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Antitumor effects of a purified polysaccharide from *Rhodiola rosea* and its action mechanism

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ABSTRACT

In the last three decades, numerous polysaccharides and polysaccharide–protein complexes have been isolated from plant or animal and used as a promising source of therapeutic agents for cancer. In this study, we prepared a homogeneous polysaccharide (RRP-ws) from *Rhodiola rosea* and tested its immunomodulation and anti-cancer activity in vitro and in vivo experiments using Sarcoma 180 (S-180) cells. Preliminary physicochemical analysis identified that RRP-ws was composed of Glc, Gal, Man and Rha with a relative molar ratio of 4.2:2.4:1.6:1.0, and contained 95.14% of total carbohydrate, 2.08% of protein and no sulfate. In vitro experiment showed that RRP-ws exerted a direct cytotoxic effect on the growth of S-180 cells. In vivo experiment, RRP-ws could inhibit tumor growth of S-180 tumor transplanted in mice, and increase the relative spleen/thymus indexes and body weight. Furthermore, RRP-ws also increased the production of IL-2, TNF-α and IFN-γ in serum, and elevated the ratio of CD4+/CD8+ on peripheral blood T-lymphocyte in tumor bearing mice. The overall findings indicated that RRP-ws could be used as a novel promising immunotherapeutic agent in cancer treatment.

1. Introduction

The rhizome and roots of *Rhodiola rosea* L. has been widely used for a long time in Russian and Chinese folk medicine to increase human physical and mental performance, longevity, and resistance to high-altitude sickness and to treat fatigue, anaemia, cancer, bacterial infection, impotence, nervous system disorders and cardiovascular diseases (Brown, Gerbarg, & Ramazanov, 2002; Olsson, von Schéele, & Panossian, 2009; Pooja, Anilakumar, & Bawa, 2006). *R. rosea* belongs to the family Crassullaceae that grows in the Arctic and in the mountainous regions of Europe, Asia, and North America (Olsson et al., 2009). Recently phytochemical studies of *R. rosea* have revealed the presence of glycosides, flavonoids, essential oils, fats, waxes, sterols, organic acids (oxalic, citric, malic, gallic, and succinic), tannins and proteins (Kurkin, Zapiesocznaja, Szczavlinskij, Nuhimovskij, & Vandysziev, 1985). Extensive studies proved *R. rosea*'s anticancer role (Kelly, 2001) and showed its positive effect on Ehrlich, B 16 and Levis cancers (Razina, Zueva, Amosova, & Krylova, 2000). Udintsev and Schakhov (1991) demonstrated that the extract of *R. rosea* could synergize the antitumor activity of cyclophosphamide, and lower its hepatotoxicity. In addition, a study by Liu, Li, Simoneau, Jafari, and Zi (2012), showed that both *R. rosea* extracts and salidroside could decrease the growth of bladder cancer cell lines via inhibition of the mTOR pathway and induction of autophagy. It has been also found that the extract of *R. rosea* rhizomes induced apoptosis and necrosis in HL-60 cells by an accumulation of cells at the prophase stage (Majewska et al., 2006). In spite of these tumor growth inhibitory effects of *R. rosea* extract, little is known about the beneficial health effect of the polysaccharide from this plant on tumor treatment. Recently, research interest has focused on polysaccharide from various herbs with immune-stimulating properties, which may be useful complementary or alternative medicine in helping human to reduce the risk of infectious disease and cancer. For an effective exploitation of this unique plant, it is necessary to understand the composition of the polysaccharide and its antitumor potential. In addition, the toxicity of polysaccharide on the liver and kidney of tumor bearing mice were also assayed.

2. Materials and methods

2.1. Reagents

The plant materials were bought from local medical market in Changchun City (China), and were identified according to the identification standard of the Pharmacopoeia of the People's Republic

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of China (PPRC). Cyclophosphamide (CTX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trifluoroacetic acid (TFA), the standard monosaccharides (rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose) were from Sigma Chemical Co. (St. Louis, MO). DEAE Sepharose Fast Flow, Sepharose 6 Fast Flow, and Sephacryl S-200 HR were from Amersham (Sweden). All the other chemical reagents were analytical reagent grade.

