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Extraction, purification and antitumor activity of a water-soluble polysaccharide from the roots of *Polygala tenuifolia*

Tao Xin[a], Fubin Zhang[b,*], Qiuying Jiang[a], Chunhong Chen[a], Dayong Huang[a], Yanju Li[a], Weixi Shen[a], Yinghua Jin[a]

[a] Department of Medical Oncology, The Second Affiliated Hospital, Harbin Medical University, Harbin 150081, China
[b] Department of Gynecology, The Third Affiliated (Tumor) Hospital, Harbin Medical University, Harbin 150040, China

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ABSTRACT

One polysaccharide PTP was isolated and purified from the roots of *Polygala tenuifolia*. It consisted of galactose, glucose and galactose in the ratio of 3.1:3.7:2.5, and a small amount of rhamnose, mannose and xylose. 17 general amino acids were identified to be components of the protein-bound polysaccharide analyzed by automatic amino acid analyzer. In order to test the anti-cancer activity of PTP, we investigated its effect against the growth of human ovarian cancer cells SKOV3 in vitro and in ovarian cancer rats. The intracellular reactive oxygen species (ROS) and glutathione (GSH) in SKOV3 cells following PTP treatment were also quantified to explore the possible mechanism underlying the antitumor activity of the polysaccharide. The result showed that PTP is effective on inhibiting the proliferation of SKOV3 cells in a concentration-dependent manner. Furthermore, treatment with PTP caused a rapid depletion of intracellular GSH content and accumulation of intracellular ROS, thus resulting in the apoptosis, which may prove to be a pivotal mechanism for its cancer protection action. In addition, a significant tumor growth inhibition effect was observed in nude mice after PTP administration for 7 weeks. All above indicated PTP could be beneficial towards ovarian cancer therapy.

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1. Introduction

Currently chemotherapy is the main tool for cancer treatment, but it induces severe side effects in many cases. Extensive studies to search for new active extracts or components derived from various plants that can be used in the treatment of cancer have been carried out, in attempts to discover additional therapeutic drugs that possess both high efficacy and safety (Gao et al., 2005; Ni et al., 2010). In the last decades, many polysaccharides and polysaccharide–protein complexes extracted from mushrooms, fungi, yeast, algae, lichens, plants and animals have been reported to exhibit a variety of biological activities, especially antitumor and immunomodulatory activities (Cao et al., 2010; Liu, Li, Yang, Zhang, & Cao, 2012; Wang, Huang, Wei, Li, & Chen, 2009; Wang et al., 2010; Zhou & Chen, 2011). They cause no harm and place no additional stress on the body, but help the body to adapt to environmental and biological stress (Mizuno et al., 2000). Therefore it is essential to explore the pharmacological research on the polysaccharide from natural source for the access to safe and potent antitumor agents that protect cancer patients from the harmful side effects of chemotherapy.

Polygala tenuifolia Willd. is distributed in northeast China. The root of *P. tenuifolia*, a Chinese traditional medicine named 'Yuan Zhi,' has been recorded in clinics with the actions of tranquilizing the mind, eliminating phlegm for resuscitation, and dissipating carbuncles. Accumulating evidence suggests that it has sedative, antipsychotic, cognitive improving/neuroprotective and anti-inflammatory therapeutic effects on the central nervous system (Chung, Kim, & Kim, 1992; Chung et al., 2002; Jang et al., 1997; Kim et al., 1998; Park et al., 2002; Zhang et al., 2008). It has also been used for insomnia, neurasthenia, amnesia, palpitations with anxiety, restlessness, and disorientation, and to prevent dementia and memory failure. Various xanthones, saponins, and oligosaccharide esters have been reported from this plant (Cheng et al., 2006; Lin, Chen, Ko, Ko, & Wu, 2008; Liu et al., 2007). However there is not a report published about the extraction and isolation of polysaccharides from the root of *P. tenuifolia*, let alone its antitumor activity. Therefore, in this study we report on the isolation and physicochemical characterization of a water-soluble polysaccharide from the root of *P. tenuifolia*. In addition, the effect of this polysaccharide against SKOV3 cell, a human ovarian cancer cell line, was also evaluated in vitro and in vivo.

* Corresponding author. Tel.: +86 0451 86641537; fax: +86 0451 86641537.
E-mail address: fubinzhang_hrbmu@hotmail.com (F. Zhang).

