

Full Length Research Paper

Evaluation of anti-tumor activity of water-soluble polysaccharides from *Dendrobium denneanum*

Yijun Fan and Aoxue Luo*

Chengdu Campus of Sichuan Agriculture University, Chengdu, PR, China.

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Three water-soluble polysaccharides (DDP1-1, DDP2-1 and DDP3-1) were obtained from the aqueous extracts of the stems of *Dendrobium denneanum* by hot water extraction, ethanol precipitation, and fractionated by DEAE-cellulose ion exchange chromatography and Sephadex G-200 gel filtration chromatography. The main structure characterizations and anti-tumor activities of three fractions were evaluated in this paper. The results showed DDP1-1 with 12.50 mg/kg could significant inhibit the increment of the tumor, increase immune index of S180 mice, and also strongly promote the secretion of IL-2, TNF- α and IFN- γ . Therefore, results of these studies demonstrated the polysaccharide DDP1-1 has strong anti-tumor and immunomodulation abilities.

Key words: Immunomodulating, *Dendrobium denneanum*, polysaccharide, anti-tumor.

INTRODUCTION

Dendrobium denneanum, belongs to the Orchidaceae family, a traditional Chinese herb, has been used in the preparation of herbal medicines in many oriental countries for a long time. Because the dried stems could be used widely for the treatment of salivary, ophthalmic disorders, fever and chronic superficial gastritis, *D. denneanum* has been archived in the Pharmacopoeia of the People's Republic of China. To elucidate the pharmacological mechanism of *Dendrobium* species, much research has been carried out on the low molecular compounds, such as bibenzyls (Majumder et al., 1999; Zhang et al., 2007), coumarins (Zheng et al., 2009), alkaloids (Wang et al., 2010) and phenanthrenes (Yang et al., 2006). As for the compounds of polysaccharides from *D. denneanum*, little information was obtained (Luo et al., 2011). Polysaccharides is some of its main bioactive compounds that possesses immunoregulatory (Shao et al., 2004; Chen et al., 2007; Feng et al., 2010), anti-inflammatory (Hitoshi et al., 1974; Popov et al., 2005), anti-virus (Wang et al., 2009) and antioxidant (Wu et al., 2007; Luo et al., 2009, 2010) activities. Most polysaccharides derived from higher plants are relatively nontoxic and do not cause significant side effects, which is a major problem associated with immunomodulatory

polysaccharides. Thus, plant polysaccharides are ideal candidates for therapeutics with immunomodulatory and antitumor effects and low toxicity (Schepetkin et al., 2006). Polysaccharides from some *Dendrobium* species exhibited strong antitumor activities and immunostimulating, such as *Dendrobium huoshanense* (Zha et al., 2007; Yves et al., 2008) and *Dendrobium nobile* Lindl (Wang et al., 2010). In order to investigate the bioactive of the polysaccharides from *D. denneanum*, the isolated and purified procedure and antitumor and immunopotentiating activities using a tumor inhibitory assay system and immunepotentiating assay system were carried out in the present study.

MATERIALS AND METHODS

Drugs and reagents

T-series Dextran and Sephadex G-200 were purchased from Pharmacia Co. (Uppsala, Sweden). Cyclophosphamide (CTX) was purchased from Hengrui medicine co.(Jiangsu China). The standard monosaccharides (glucose, mannose, rhamnose, galactose, xylose and arabinose) were purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The tumor necrosis factoralpha (TNF- α), interferon-gamma (IFN- γ) and interleukin-2 (IL-2) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai Senxiong Biotech Co. (Shanghai, China). All the other chemical reagents were analytical reagent grade. The plant materials were

*Corresponding author. E-mail: aoxueluo@hotmail.com.

bought from the market of traditional Chinese medicinal materials in Chengdu (China), and were identified according to the identification standard of the Pharmacopoeia of the People's Republic of China (PPRC).

