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Anti-ovarian cancer potential of two acidic polysaccharides from the rhizoma of *Menispermum dauricum*

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ABSTRACT

Two acidic polysaccharides (MDP-A1 and MDP-A2) were isolated from the rhizome of *Menispermum dauricum* and their apparent molecular weight are 9.1×10^4 and 5.8×10^4 Da, respectively. Both contained glucose, mannose, galactose, arabinose, glucuronic acid and galacturonic acid, but differed in the molar ratio. We also investigated the antitumor activities and mechanisms of MDP-A1 and MDP-A2 in human ovarian carcinoma SKOV3 cells. The MTT assay showed that MDP-A1 and MDP-A2 were able to inhibit cell proliferation of SKOV3 cells in a bell-shaped concentration–response manner, due to a significant increase in the number of apoptotic cells. Furthermore, treatment with MDP-A1 or MDP-A2 caused significant induction of caspase-3 and caspase-8, but slightly affected caspase-9 activity. In addition, a significant reduction in tumor volume was observed in mice treated with MDP-A1 or MDP-A2. Taken together, these results suggested that MDP-A1 and MDP-A2 had a potential application as natural antitumor drugs.

1. Introduction

Menispermum dauricum DC. (Menispermaceae), called Bian Fu Ge in Chinese, distributes widely in China and its rhizome is a traditional Chinese medicine officially listed in the Chinese Pharmacopeia for treating sore throats, colitis, dysentery, and rheumatic arthralgia (Anon, 1977). Previous phytochemical studies on this herb have revealed that alkaloid were main components, which possessed various bioactivities including cardiovascular, anti-arrhythmic and anti-tumor effects (Liu, Zhang, & Hong, 2005; Shan et al., 2006; Yu, Fu, & Tao, 2005; Zhou, Qu, & Wang, 2005). Moreover, the injection of its total alkaloids has been applied clinically for a long time to treat chronic tracheitis, throat-sore and arthralgia (Chen, Xie, Zhou, & Qin, 2012). The bioactive components of *M. dauricum* have been mainly attributed to its alkaloids. However, only a few reports have focused on the carbohydrate constituents from this species. Wang, Xue, Jiang, and Zhang (2011) found that *M. dauricum* alkali polysaccharides had significant inhibiting effect on Hela cell proliferation. Until now, no investigation has been carried out on acidic polysaccharides from *M. dauricum* concerning anticancer activities. Therefore, for the first time, the aim of present study is to isolate and characterize the acidic polysaccharides from the rhizome of *M. dauricum* and evaluate their anti-ovarian cancer activities.

2. Materials and methods

2.1. Materials and reagents

The rhizome of *M. dauricum* was purchased from Tonghua, Jilin Province, China. The DEAE Sepharose fast flow and Sepharose 6 Fast Flow resin for chromatography were purchased from Amersham Biosciences. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trifluoroacetic acid (TFA), dimethylsulfoxide (DMSO), methyl iodide, and different monosaccharide standards were purchased from Sigma Chemical Co. All other chemicals and reagents used were of analytical reagent grade and obtained locally.

2.2. Isolation and purification of acidic polysaccharides

Ground rhizome of *M. dauricum* was soaked with 95% ethanol at 35 °C for 6 h to remove lipophilic components and inactivate enzymes, and then the dried residue was refluxed by hot distilled water for 3 h at 80 °C. The combined extract was filtered through gauze and centrifuged to separate from water-insoluble materials. The aqueous extract was concentrated to 30% of the original volume under reduced pressure in a rotary evaporator, and proteins were

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removed with Sevag method (Staub, 1965). The obtained aqueous fraction was dialyzed, concentrated and precipitated with 4-fold volume of anhydrous ethanol. The precipitate was washed with acetone and ethyl ether four times to afford the crude polysaccharide (CMDP, 8.5 g), which were further purified and isolated according to their different molecular weights and sizes, or based on their different ionic properties.