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2. Experimental

2.1. Materials and chemicals

P. tenuifolia was harvested in October from a farm in Northeast China. It was dried in sunlight, cut into small pieces and then dried at an oven for 16 h before use. The material, which passed through 160-mesh screen and was retained on an 80-mesh screen, was collected for subsequent experiment. 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR). DEAE-Sephacel and Sephadex G-100 were purchased from Amersham Pharmacia Biotech. Bovine serum albumin (BSA), CF_3COOH (TFA), 1-phenyl-3-methyl-5-pyrazolone (PMP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the monosaccharides (mannose, glucosamine, ribose, rhamnose, glucuronic acid, galacturonic acid, galactosamine, glucose, galactose, xylose, arabinose, and fucose) were purchased from Sigma. All other reagents were of the highest available quality.

2.2. Isolation and purification of polysaccharide fractions

The dried *P. tenuifolia* (200 g) was first treated by refluxing in a Soxhlet apparatus with toluene–ethanol (1:1, v/v) for 6 h to remove fats and waxes. The dewaxed material (178 g) were washed quickly with water and decocted twice in water for 2 h. After the extraction solution was filtrated, concentrated and cooled down, 95% ethanol was added slowly until the end concentration of 50% (v/v) and was kept for 1 h. Then 10% $CaCl_2$ was added and kept overnight to precipitate the tannin. The supernatant was obtained by centrifugation at 20,000 rpm for 5 min and successively ultrafiltrated with membrane (MWCO: 1 k) and membrane (MWCO: 10 k) on an ultrafiltration apparatus. The ultrafiltrate (outside of membrane of 10 k) was concentrated by heating and lyophilized to obtain crude polysaccharide (cPTP). The cPTP dissolved in distilled water was loaded on DEAE-Sephacel column, being first washed with deionized water, then with increasing ionic strength of NaCl solution (0→2 M) at a flow rate of 1 mL/min. In this procedure, two main fractions contained polysaccharide were acquired in deionized water and gradient NaCl elution part. The fraction containing polysaccharide eluted by deionized water was further applied to a column (2.5 cm × 100 cm) of Sephadex G-100 equilibrated with 50 mM acetate buffer (pH 5.2), and was eluted with the same buffer at a flow rate of 1 mL/min. In order to detect polysaccharides, a 0.2 mL sample collected from each eluted fraction (6 mL/tube) was mixed with sulfuric acid and phenol to produce color reaction. The fractions with rose color were combined, concentrated under vacuum, dialyzed with membrane (MWCO: 1 k) to desalinate and lyophilized to obtain the light yellow purified polysaccharide (PTP). The purified polysaccharide was kept in dryer for further analysis.

2.3. Polysaccharide content and monosaccharide composition analysis by HPLC

Total carbohydrate content in PTP was measured by phenol–sulfuric acid colorimetric method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The monosaccharide components of the polysaccharide were analyzed by HPLC method based on Chen, Zhang, Jiang, Mu, and Miao (2012), with some modifications. Briefly, the protein-bound polysaccharide sample (5 mg) was first methanolyzed using 0.5 mL anhydrous methanol containing 2 M HCl at 80 °C for 16 h and then completely hydrolyzed with 0.5 mL of 2 M TFA to monosaccharide at 105 °C for 4 h. The hydrolytic product of polysaccharide and standard monosaccharide mixture (mannose, glucosamine, ribose, rhamnose, glucuronic acid, galacturonic acid, galactosamine, glucose, galactose, xylose, arabinose, and fucose) were derivatized to be PMP derivatives and subsequently subjected to HPLC (ZORBAX Eclips XDB-C18, 5 μm, 250 mm × 4.6 mm column) analysis by UV absorbance at 245 nm. The mobile phase was 0.1 M NaCl buffer with flow rate of 0.5 mL/min and the column temperature was 50 °C.

2.4. Analysis of protein, uronic acid contents and amino acid composition

The proteins in the polysaccharides were quantified according to the Bradford method using BSA as the standard (Bradford, 1976). Uronic acid contents were determined by measuring the absorbance at 523 nm using the m-hydroxybiphenyl colourimetric procedure and with D-glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991). The amino acids in polysaccharide were determined with a Hitachi 835-50G automatic amino acid analyzer (Hitachi Ltd., Tokyo, Japan). The PTP (5 mg) was hydrolyzed in a sealed tube for 24 h at 110 °C under vacuum. The hydrolysate was evaporated with a speedvac concentrator and the dried residue was redissolved in 0.02 M HCl solution. The sample was passed through a 0.45-μm nylon filter before being injected into the amino acid analyzer.