Preparation for polysaccharides

D. denneanum polysaccharides were isolated and purified as described previously (Fan et al., 2009). Briefly, the stem of *D. denneanum* was triturated into powder and boiled in petroleum ether for 2 h. After filtration to remove the petroleum ether, the residue was further extracted successively with 80% ethanol and double-distilled water. Then the aqueous extracts were collected. After combined and concentrated, the extract was precipitated with four times volumes ethanol for 24 h at 4°C. Protein was removed with the sevag method (Alum et al., 1986). Then the precipitate was dissolved with distilled water, after dialyzed against deionized water for 72 h, it was applied to a DEAE-cellulose (26 x 300 mm). The column was eluted with distilled water, followed by 0.05 M, 0.1 M NaCl, sequentially. From this isolation procedure, the fraction DDP1 (eluted with water), DDP2 (eluted with 0.05 M NaCl) and DDP3 (eluted with 0.1 M NaCl) were collected. Sephadex G-200 (16 x 600 mm) was used to identify the purity of the polysaccharide. Fractions were collected by a fraction collector. Three major polysaccharide peaks, DDP1-1 (From DDP1), DDP2-1 (From DDP2) and DDP3-1 (From DDP3) were obtained and then freeze-dried.

The further identified of three major polysaccharide peaks were carried out in the present study. The average molecular weights (Mws) of these fractions were 51.5, 26.1 and 6.95 kDa, respectively. Monosaccharide components analysis indicated that DDP1-1 and DDP2-1 were composed of arabinose, xylose, mannose, glucose and galactose in a molar ratio of 1.00:2.82:57.11:140.82:7.76 and 1.00:1.62:1.18:77.5:7.79. DDP3-1 was composed of arabinose, mannose, glucose and galactose in a molar ratio of 1.00:1.03:8.84:2.00. These data are same to our previous results (Fan et al., 2009).

In vivo antitumor test

Animals and treatment

Kunming mice between 6 and 8 weeks old (weight: 20.0±2.0 g) were purchased from the Experimental Animal Center of Sichuan Academy of Medical Sciences (Chengdu, China. Quality Certificate Number: SCXK 2008-24). The mice were housed under normal laboratory conditions, that is, room temperature, 12/12 h light–dark cycle with free access to standard rodent chow and water. The experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Sarcoma 180 cells (S180) were generously donated by Dr. Yu of Sichuan Academy of Chinese Medicine Sciences. Sarcoma 180 cells were passed into mice ascites. Then, ascites was inoculated subcutaneously 0.2 ml (5.0×10^7 cells/ml) into the sword arm of each experimental mouse. Normal control mice were not inoculated Sarcoma 180. The mice were treated as following: normal control (normal saline), positive control (Cyclophosphamide, 12.5 mg/kg body weight), model control group (normal saline) and the polysaccharide (50, 25 and 12.5 mg/kg body weight). All the groups were administered daily by intraperitoneal injection (0.2 ml).

Tumor inhibition effect

The tested samples (12.5, 25 and 50 mg/kg body weight of each polysaccharide) and CTX (12.5 mg/kg body weight) were dissolved in saline, and then injected intraperitoneally (i.p.) once a day for

14 days, starting 24 h after tumor inoculation. The normal control and model control mice received an equal volume of saline (0.2 ml). 24 h after last tested sample administration. All animals were weighted and sacrificed. The inhibitory rate was calculated as $[(A-B)/A] \times 100\%$ (Furukawa et al., 2000), where A was the tumor weight of the model group, and B was the tumor weight of the tested group.

Analysis of immune index

Spleen index was determined from the weight of the spleen of the mice surviving up to 15 days. Twenty-four hours after the last administration, these mice were anatomized by cervical dislocation, and the spleens were recovered and weighed. The results are expressed as the spleen index using the formula: weight of spleen (mg)/ body weight (g). The thymus index and the tumor weights of the mice were measured by using the same method.

Determination of IL-2, TNF- α , and IFN- γ by the ELISA method

These mice were anatomized after 14 days, and their blood samples were obtained from eye orbitae. Serum was collected 4 h after administration of the polysaccharide. The interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF- α), and interferon-gamma (IFN- γ) concentration were measured with an enzyme-linked immunosorbent assay (ELISA kit, Shanghai Senxiong Biotech) according to the indication of the manufacturer.

Analysis of the drug synergism between CTX and polysaccharide

The mice inoculated Sarcoma 180 was divided into experimental mouse groups, model control group and positive control group. Normal control mice were not inoculated Sarcoma 180. The normal control received normal saline. Model control received normal saline. Experimental mouse groups received CTX (12.5 mg/kg body weight) by injected intraperitoneally at 1,3,5,7,9,11 and 13 days, and also received polysaccharide (12.5 mg/kg body weight) by injected intraperitoneally at 2,4,6,8,10,12 and 14 days, 24 h after last tested sample administration. All animals were weighted and sacrificed. The inhibitory rate was calculated as $[(A-B)/A]$ and 100%, where A was the tumor weight of the model group, and B was the tumor weight of the tested group.