2.2. Purification and chemical properties of polysaccharides

Powdered *R. rosea* (200 g) was homogenized and extracted three times in a blender with 2 l of distilled water for 3 h at room temperature and then at 100 °C for 3 h. The whole extract was filtered and centrifuged at 10,000 rpm for 30 min at 4 °C to obtain a clear soln. The supernatant was concentrated to 200 ml and precipitated by the addition of aq. EtOH (1:5, v/v) at room temperature, and kept overnight. After overnight precipitate, the sample was centrifuged as described above, and the precipitation was dissolved in water to sufficiently mix with Sevag reagent for removing the free protein and combined protein (Staub, 1965) in this precipitate. After the disappearance of protein, the remaining liquid was dialyzed through a cellulose bag for 24 h to remove the low molecular weight materials, and freeze dried, giving 8.6 g of crude polysaccharide. The crude polysaccharide was fractionated on a DEAE Sepharose Fast Flow column (Cl^- form, 50 cm × 2.6 cm), and eluted stepwise with distilled water, 0.1, 0.3, and 0.5 M NaCl. After dialysis and lyophilization, the fraction of elution with distilled water was purified on a Sepharose 6 Fast Flow column (100 cm × 2.6 cm), eluting with 0.15 M NaCl. The portion of elution with 0.15 M NaCl was collected, dialyzed, lyophilized and further fractioned on Sephacryl S-200 HR columns (100 cm × 2.6 cm) with 0.15 M NaCl as eluent, yielding the purified polysaccharide (RRP-ws). The purification process was carried out several times and a total of 200 mg polysaccharide was collected. All the fractions were collected by the automated fraction collector, and monitored with the phenol–sulfuric acid method at 490 nm absorbance for polysaccharides. Total carbohydrate content in polysaccharide was determined by phenol–sulfuric acid colorimetric method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The concentration of total protein was estimated by the folin–phenol method of Lowry, Rosebrough, Farr, and Randall (1951) using BSA as standard. The sulfate content of the polysaccharide was measured using a modification of $BaCl_2$ turbidimetric method described by Craige, Wen, and Meer (1981).

2.3. Monosaccharide composition analysis

The identification and quantification of the monosaccharides of polysaccharide was achieved by gas chromatography (GC) analysis (Liu et al., 2011). The polysaccharides (10 mg) were hydrolyzed with 2 M TFA at 100 °C for 2 h, followed by evaporation to dryness and successive reduction with $NaBH_4$ and acetylation with Ac_2O–NaOAc at 120 °C for 1 h. The Ac_2O was destroyed with ice-water, and the resulting alditol acetates extracted with $CHCl_3$ and analyzed by GC. The following neutral monosaccharides were used as references: rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose. GC was performed on a Varian model 3300 instrument equipped with a DB-225 capillary column (30 m × 0.25 mm i.d.) and detected with a flame ionization detector (260 °C), the column temperature was increased from 150 to 200 °C in a rate of 4 °C/min then hold on 5 min. Quantification was carried out from the peaks area, using response factors from standard monosaccharide (Table 1).

2.4. UV analysis

Spectrophotometer (Shimadzu MPS-200) was used to detect UV–vis absorption spectra between 190 and 290 nm.

2.5. In vitro antitumor activity against S-180 tumor cell

The tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan product (Mosmann, 1983). Sarcoma 180 (S-180) cells (donated by Pharmacology Experimental Center of Jilin University) from peritoneal cavity of tumor inoculated mice were washed twice with PBS and resuspended in PBS. The S-180 tumor cells were inoculated on a 96-well cultivation plate (100 μl/well) at a density of 1×10^4 cells/ml and after 24 h incubation at 37 °C they were exposed to various concentrations (25, 50 and 100 μg/ml) of the tested compounds for 24 h, 48 and 72 h. MTT dissolved in PBS was added to the cultures at a final concentration of 0.5 mg/ml. The supernatant was removed by centrifuging, and then 100 μl DMSO was added to dissolve formazan crystals. The absorbance at 490 nm was measured on a micro-plate Reader (Bio-rad, USA) with DMSO as a blank control. Compared with the control groups, the antitumor activity of the tested samples was expressed as an inhibition ratio (percent) calculated as: [1 − (A_{490} of treated samples/A_{490} of untreated samples) × 100%]. All tests were run in triplicate.

