4 when compared with DNP-OH and DNP-H. According to six sub-fractions from DNP-W, DNP-W1 has the smallest Mw 9.2×10^4 and relatively high viscosity 28.6 $cm^3\,g^{-1}$, which indicates that DNP-W1 has a relatively more expanded flexible chain conformation (Jin, Zhang, Zhang, Chen, & Cheung, 2003). Moreover, compared with other polysaccharide fractions, DNP-W3 has the highest η 41.3 $cm^3\,g^{-1}$ and Mw 107.3×10^4.

3.3. In vivo antitumor activities

Table 2 showed the results from in vivo assay of the antitumor activities. Among three extracting polysaccharides, no obvious antitumor activities were observed in DNP-OH and DNP-H, which indicated that they were ineffective in suppressing the growth of the Sarcoma 180 tumor cells. DNP-W had bioactivity with an inhibition ratio of 31.3%. For finding higher antitumor activities source, six sub-fractions were obtained from DNP-W by anion-exchange chromatography. The results of antitumor activities from these fractions indicated that DNP-W1 and DNP-W3 exhibited significantly higher antitumor activities against the growth of Sarcoma 180 tumor cells, showing the inhibition ratios of 65.3% and 61.2%, respectively. DNP-W2 and DNP-W4 exhibited similar antitumor activity with DNP-W, showing the inhibition ratios of 33.7% and 33.4%, respectively. The antitumor activity data for DNP-W5 and DNP-W6 were exhibited very low antitumor activities against the growth in vivo experiments, showing the inhibition ratios of 10.3% and 8.7%, respectively. Moreover, thymus index and spleen index of the mice were calculated to explore the mechanism of antitumor (Table 2). The results showed that the polysaccha-

Table 2
Antitumor activities of polysaccharides against Sarcoma 180 solid tumor grown in BALB/c mice.

Sample	$[\eta]$ ($cm^3\,g^{-1}$)	Mw $\times 10^4$ ($g\,mol^{-1}$)	Inhibition ratio (%)	Complete repression	Thymus index (mg/10 g)	Spleen index (mg/10 g)
Control	n	n	n	0/10	89.1 ± 9.4	42.2 ± 5.1
DNP-W	27.1	41.3	31.3	1/10	$143.2 \pm 11.2^{**}$	44.2 ± 1.3
DNP-OH	11.1	17.6	9.7	0/10	$110.4 \pm 11.4^{*}$	41.8 ± 3.7
DNP-H	10.7	21.9	5.2	0/10	94.9 ± 18.5	42.5 ± 8.4
DNP-W1	28.6	9.2	65.3	1/10	$153.2 \pm 11.2^{**}$	44.2 ± 1.3
DNP-W2	11.2	10.1	33.7	1/10	$120.4 \pm 11.4^{*}$	41.8 ± 3.7
DNP-W3	41.3	107.3	61.2	2/10	$144.9 \pm 18.5^{**}$	42.5 ± 8.4
DNP-W4	15.2	42.1	33.4	0/10	$124.4 \pm 10.8^{*}$	42.7 ± 9.6
DNP-W5	26.1	46.3	10.3	0/10	$116.1 \pm 17.6^{*}$	44.2 ± 11.5
DNP-W6	18.1	54.1	8.7	0/10	$107.8 \pm 9.6^{*}$	42.7 ± 8.5

n, not detected.
* $p < 0.05$.
** $p < 0.01$.

rides significantly increase thymus index in mouse, but little effect on spleen index (Table 2). Thus immunostimulation maybe the way to against the growth of Sarcoma 180 tumor cells.

3.4. In vitro antitumor activities

The inhibition ratios of tumor cell (suspended HL-60 leukemic cell and adherent HepG2 cell) growth by the polysaccharide fractions were showed in Figs. 2 and 3. According to in vitro inhibition ratios of HL-60 leukemia cells by the polysaccharide fractions at different concentrations (25, 50, 100 and 200 μg/ml) (Fig. 2), all polysaccharides exhibited strong inhibition against cell growth and there was a dose–response relationship between concentration of polysaccharides and suppression of HL-60 cell proliferation. In particular, the DNP-W1 and DNP-W3 presented significantly high inhibition ratio of more than 80% at the concentration of 200 μg/ml, which suggested that they had potential for investigation for leukemia therapy. The results also suggested that DNP-W1 and DNP-W3 could be considered as an effective natural antitumor source.