Statistical analysis

The data were expressed as means \pm SD. Data were analyzed by an analysis of variance ($P < 0.05$) and the results were processed by SPSS software.

RESULTS AND DISCUSSION

In vivo tumor inhibition effect of the polysaccharide fractions

Although, any reduction of the tumor volume was not the only standard of anti-tumor, the tumor masses reduction often indicate the strong anti-tumor effect. So, tumor inhibition rate was usually used as an index of screening the anti-tumor drug. Cyclophosphamide is generally used for treatment of various types of cancers. It is a clinically

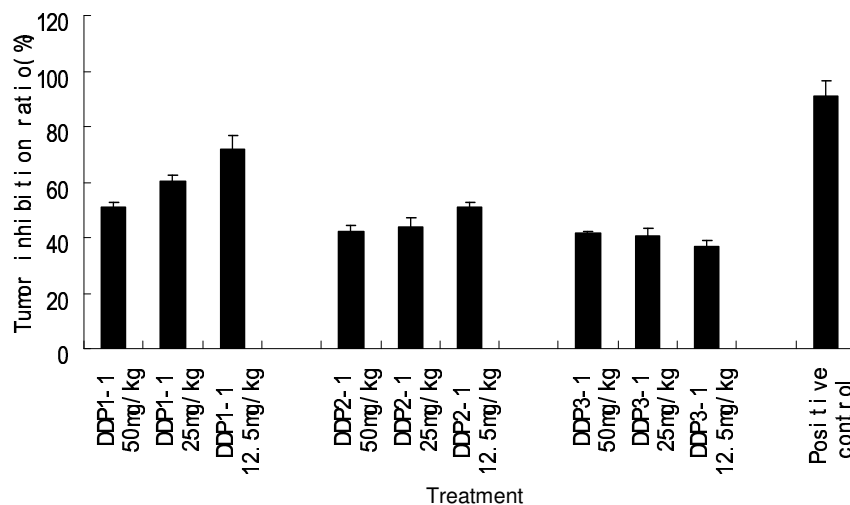


Figure 1. Tumor inhibitory rate was calculated as $[(A-B)/A] \times 100\%$, where A and B were the average tumor weight of the model control and experimental groups, respectively. Values of tumor inhibitory rate are means \pm S.D, (n=10).

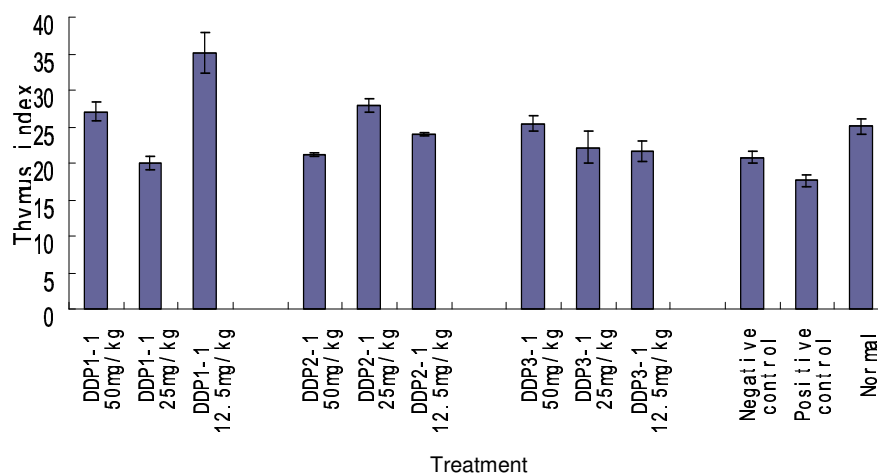


Figure 2. Thymus index was measured in the ratio of the thymus weight (mg) to body weight (g). Values are means \pm S.D, (n=10). *p<0.05, **p<0.01 vs. negative control.

approved anticancer agent that works by slowing or stopping cell growth. Figure 1 exhibited the tumor inhibition rates of different polysaccharide fractions and CTX. From the figure, the positive control exhibited significant anti-tumor activity with a high tumor inhibition rate of 90.96%. Meanwhile, different doses of three polysaccharide fractions exhibited different anti-tumor activities. The anti-tumor effects of DDP2-1 and DDP3-1 were weakly. On the other hand, DDP1-1 showed high anti-tumor activity, the effect was decreasing with the increasing dose, at the low dose of 12.5 mg/kg, the anti-tumor activity was 72.04%, which was closed to CTX. Therefore, a significant tumor inhibition was observed at

12.5 mg/kg of DDP1-1.