CMDP was dissolved in distilled water, and filtered through 0.45-μm Millipore filter, and then the solution was subjected to a DEAE Sepharose fast flow anion exchange column pre-equilibrated with 5 mM Tris–HCl (pH 8.0). The unabsorbed fraction (WCMDP), namely neutral polysaccharides, was eluted with 5 mM Tris–HCl (pH 8.0). Then the absorbed fraction (acidic polysaccharides) was eluted with NaCl gradients (0–2.0 M) to afford two acidic fractions (CMDP-A1 and CMDP-A2). The elution fraction (5 ml) were collected and monitored for carbohydrate content based on phenol–sulfuric acid method at 492 nm absorbance. After dialyzing and concentrating, CMDP-A1 and CMDP-A2 were further purified with a gel filtration column of Sepharose 6 Fast Flow with 5 mM Tris–HCl as eluant at the flow rate of 1 ml/min to yield two main fractions, coded as MDP-A1 and MDP-A2, respectively.

2.3. Molecular weight determination

The molecular weight of fractions were evaluated and determined by the high performance gel permeation chromatography with a Agilent 1100 HPLC apparatus. The sample (2.0 mg) was dissolved in distilled water (2 ml) and passed through a 0.45-μm Millipore filter. The filtrate was applied to a gel-permeation chromatographic column of TSK-gel G4000PW$_{XL}$ (7.8 mm × 300 mm, 5 μm), maintained at a temperature of 35 °C, eluted with 0.05 M Na_2SO_4 solution at a flow rate of 0.5 ml/min and detected by a RID-10A refractive index detector. A 20 μl sample was injected in each run. The columns were calibrated with T-series dextran (T-500, T-200, T-70, T-40, T-20 and T-10). The molecular weight of two acidic polysaccharides was estimated by the calibration curve made above.

2.4. Chemical properties

The yield of crude polysaccharides was calculated as a percentage of the total weight of sample used. The neutral carbohydrate content was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid content was determined by measuring the absorbance at 525 nm using the m-hydroxybiphenyl colourimetric procedure and with D-glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991). Protein content was measured according to Coomassie brilliant blue G-250 method, using BSA as the standard (Pierce & Suelter, 1977). The monosaccharide composition of WMDP was determined according to the method reported by Zhang, Xiao, Deng, He, and Sun (2012), with some modification. Briefly, polysaccharides (5 mg) were hydrolyzed with 4 ml of 2 M TFA at 120 °C for 2 h into monosaccharide under airtight condition, and the excess TFA was removed through decompression and distillation with methanol reiteration at a temperature of 40 °C. Then the hydrolyzed products were reduced with $NaBH_4$ (20 mg) and acetylated with acetic anhydride (Albersheim, Nevins, English, & Karr, 1967). The resulting alditol acetates were analyzed by gas chromatography (GC) using an Agilent 7890N instrument equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm) and a flame-ionization detector (FID), using inositol as the internal standard.

2.5. Cell lines and cell cultures

Human ovarian cancer cells SKOV3, human breast cancer cells MCF7 and hepatocellular carcinoma cells HepG2 were obtained from American Type Cell Culture (ATCC) and they were grown at 37 °C in a 5% (v/v) CO_2 atmosphere. The cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) plus 100 U/ml of penicillin and 100 μg/ml of streptomycin.

2.6. Cell viability assay

Cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Wu, Cui, Zhang, & Li, 2012). Briefly, cancer cells were plated at a density of 1×10^5 cells/ml into 96-well plates. After overnight growth, cells were pretreated with a series of concentrations of polysaccharides (50, 100, 200, 400 and 800 μg/ml) or medium for 24 h. At the end of treatment, 20 μl of MTT (5 mg/ml) was added to each well, and the cells were incubated for a further 4 h. Cell viability was determined by scanning with an ELISA reader with a 570-nm filter. The absorbance of the untreated cells (negative control) was set at 100% and percentage survival was calculated as the fraction of the negative control.

2.7. Flow cytometry detection of apoptosis

Detection of apoptosis was performed by staining cells with Annexin V-FITC and propidium iodide (PI) labeling as follows (Bharti, Takada, Shishodia, & Aggarwal, 2004). Briefly, SKOV3 cells were plated at a density of 2×10^5 cells/ml and were incubated with samples (100, 200 and 400 μg/ml) or medium (Control). After 24 h incubation, the treated cells were harvested and separated by gentle centrifugation and cell pellet was suspended in binding buffer. For each sample, the staining solution (5 μL Annexin V-FITC, 10 μL PI) was added and cells were incubated in the dark for 15 min at room temperature. Finally, the samples were analyzed by flowcytometry and evaluated based on the percentage of cells for Annexin V positive.