2.5. Cell proliferation assay

Cell proliferation was determined by MTT assay. Cells in their exponential growth phase were seeded into flat-bottomed 96-well plates at a density of 1×10^5 cells per well and incubated for 24 h at 37 °C in CO_2 incubator. PTP ranging from 0 to 40 μg/mL was added to the wells and the plates were incubated for additional 48 h at 37 °C. After removing the supernatants, 20 μl of MTT and 180 μl of PBS were added to each well and the plates were incubated at 37 °C for 4 h in a humidified chamber. Again the supernatant was carefully removed and 150 μl of dimethyl sulphoxide (DMSO) was added into each well to dissolve the MTT formazan at the bottom of the wells. After 10 min, the absorbance was read at 570 nm using enzyme-linked immunosorbentassay (ELISA) plate reader. The inhibition ratio of the treated cells was calculated based on the following formula: (1 − the A_{570} value for treated cells/the A_{570} value of untreated cells) × 100%. All experiments were carried out in triplicate.

2.6. DCF method for detection of intracellular ROS

Measurements of intracellular ROS levels in SKOV3 cells were made using DCFH-DA, which is able to diffuse through the cell membrane and become enzymatically hydrolyzed by intracellular esterases to produce non-fluorescent DCFH. Intracellular ROS mainly H_2O_2, $HO^•$, $ROO^•$, $NO^•$ and $ONOO^-$ can oxidize DCFH to fluorescent DCF which stains the cells (Wang & Joseph, 1999). Thus, the fluorescence intensity is proportional to the amount of ROS produced by the cells (Pan et al., 2006). SKOV3 cells were seeded in a 96-well plate and incubated with the polysaccharide at the concentrations of 10, 20 and 40 μg/mL. After 24 h incubation, cells were incubated with 5 μM fluorescent dye DCFH-DA at 37 °C for 30 min, then were washed twice with phosphate buffered saline (PBS) and centrifuged at 1200 rpm to remove the extracellular DCFH-DA to detect the intracellular levels of ROS (λ_{ex} = 485 nm and λ_{em} = 530 nm). Measurements were done in triplicates for each concentration for a total of at least 3 independent experiments. Results are expressed as mean relative fluorescence intensity (RFI) ± SD.

2.7. Measurement of intracellular glutathione (GSH)

The intracellular content of GSH was assessed using a GSH assay kit (Shanghai Qianchen Biotech Co., China). After SKOV3 cells exposed to different concentrations of polysaccharide (10, 20 and 40 μg/mL) for 24 h, the cells were collected, and then centrifuged at 10,000 × *g* for 10 min. The supernatants were then added to a 96-well plate, and the assay was performed according to the manufacturer's instructions.

2.8. In vivo antitumor activity

Female congenital athymic BALB/c nude (nu/nu) mice (6–8 weeks) weighing 18–22 g were obtained from the Vital River Laboratories (Peking, China) and housed in a rodent facility at 22 ± 1 °C with a 12-h light/12-h dark cycle. They were fed a standard diet and water ad libitum according to institutional guidelines. All procedures were in accordance to the Institute Ethical Committee for Experimental Use of Animals.

SKOV3 cells (2×10^6 cells in 0.1 mL of PBS) were injected subcutaneously at one site of the right flank of female nude mice. When the tumor reached approximately 3 mm × 3 mm in size, animals were divided randomly into four groups (ten mice per group) and then treated with PTP intraperitoneally (i.p.) for 7 weeks. Administration of PTP (10, 20 and 40 mg/kg), dissolved in a vehicle of PBS, was performed 5 days per week and tumor size was determined once a week for 7 weeks by measurement of the length (L) and width (W) of the tumor. Tumor volumes were calculated as TV (mm^3) = ($L \times W^2$)/2, as described previously (Wang et al., 2006). The control group was treated with vehicle only.

2.9. Statistical analysis

All determination was performed in triplicate, and data were expressed as means ± SD. The data were subjected to an analysis of variance (ANOVA, $p < 0.05$), and Duncan's multiple range tests. A significant difference was judged to exist at a level of $p < 0.05$.