In the MTT assay, the polysaccharide fractions DNP-OH, DNP-W4 and DNP-W5 showed very low inhibitions against the proliferation of adherent HepG2 tumor cells, only DNP-W, DNP-W1 and DNP-W3 had an inhibition ratio of less than 30% at the concentration of 200 μg/ml. In the case of DNP-H and DNP-W6, negative proliferation of the cancer cells was observed. The foregoing results indicated that the heteropolysaccharides from *D. nobile* exhibited significant inhibition against the proliferation of suspended HL-60 tumor cells when compared to adherent HepG2 tumor cell proliferation. It was concluded that the *D. nobile* polysaccharides were potent tumor cell growth inhibitors that showed selectively higher antitumor activities against suspended cells than adherent ones.

The foregoing differences in antitumor activity among the various polysaccharide fractions were probably due to their different monosaccharide compositions, originating from the different exactions, as shown in the case of DNP-W, DNP-OH and DNP-H. The antitumor activities of polysaccharide fractions were found to correlate positively with the amount of mannose, more expanded flexible chain conformation, and presence of glucose and galactose, as shown in the case of DNP-W1. In addition, relatively higher molecular mass and better water solubility seemed to increase the antitumor activities, as shown in the case of DNP-W3. Many factors affected the activities of polysaccharide fractions, such as monosaccharide composition, molecular mass, glycosyl residues, viscosity and chain conformation (Bao, Liu, Fang, & Li, 2001; Hua et al., 2004; Huang, Jin, Zhang, Cheung, & Kennedy, 2007; Jin et al., 2003; Ye, Wang, Zhou, Liu, & Zeng, 2008). Polymers having branches of (1 → 3)-linked or (1 → 6)-linked glycosyl residues, more order conformation in aqueous solution, and high molecular weight were often reported to be pharmacologically activities (Bao et al., 2001; Hua et al., 2004; Huang et al., 2007). The antitumor activities of high molecular weight polysaccharides were consid-

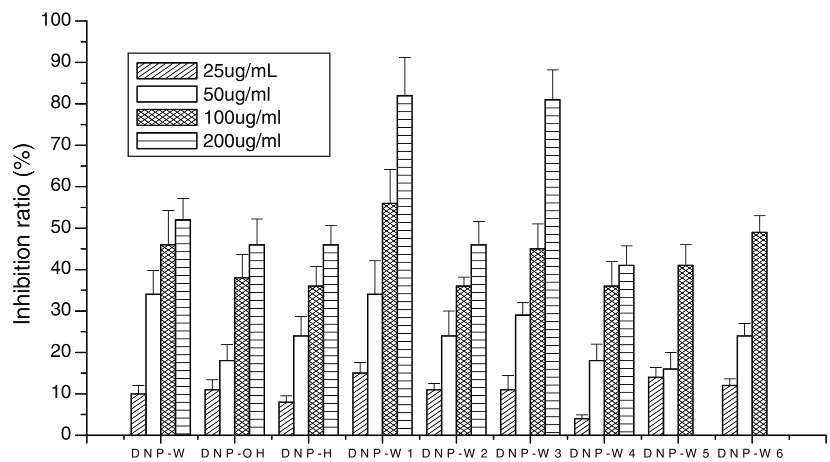

Fig. 2. Inhibition of proliferation of HL-60 leukemic cells by different concentrations of polysaccharide fractions.

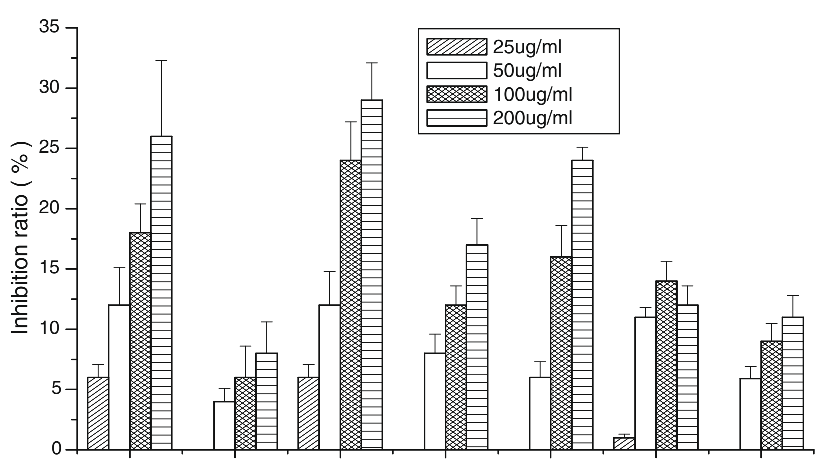

Fig. 3. Inhibition of proliferation of HepG2 liver cancer cells by different concentrations of polysaccharide fractions.

ered to be a consequence of stimulation from the immune response in the host, rather than direct killing tumor cells, as reported by Wasser (2002) and Zjawiony (2004). In general, polysaccharides isolated from the stems of *D. nobile* was found to have different in vivo and in vitro antitumor activities, depending on their monosaccharide composition, protein content, molecular mass and chain conformation.

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