2.8. Caspase activities

Caspases 3, 8 and 9 activities were quantified using a colorimetric assay kit (Hushang Inc., Shanghai, China) according to the manufacturer's instructions. Briefly, cells were incubated with samples at indicated concentrations (100, 200 and 400 μg/ml) for 24 h. Cell lysates were collected in lysis buffer and centrifuged at 12,000 × *g* for 15 min at 4 °C. The resulting supernatants were added to the reaction buffer containing caspase substrate and incubatied for 2 h at 37 °C. Finally, absorbance was measured at 405 nm. The results were represented as the percentage of absorption value of treated group compared to the untreated control.

2.9. Animal experiment

Female congenital athymic BALB/c nude (nu/nu) mice (18–22 g) were provided by Experimental Animal Center of Harbin Medical University. The animals were maintained in cages under pathogen-free conditions at 23 ± 1 °C, with relative humidity of 55 ± 10%, light/dark cycle of 12/12 h and had free access to standard pellet diet and water. The animal care, use and experimental protocols were approved by the Animal Care Committee of Harbin Medical University.

Antitumor activities of MDP-A1 and MDP-A2 were determined using solid tumor models. Tumor was induced by subcutaneous

(s.c.) injection of SKOV3 cells (2×10^6 cells in 0.1 ml of PBS) at one site of the right flank of BALB/c nude mice. When the subcutaneous tumors became distinctively visible (approximately 3 mm × 3 mm in size) 14 days post inoculation, the mice were randomly divided into five groups (ten mice per group): a control group, which received vehicle (physiological saline); and four drug-treated groups, which received 25 and 50 mg/kg body weight of MDP-A1 or MDP-A2 in physiological saline. Daily administration of vehicle or two polysaccharides via intraperitoneally (i.p.) injection continued for 7 weeks. At the end of experiment, the mice were sacrificed by cervical dislocation and the tumor volume (TV) was calculated by measurement of the length (L) and width (W) of the tumor mass with the following formula: TV (mm^3) = $L \times W^2/2$, as described previously (Yawata et al., 1998). TV was recorded every week until animals were sacrificed.

2.10. Statistical analysis

Data are expressed as the mean ± S.D. and statistically analyzed using Student's t-test. A p value <0.05 is considered as statistically significant.

3. Results and discussion

3.1. Extraction and purification of two acidic polysaccharides

After removing lipophilic components, hot water extraction, deproteination and ethanol precipitation, crude polysaccharide (CMDP) was obtained from the rhizome of *M. dauricum*, and then separated and sequentially purified through DEAE Sepharose fast flow and Sepharose 6 Fast Flow column chromatography. According to the ionic strength difference, acidic polysaccharides were separated from neutral polysaccharides by eluting with 5 mM Tris–HCl and different NaCl solution (0–2 M), respectively. Two acidic polysaccharides fractions, named as CMDP-A1 and CMDP-A2, were collected from a gradient of NaCl aqueous solutions as monitored by phenol–sulfuric acid method. On account of molecular weight difference, CMDP-A1 and CMDP-A2 were further purified by size-exclusion chromatography on a Sepharose 6 Fast Flow column, giving two purified acidic polysaccharides which were named as MDP-A1 and MDP-A2, respectively (Fig. 1).