2.6. In vivo antitumor activity against S-180 tumor

Kunming mice between 6 and 8 weeks old (weight: 20.0 ± 2.0 g) were purchased from the Experimental Animal Center of Jilin University (Changchun, China). The mice were housed under normal laboratory conditions, namely 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.

Fifty mice were randomly divided into five groups of 10 each. Under the sterile condition, 0.2 ml of S-180 cells (1×10^7 cells/ml) were subcutaneously inoculated into Kunming mice (Day 0). After 24 h of tumor inoculation, the RRP-ws, dissolved in phosphate suffer saline (PBS), was given (25, 50 and 100 mg/kg) once daily for 10 days. The group received the same volume of PBS serve as the control group and the group treated with CTX (25 mg/kg) was considered as the standard reference drug. All groups were continuously treated for 10 days and were administered daily by intraperitoneal (i.p.) injection (0.2 ml). By the end of the experiment on day 10, the tumor mass, thymus, spleen and peripheral blood of all groups were collected for examination immediately after the mice were weighted and killed. Spleen index were expressed as the weigh of spleen (mg)/body weight (g). The thymus index was measured by using the same method. The body weight ratio was calculated as the average percentage of the final body weight to initial body weight. The antitumor activity of the tested samples was expressed as an inhibition ratio (percent) calculated as $[(A - B)/A] \times 100\%$; where A and B are the average tumor weights of the control and treatment groups, respectively.

2.7. Flow cytometric assays for peripheral blood T-lymphocyte subpopulations of tumor bearing mice

The collected anticoagulated blood was diluted to 1×10^6 cell/ml, labeled with anti-mouse monoclonal antibodies for 30 min, washed with 10 ml PBS and then incubated in the dark for 30 min at 4 °C with FITC-conjugated rabbit anti-mouse CD4+ and CD8+ monoclonal antibodies (mAbs). Stained cells were

Table 1
Components of monosaccharides and properties of the water-soluble polysaccharide from *R. rosea*.

Sample	Carbohydrate (wt%)	Protein (wt%)	Sulfate group	Monosaccharide composition (mol%)			
				Glc	Gal	Man	Rha
RRP-ws	95.14	2.08	–	4.2	2.4	1.6	1.0

examined with a FACScan cytofluorimeter (EPICS XL, Beckman Coulter, USA).

2.8. IL-2, TNF-α, and IFN-γ determination by the ELISA method

The interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) concentration were measured with an enzyme-linked immunosorbent (ELISA) kits (Shanghai Qianchen Biotech Co., China) according to the indication of the manufacturer.

2.9. Histopathological examination of liver and kidney tissue of S-180 tumor-bearing mice

The liver and kidney of treated and control mice were excised and fixed in 4% formalin, embedded in paraffin, cut in 4 μm sections and stained with hematoxylin and eosin for histology study.

2.10. Statistical analysis

All results were expressed as mean ± SD. Data were analyzed by standard *t*-test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Isolation of a water-soluble polysaccharide from R. rosea and its composition

RRP-ws was obtained from *R. rosea* through a series of DEAE anion exchange and gel-permeation chromatography. This polysaccharide showed a single and symmetrical peak on Sephacryl S-200 HR column, indicating its homogeneity. The result of ultraviolet scanning showed that the maximal absorbance of RRP-ws was around 210 nm, but the absorbance at 280 nm for RRP-ws was very limited, which was well in agreement with the fact that RRP-ws contained 95.14% of total carbohydrate and only 2.08% of protein. Sulfate content determination indicated that absence of sulfate group in RRP-ws. Analysis by GC indicated that RRP-ws was composed of Glc, Gal, Man and Rha with a relative molar ratio of 4.2:2.4:1.6:1.0.

3.2. In vitro tumor inhibition effect of the polysaccharide

The cytotoxicity of RRP-ws was determined in S-180 cells. After the treatment of the cells with increasing concentrations of RRP-ws for 24, 48 and 72 h, cell proliferation was evaluated using MTT assay. As seen in Fig. 1, all RRP-ws demonstrated a dose-dependent inhibition on the growth of S-180, especially 48 h later. Even at a low concentration of 25 μg/ml, RRP-ws still had a growth inhibition percentage above 35.4% on S-180 cells at 48 h. These results suggested RRP-ws had an acute cytotoxic effect on the S-180 cells.