3. Results and discussion

3.1. Isolation and purification of polysaccharides

Crude polysaccharide cPTP was prepared from *P. tenuifolia* by hot-water extraction, ultrafiltration and EtOH precipitation. cPTP yield was 5.27% of the dry weight. Total cPTP was fractioned by chromatography on DEAE-Sephacel column and two fractions obtained were named as cPTP-I and cPTP-II (Fig. 1A), respectively. The main fraction cPTP-I was further purified on Sephadex G-100 column (Fig. 1B). The final purified polysaccharide was named as PTP, a light yellow powder.

3.2. Physicochemical properties and chemical compositions

The PTP had an obvious absorption at 280 nm in the UV spectrum, supporting the fact that 30.2% protein was present in PTP evaluated by Bradford method. Results from phenol–sulfuric acid assay showed that PTP contained 68.4% carbohydrate and no uronic acid was detected by m-hydroxybiphenyl colourimetric method. Monosaccharide composition analysis, carried out by HPLC following acid hydrolysis and derivatization with PMP, showed that PTP was composed of primarily of galactose (31%), glucose (37%) and galactose (25%), whereas others (e.g. rhamnose, mannose and xylose) were in a minor content, below 7%. The amino acid composition in protein-bound polysaccharide was analyzed by automatic amino acid analyzer. As shown in Table 1, 17 general amino acids were identified to be components of the macromolecule. PTP

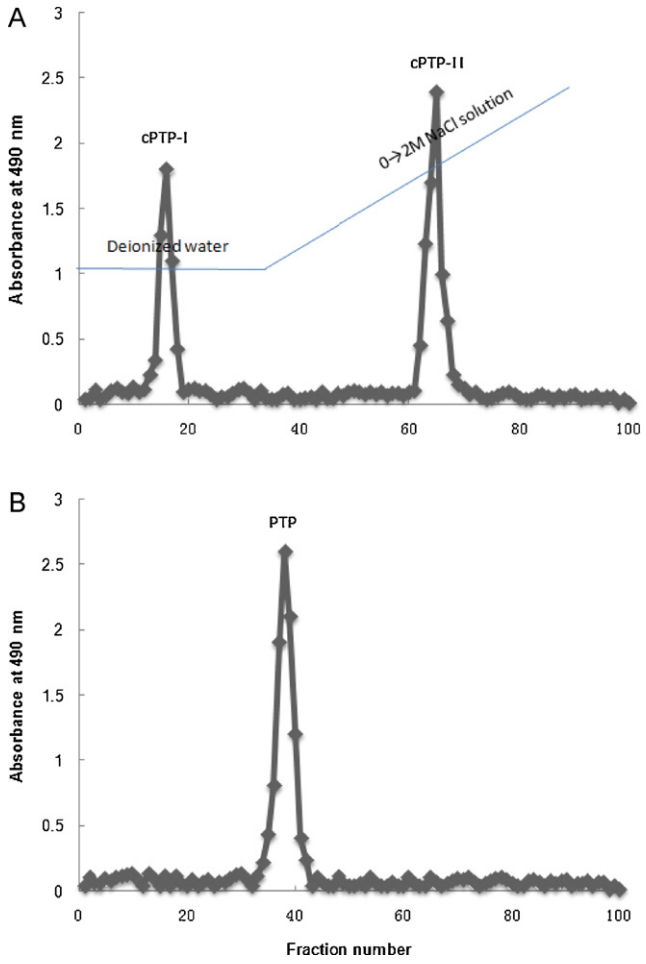

Fig. 1. (A) Anion-exchange chromatography of the water-soluble polysaccharide (PTP) from *P. tenuifolia* on DEAE-Sephacel column. (B) Gel filtration chromatography of the water-soluble polysaccharide (PTP) from *P. tenuifolia* on Sephadex G-100 column.

was rich in aspartic acid (13.2%), followed by threonine (10.3%), glutamic acid (9.4%), serine (8.1%) and alanine (7.2). The high concentration of threonine and serine indicated the possibility of the existence of the O-glycosidic linkages (Chen, Xie, Nie, Li, & Wang, 2008).

Table 1
The amino acid composition of the water-soluble polysaccharide (PTP).