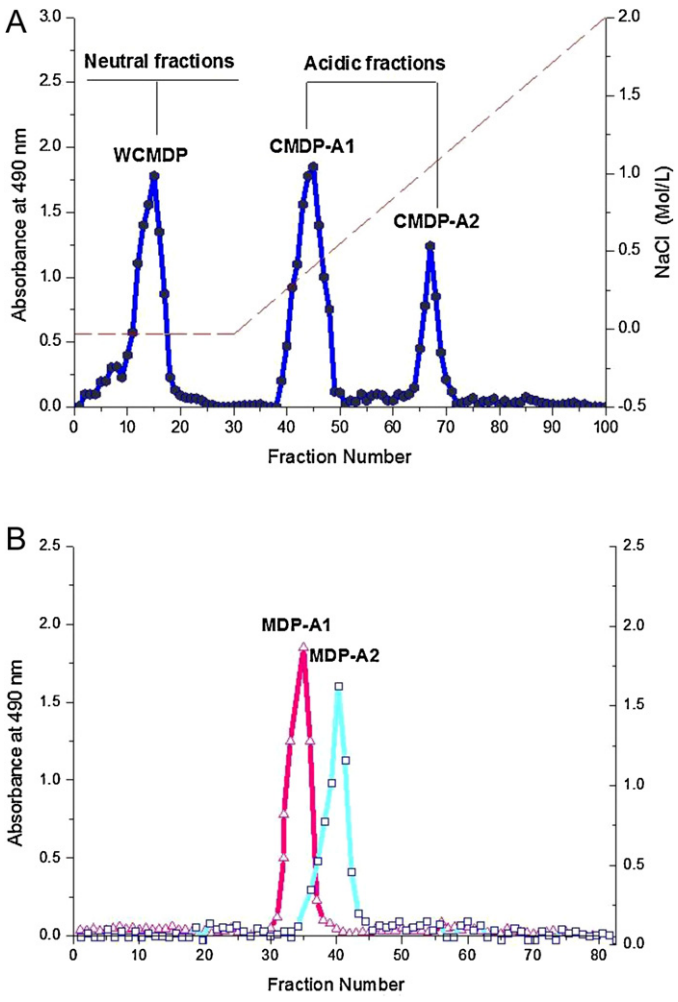

Fig. 1. (A) Elution curve of crude polysaccharide extracted from the rhizome of *M. dauricum* on a DEAE Sepharose fast flow anion-exchange column. (B) Purification of acidic polysaccharide fractions CMDP-A1 and CMDP-A2 on a Sepharose 6 Fast Flow column.

3.2. Analysis of two acidic polysaccharides

The total sugar, protein, uronic acid contents, molecular weight and monosaccharides composition of the polysaccharide fractions are summarized in Table 1. The total sugar contents MDP-A1 and MDP-A2 were found to be 67.4% and 58.8%, respectively, according to the phenol–sulfuric acid method. As determined by metahydroxydiphenyl colorimetric method, MDP-A1 and MDP-A2 contained 23.4% and 35.9% uronic acid, respectively. Although the Sevag method had been repeated for many times to remove free proteins, the combined protein was also found in all fractions. A small amount of 12.8% proteins existed in the MDP-A1, while MDP-A2 had a high protein content of 21.4%. Therefore two polymers could be protein-bound acidic polysaccharides. High performance size-exclusion chromatography of the MDP-A1 and MDP-A2 on Agilent 1100 HPLC system suggests that the polymers are homogeneous (data not shown). The apparent molecular weight of the polysaccharides would be 9.1×10^4 Da and 5.8×10^4 Da in reference to standard T-series Dextrans. According to GC analysis, MDP-A1 and MDP-A2 were composed of glucose, mannose, galactose, arabinose, glucuronic acid and galacturonic acid, with molar percent of 31.1%, 19.5%, 17.1%, 9.7%, 16.4% and 6.3% for MDP-A1 and 15.7%, 40.9%, 8.5%, 3.0%, 20.2 and 12.1% for MDP-A2, respectively. Glucose was the dominant monosaccharide of MDP-A1 with exceeding 30% (molar ratio) of the total sugars and mannose content is greater than 40% in MDP-A2.

3.3. Effect of two acidic polysaccharides on cell viability

To determine whether two acidic polysaccharides have growth inhibitory effect on human cancer cells, we performed MTT assays using three human cancer cell lines. As shown in Fig. 2, human ovarian cancer cells SKOV3 exhibited significant bell-shaped dose-response sensitivity to MDP-A1 or MDP-A2. The cell survival rate of SKOV3 cells treated by MDP-A2 was lower than that by MDP-A1 treatment, especially at the concentration of 400 μg/ml. In addition, MDP-A1 or MDP-A2 also killed other human tumor cell lines such as breast cancer cell line MCF7 and hepatoma cell line HepG2 cells in a dose-dependent manner, although the effect was not significant from the control except for MDP-A2 treatment for MCF7 cells at the concentration of 400 and 800 μg/ml. The results suggest that MDP-A1 and MDP-A2 have promising anti-ovarian cancer activities in vitro. Therefore, we further examined the cytotoxic effects of MDP-A1 or MDP-A2 in human ovarian cancer SKOV3 cells.

Table 1
Monosaccharide composition, sugar content, protein content, uronic acid, and average molecular weight of two acidic polysaccharides.