Analysis of immune index

To evaluate the effect of three polysaccharide fractions on the immune system, the experiment evaluated the effects on spleen and thymus index of different drugs. The results showed in Figures 2 and 3. Figure 2 indicated that the dose of 50 and 12.5 mg/kg of DDP1-1 caused a significant increase in the thymus index compared with S180 negative control group. DDP2-1 and DDP3-1 with different doses exhibited low effects on the thymus index

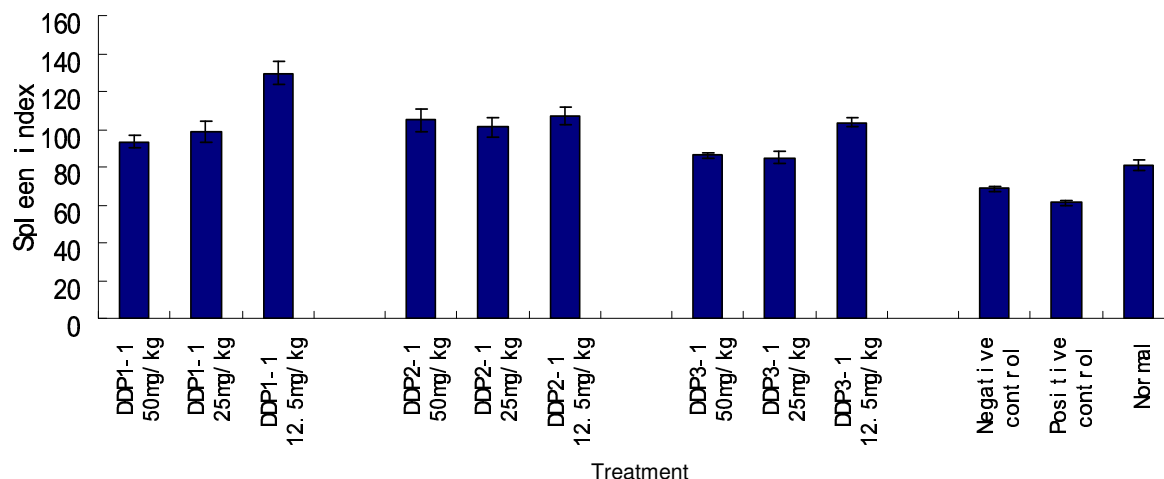


Figure 3. Spleen index was measured in the ratio of the spleen weight (mg) to body weight (g). Values are means \pm S.D, (n=10). *p<0.05, **p<0.01 vs. Negative control.

of S180 mice. On the other hand, no significant increase was observed in CTX-treated animals. There was significant increase on spleen index of 12.5 mg/kg DDP1-1 compared with S180 negative control group. The effect was much higher than that of DDP2-1 and DDP3-1. There was also no significant effect was observed in CTX-treated animals (Figure 3). The results indicated that the polysaccharide fraction DDP1-1 could significantly increase the immune system of S180 mice, suggesting that immunomodulation may be the mechanism of the anti-tumor activity of 12.5 mg/kg DDP1-1.

Determination of IL-2, TNF- α , and IFN- γ by the ELISA method

Serum from each groups were collected 4 h after administration of the drugs. The interleukin-2, tumor necrosis factor-alpha and interferon-gamma concentration were measured with an enzyme-linked immunosorbent assay according to the indication of the manufacturer. IFN- γ is an important immunoregulatory molecule. It induces the generation of T cells, activates macrophages, and regulates crossly Th1 and Th2 cells. TNF- α and IFN- γ can enhance immunoregulatory ability each other towards tumor.

From the results of Table 1, the concentrations of TNF- α , IFN- γ and IL-2 in the group with CTX-treated were significant low, which indicated CTX could not promote the secretion of the three cytokines. However, the concentrations of TNF- α with different doses polysaccharide-treated have different degrees increase. Especially 25.00 mg/kg DDP2-1, 25.00 and 12.50 mg/kg DDP1-1 have strongly promoted the secretion of the three cytokines (p<0.01). Compared with negative control, there were higher concentrations in the serum of

12.50 mg/kg DDP1-1-treated and DDP2-1-treated groups. As for IL-2, only 12.50 mg/kg DDP1-1-treated group exhibited strong promoted effect. Therefore, the results indicated that the level of three cytokines were significant increase compared with the control, that was 12.50 mg/kg DDP1-1-treated could augment well IL-2, TNF- α and IFN- γ production.