No.	Name of amino acid	Content (%)
1	Aspartic acid	13.2
2	Threonine	10.3
3	Glutamic acid	9.4
4	Serine	8.1
5	Alanine	7.2
6	Glycine	6.8
7	Arginine	6.4
8	Histidine	5.9
9	Tyrosine	5.7
10	Cystine	5.4
11	Isoleucine	5.2
12	Lysine	5.1
13	Valine	4.8
14	Methionine	2.2
15	Proline	1.9
16	Phenylalanine	1.3
17	Leucine	1.2

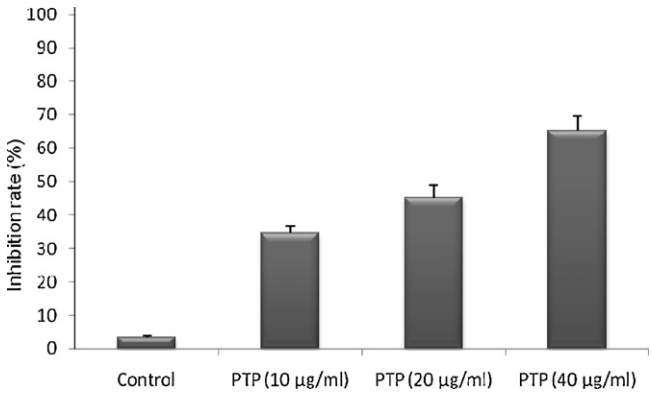

Fig. 2. Antiproliferative action of the water-soluble polysaccharide (PTP) from *P. tenuifolia* in SKOV3 cells. Data are presented as mean ± SD values based on three independent experiments.

3.3. Antitumor activity of PTP in vitro

MTT method was used to evaluate the antitumor properties of PTP against SKOV3 cells and growth inhibition was calculated comparing to a negative control growth. The reduction of yellow MTT to purple formazan takes place only when mitochondrial reductase enzymes are active, and therefore the amount of conversion can be directly accounted for the percentage of viable (living) cells (Slater, Sawyer, & Straeuli, 1963). As shown in Fig. 2, when cells were exposed to polysaccharide (10, 20 and 40 μg/mL) for 2 days, the tumor cell growth inhibition rates progressively increased in a dose-dependent manner. The result indicated that PTP had a potent anti-proliferation effect on SKOV3 cells.

3.4. The effect of PTP on the content of intracellular ROS and GSH in SKOV3 cells

Many drugs induce apoptosis through an increased intracellular ROS event (Hsieh, Liu, Lu, Hsieh, & Chen, 2006). So we examined the effect of PTP stimulation on intracellular ROS production by flow cytometry using DCFH-DA. As anticipated, SKOV3 cells exposed to PTP at the concentrations of 10, 20, and 400 μg/mL, resulted in a burst of ROS accumulation (Fig. 3), as measured by DCF fluorescence, a fluorophore that has been extensively used as a general marker of overall intracellular ROS production (Matés & Sánchez-Jiménez, 2000). The significance of P value was less 0.05. This result demonstrated that apoptosis of SKOV3 cells induced by PTP might be mediated by increasing intracellular ROS creation.

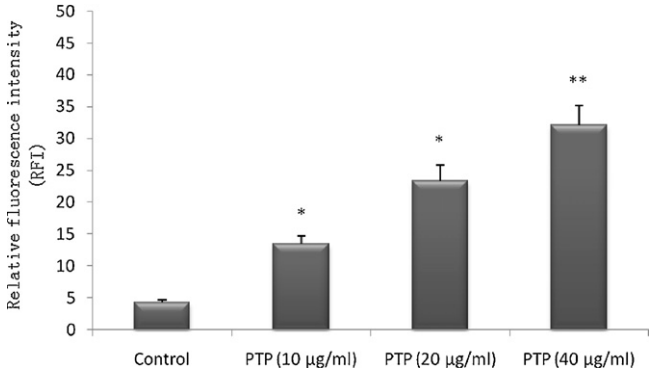

Fig. 3. Quantification of intracellular ROS was detected by flow cytometry using DCFH-DA. Data are presented as mean ± SD values based on three independent experiments.