Fractions	Molecular weights ($\times 10^4$ Da)	Total sugar (w/w%)	Uronic acid (w/w%)	Protein (w/w%)	Contents of the sugar residues (mol.%)					
					Glucose	Mannose	Galactose	Arabinose	Glucuronic acid	Galacturonic acid
MDP-A1	9.1	67.4	23.4	12.8	31.1	19.5	17.1	9.7	16.4	6.3
MDP-A2	5.8	58.8	35.9	21.4	15.7	40.9	8.5	3.0	20.2	12.1

3.4. Effect of two acidic polysaccharides on apoptosis in SKOV3 cells

To determine whether the inhibition of cell growth by MDP-A1 or MDP-A2 resulted from the induction of apoptosis, Annexin V-FITC/PI double staining was performed. Compared with 1.9% of apoptotic cells in vehicle treated group, pretreatment with 100, 200 and 400 μg/ml of two acidic polysaccharides significantly increased the number of apoptotic cells to 32.17%, 41.54% and 35.22% for MDP-A1, and 42.34%, 56.54% and 40.02% for MDP-A2 (Fig. 3), respectively. These observations suggested that SKOV3 cells undergo apoptosis after two acidic polysaccharides treatment.

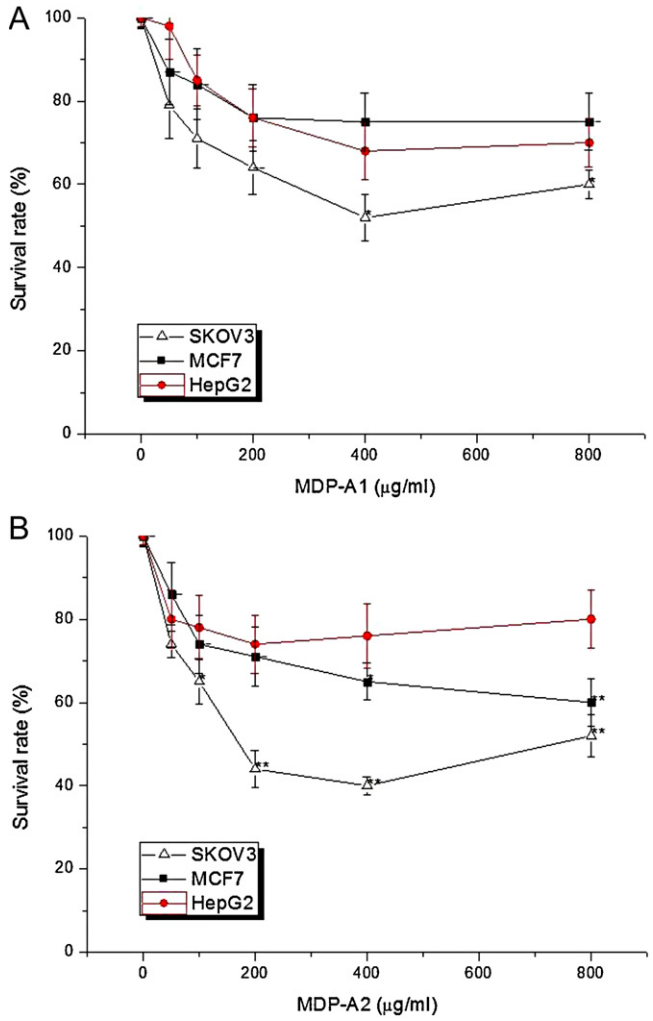

Fig. 2. Effects of MDP-A1 or MDP-A2 on the proliferation of SKOV3 cells evaluated by MTT assay. The results are presented as means ± S.D. of three independent experiments. $^{*}P < 0.05$ or $^{**}P < 0.01$ vs control values.

3.5. Effect of two acidic polysaccharides on caspase activities in SKOV3 cells

The activities of caspases 3, 8 and 9 in SKOV3 cells were determined following 24 h treatment with MDP-A1 or MDP-A2 at the concentration of 100, 200 and 400 μg/ml using colorimetric assay kits (Fig. 4). Compared with the control, treatment with two acidic polysaccharides at the concentration of 100, 200 and 400 μg/ml significantly increased the activity of caspase-3 to 1.7-, 2.8- and 2.1-fold for MDP-A1, and 2.1-, 3.5- and 2.8-fold for MDP-A2, respectively. A remarkable increase in the caspase-8 activity was also observed in a dose-dependent manner after the cells were exposed to MDP-A1 or MDP-A2 for 24 h, compared with the control. However caspase 9 activity was not sensitive to the lower concentration of two acidic polysaccharides and only increased mildly from 400 to 800 μg/ml. These data suggest MDP-A1 and MDP-A2 induced apoptosis of SKOV3 cells are more dependent on caspase 3 and caspase 8, and less dependent on caspase 9.