Analysis of the drug synergism between CTX and polysaccharide

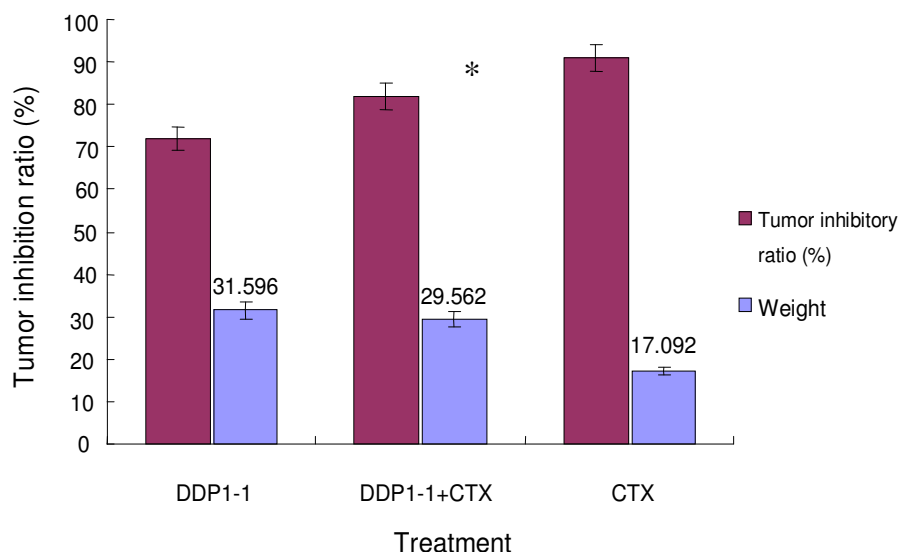
From the results of above, the polysaccharide DDP1-1 could anti-cancer by improving the immune system. To investigate the ability of DDP1-1 on reducing toxicity and promoting curative effect, further study was carried out. The results were showed in Figure 4, in the group of CTX-treated, all mice grew badly, the average weights of the mice was 17.092 g, which indicated that CTX has strong toxicity on the mice. On the other hand, the mice in the polysaccharide-treated group grew well. When reduce the dose of CTX and increase DDP1-1, the average weights of the mice was 29.562 g, which closed to DDP1-1-treated group. At the same time, the increase of tumor inhibitory rate in DDP1-1 and CTX treated group was significant observed, which is closed to that of CTX-treated group (p<0.05). Therefore, these results clearly showed that DDP1-1 has potential ability of reducing toxicity and promoting curative effect.

Conclusions

In the present study, three polysaccharide fractions were obtained from *D. denneanum*. Through anti-tumor *in vivo*, DDP1-1 exhibited strong anti-tumor ability, meanwhile,

Table 1. Effects of different polysaccharides and CTX on the secretion of the three cytokines (IL-2, TNF- α and IFN- γ). * $p < 0.05$, ** $p < 0.01$ vs. negative control.

Groups	Dose (mg/kg)	Concentration (pg/ml)		
		TNF- α	IFN- γ	IL-2
Negative control	-	65.00	7.96	13.23
	50.00	55.00	8.96	17.67
DDP1-1	25.00	780.00 **	5.93	27.88 *
	12.50	645.00 **	18.88 **	41.65 **
	50.00	33.00	7.77	8.70
DDP2-1	25.00	170.00 **	9.27	24.62
	12.50	60.00	15.58 *	14.90
	50.00	55.00	7.08	16.53
DDP3-1	25.00	50.00	13.71	19.28
	12.50	31.00	6.97	16.06
Positive control	12.50	32.00	3.28	6.58

**Figure 4.** The ability of DDP1-1 on reducing toxicity and promoting curative effect. Values are means \pm S.D, (n=10). * $p < 0.05$, ** $p < 0.01$ vs. positive control.

DDP1-1 with 12.50 mg/ kg also significant increased immune index of S180 mice, and strongly promoted the secretion of IL-2, TNF- α and IFN- γ . IL-2 could promote the long-term proliferation of T cells, TNF- α and IFN- γ can enhance immunoregulatory ability each other towards tumor. Therefore, enhancement of immunoregulatory ability was assumed to be the possible mechanism of DDP1-1 on inhibition of tumor. In addition, DDP1-1 has strong ability of reducing toxicity and promoting curative effect. With the results above, DDP1-1 could be explored as a novel potential anti-cancer drug.

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