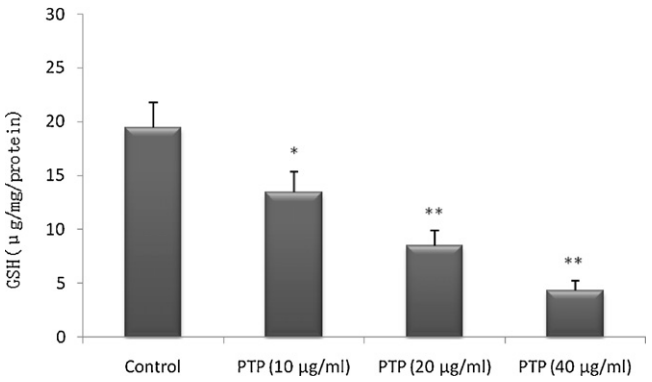

Fig. 4. The content of intracellular GSH influenced by the water-soluble polysaccharide (PTP) in SKOV3 cells. Data are presented as mean ± SD values based on three independent experiments.

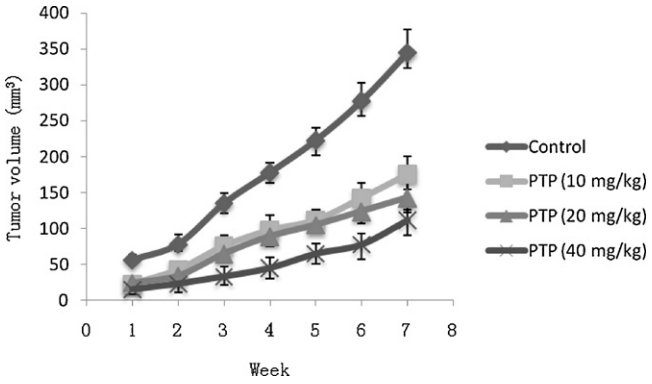

Fig. 5. Inhibitory effect of the water-soluble polysaccharide (PTP) from *P. tenuifolia* on tumor growth in mice. Data are presented as mean ± SD values based on 10 mice in each group.

GSH is an intracellular nonenzymatic antioxidant, a major cellular reductant found in all eukaryotic cells. Depletion of glutathione pools is suggested to be part of cell death effector machinery, and accompanies ROS production during apoptosis in relevant systems (Macho et al., 1997). As shown in Fig. 4, PTP treatment significantly reduced glutathione in SKOV3 cells compared to control cells after 24 h incubation. Collectively, the accumulation of intracellular ROS and depletion of glutathione suggests that PTP could cause oxidative stress in SKOV3 cells, thus leading to the cell death or apoptosis.

3.5. Antitumor activity of PTP in vivo

To evaluate the antitumor activity of PTP in vivo, human ovarian cancer xenografts were established by i.p. injection of SKOV3 cells (2×10^6 cells in 0.1 mL of PBS) at one site of the right flank of female nude mice. After the tumor reached about 3 mm × 3 mm in volume, mice were randomized into vehicle control and treatment groups (ten animals each) and given i.p., injections of either PBS (control group), or 10, 20 and 40 mg/kg of PTP (treatment groups) for five days every week until they were sacrificed. After 7 weeks of PTP treatment, the tumor growth was significantly suppressed at three doses in a dose-dependent fashion, whereas the tumors continued to grow in the control group (Fig. 3). And no observable histological changes happened in various normal tissues including brain, liver and kidney (data not shown) (Fig. 5).

4. Conclusions

In the current study, we isolated a water-soluble polysaccharide from the root of *P. tenuifolia*, which is a protein-bound

polysaccharide consisting of galactose, glucose and galactose in the ratio of 3.1:3.7:2.5, and a small amount of rhamnose, mannose and xylose. The total carbohydrate content of PTP was 68.4% determined by phenol–sulfuric acid method and 30.2% protein as evaluated by Bradford method. There are total 17 general amino acids identified in PTP. MTT assay showed that PTP treatment had a obvious inhibitory effect on the proliferation of SKOV3 cells at all three concentrations. Additionally, it has been convincingly shown that PTP can induce glutathione depletion in SKOV3 cells, supporting the fact that the burst of intracellular ROS production following PTP treatment, as determined by flow cytometry using the ROS-specific probe DCFH-DA. More importantly, after 7 weeks of PTP treatment, the tumor growth was significantly suppressed in mice implanted with SKOV3 cells. Our results clearly demonstrate that the antitumor mechanism of PTP might be mediated by GSH depletion and the increase of intracellular ROS in cancer cells, thus resulting in the apoptosis. On the basis of results reported here, we are currently elucidating its mechanism of action in an elaborate way and in animal experimental model.

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