3.6. Effect of two acidic polysaccharides on tumor growth in SKOV3 carcinoma-bearing mice

To further evaluate whether two acidic polysaccharides possessed suppressive effects on tumor growth in mice, we used a nude mice xenograft model in which human ovarian cancer SKOV3 cells were injected s.c. into BALB/c nude mice. Two weeks after tumor implantation, mice were treated i.p. daily for 7 weeks with different concentrations of MDP-A1 and MDP-A2. Beginning the third week, a significant reduction in TV was observed in mice that were treated with MDP-A1 or MDP-A2 in a time and dose-dependent manner compared to that of untreated mice (Fig. 5). Moreover MDP-A2 exhibited a high inhibitory rate on tumor growth than MDP-A1

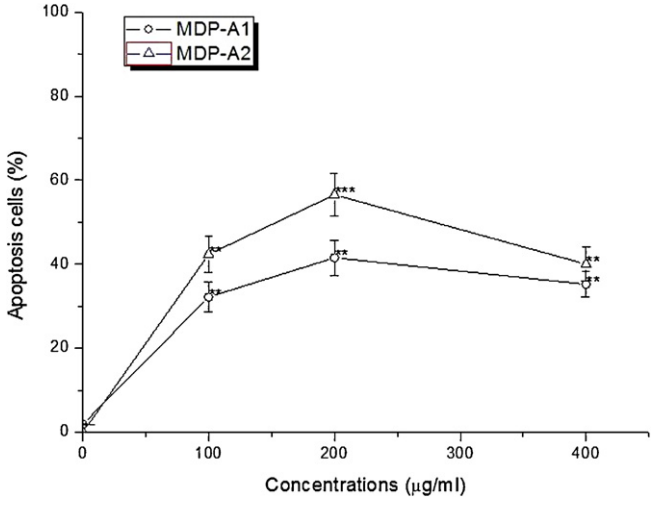

Fig. 3. Quantitative analysis of apoptotic cells after 24 h treatment with 100–400 μg/ml MDP-A1 or MDP-A2 by Annexin V-FITC/PI binding using flow cytometry analysis. The results are presented as means ± S.D. of three independent experiments. $^{**}P < 0.01$ or $^{***}P < 0.001$ vs control values.

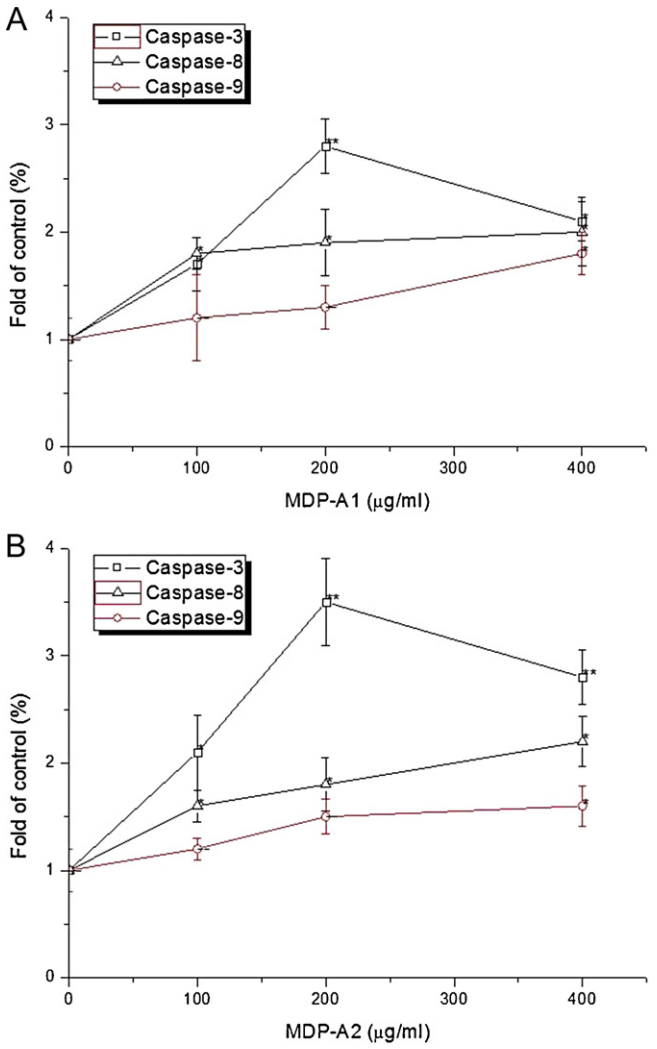

Fig. 4. Effect of MDP-A1 or MDP-A2 on caspase-3, -8 and -9 activities in SKOV3 cells. The results are presented as means ± S.D. of three independent experiments. $^{*}P < 0.05$ or $^{**}P < 0.01$ vs control values.

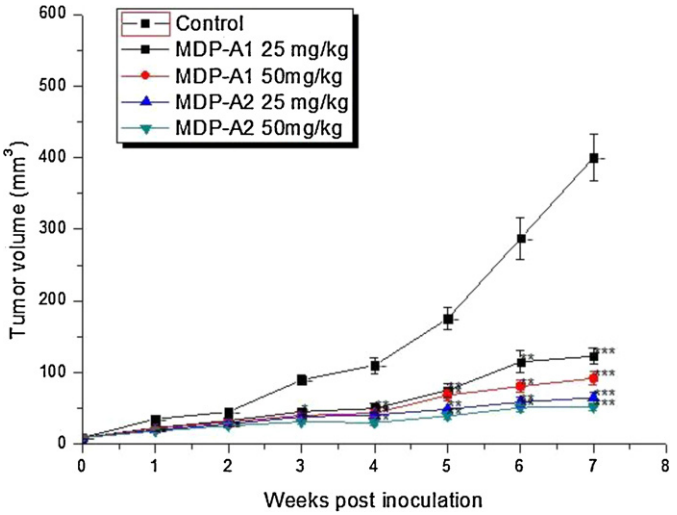

Fig. 5. Effect of MDP-A1 or MDP-A2 on the tumor growth of SKOV3 carcinoma-bearing mice. The results are presented as means ± S.D. ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ vs control values.

at every dose in each time point. These data imply MDP-A1 and MDP-A2 also inhibited the growth of transplantable tumor in vivo.

4. Conclusions

Cancer is the leading cause of mortality worldwide and a formidable problem for people. The great majority of the chemotherapeutic agents have been identified as cytotoxic to cancer cells, but also reported to exhibit undesirable side effects to normal tissues (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Fan, Ding, Ai, & Deng, 2012). Recently polysaccharides extracted from natural sources have drawn more attention of researchers and consumers due to their relatively low toxicity and obvious antitumor activities (Cao et al., 2010; Karnjanapratum & You, 2011). Therefore, it is very imperative to investigate novel antitumor strategies for eliminating the cancer cells with little toxicity to normal cells.

In this study, two purified acidic polysaccharides were successfully purified from the rhizome of *M. dauricum* by DEAE-Sepharose CL-6B and Sepharose 6 Fast Flow column chromatography. The present study demonstrated for the first time that MDP-A1 and MDP-A2 not only significantly inhibit the proliferation of SKOV3 cells in vitro, but also suppress the growth of transplantable tumor in BALB/c nude mice inoculated with SKOV3 cancer cells. An Annexin V-FITC/PI staining double staining assay revealed that antitumor activities of MDP-A1 and MDP-A2 were due to a significant increase in the number of apoptotic cells. Moreover, treatment with MDP-A1 or MDP-A2 caused different induction of caspase-3 and caspase-8 activity in SKOV3 cells, but only affected caspase-9 activity at high concentration of 800 μg/ml. Based on all the results, we found that MDP-A2 exhibited a better antitumor effect than MDP-A1, which would arise from different ratio of monosaccharide composition, average molecular weights, as well as the contents of sugar, protein and uronic acid among two acidic polysaccharides. Our findings should provide a clue for the development of novel therapeutic strategies for cancer.

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References

Albersheim, P., Nevins, D. J., English, P. D., & Karr, A. (1967). A method for the analysis of sugars in plant cell-wall polysaccharide by gasliquid chromatography. *Carbohydrate Research*, *5*, 340–345.

Anon. (1977). *Dictionary of traditional Chinese medicine*. Shanghai: Shanghai Science and Technology Publisher., pp. 2612–2614.

Bharti, A. C., Takada, Y., Shishodia, S., & Aggarwal, B. B. (2004). Evidence that receptor activator of nuclear factor (NF)-kappaB ligand can suppress cell proliferation and induce apoptosis through activation of a NF-kappaB-independent and TRAF6-dependent mechanism. *The Journal of Biological Chemistry*, *279*, 6065–6076.

Borchers, A. T., Stern, J. S., Hackman, R. M., Keen, C. L., & Gershwin, M. E. (1999). Mushrooms, tumors, and immunity. *Proceedings of the Society for Experimental Biology and Medicine*, *221*, 281–293.

Cao, W., Li, X. Q., Wang, X., Li, T., Chen, X., Liu, S. B., et al. (2010). Characterizations and anti-tumor activities of three acidic polysaccharides from *Angelica sinensis* (Oliv.) Diels. *International Journal of Biological Macromolecules*, *46*, 115–122.

Chen, J. Y., Xie, Y. F., Zhou, T. X., & Qin, G. W. (2012). Chemical constituents of *Menispermum dauricum*. *Chinese Journal of Natural Medicines*, *10*, 292–294.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Fan, L. P., Ding, S. D., Ai, L. Z., & Deng, K. A. (2012). Antitumor and immunomodulatory activity of water-soluble polysaccharide from *Inonotus obliquus*. *Carbohydrate Polymers*, *90*, 870–874.

Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, *197*, 157–162.

Karnjanapratum, S., & You, S. G. (2011). Molecular characteristics of sulfated polysaccharides from *Monostroma nitidum* and their *in vitro* anticancer and

immunomodulatory activities. *International Journal of Biological Macromolecules*, *48*, 311–318.

Liu, W., Zhang, Z. Q., & Hong, T. (2005). Determination of dauricine and daurisoline in *Menispermum dauricum* DC. with HPLC. *Journal of Chinese Medicinal Materials*, *9*, 783–784.

Pierce, J., & Suelter, C. H. (1977). An evaluation of the Coomassie brilliant blue G-250 dye-binding method for quantitative protein determination. *Analytical Biochemistry*, *81*, 478–480.

Shan, B., Ren, F. Z., Liang, W. J., Zhang, J., Li, Q. X., & Zeng, Y. P. (2006). Isolation and purification of antitumor component in Rhizoma Menispermi and analysis of its activity. *Teratogenesis, Carcinogenesis, and Mutagenesis*, *3*, 175–179.

Staub, A. M. (1965). Removal of protein-Sevag method. *Methods in Carbohydrate Chemistry*, *5*, 5–6.

Wang, Z. H., Xue, J. B., Jiang, W. Y., & Zhang, G. R. (2011). A study on effects of *Menispermum dauricum* alkali polysaccharide on Hela cell proliferation. *Dong Bei Shi Da Xue Bao*, *43*, 132–136.

Wu, B., Cui, J. C., Zhang, C. G., & Li, Z. H. (2012). A polysaccharide from *Agaricus blazei* inhibits proliferation and promotes apoptosis of osteosarcoma cells. *International Journal of Biological Macromolecules*, *50*, 1116–1120.

Yawata, A., Adachi, M., Okuda, H., Naishiro, Y., Takamura, T., Hareyama, M., et al. (1998). Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells. *Oncogene*, *16*, 2681–2686.

Yu, W. P., Fu, C. S., & Tao, L. L. (2005). Determination of Dauricine in *Rhizoma Menispermi* from different districts by HPLC. *Qilu Pharmaceutical Affairs*, *3*, 72–73.

Zhang, A. Q., Xiao, N. N., Deng, Y. L., He, P. F., & Sun, P. L. (2012). Purification and structural investigation of a water-soluble polysaccharide from *Flammulina velutipes*. *Carbohydrate Polymers*, *87*, 2279–2283.

Zhou, J., Qu, F., & Wang, X. T. (2005). Study on alkaloids in *Menispermum dauricum* DC. *Liaoning Journal of Traditional Chinese Medicine*, *11*, 1193–1194.