3.3. In vivo tumor inhibition effect of the polysaccharide

Antitumor activities of RRP-ws were examined in a system using S-180 solid tumors implanted in mice by i.p. injection at designed concentrations once daily for 10 days. After the tumor was excised from the mice and weighted, the tumor growth inhibitory rate was calculated as the average tumor weight of RRP-ws treated group compared with that of the control group. As shown in Table 2, after daily i.p. administration of CTX (25 mg/kg) and of RRP-ws (25, 50 or 100 mg/kg) for 10 days, we found that RRP-ws significantly inhibited tumor growth in a dose-dependent manner. The tumor weight of control group was 2.18 ± 0.34 g, while the tumor weight of low, medium and high dose of RRP-ws treated mice was reduced to 1.36 ± 0.23 g, 1.12 ± 0.22 g and 0.85 ± 0.13 g, respectively and tumor weight treated by CTX was 0.65 ± 0.09 g. The tumor inhibition rate was 37.61 (25 mg/kg), 48.62 (50 mg/kg), 61.01 (100 mg/kg) and 65.56% (CTX), respectively. Antitumor activities of the RRP-ws were slightly lower than that of CTX, but it was good for increasing the body weight than that of CTX. It was suggested that the polysaccharide sample might be less toxic than CTX, which kill normal cells as well as cancer cells. In addition, the relative spleen and thymus indexed were increased obviously by RRP-ws administration compared with that of control group, with a maximum value of 8.44 at the dose of 100 mg/kg for spleen index and 2.67 at the dose of 50 mg/kg for thymus index. On the contrary, it was notable that the spleen index and thymus index in CTX treated group are lower than those of the untreated mice, which implied that CTX's damage to the immunological function. Taken together, these data indicated that RRP-ws may have a direct and indirect antitumor activity on S-180 solid tumors

3.4. Effect of the polysaccharide on CD4+ and CD8+ peripheral blood T-lymphocyte subpopulations of tumor bearing mice

As we know, CD4+ and CD8+ T-lymphocyte are of extreme importance to elicit an immune response (Shiku, 2003). With the purpose of finding out the mechanism by which RRP-ws could ameliorate the immune function, these two subpopulations expressions on peripheral blood T-lymphocyte were tested. Table 3 showed the flow cytometric quantity analytic results for CD4+ and CD8+ on T-lymphocyte of peripheral blood in tumor bearing mice. The number of CD4+ T-lymphocyte in the RRP-ws-treated group was significantly increased to the value of 23.95×10^6, 27.63×10^6 and 29.65×10^6 at the dose of 25, 50 and 100 mg/kg, respectively, while the corresponding number of CD8+ T-lymphocyte was reduced remarkably to 10.36×10^6, 9.54×10^6 and 7.43×10^6, thus leading to a high ratio of CD4+/CD8+ T lymphocyte. Furthermore, the number of CD4+, CD8+ T lymphocyte subpopulation of peripheral blood was significantly decreased by CTX treatment, accompanied by a relative increased CD4+/CD8+ ratio. In addition, the ratio of

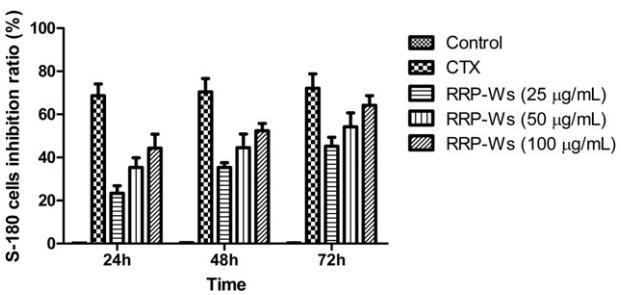

Fig. 1. In vitro antitumor effects of the water-soluble polysaccharide from *R. rosea* against Sarcoma-180 cells at different time and concentrations.

Table 2
In vivo antitumor effects of the water-soluble polysaccharide from *R. rosea* in S180 tumor-bearing mice.

Groups	Dose (mg/kg)	Body weight ratio (%)	Tumor weight (g)	Inhibition rate (%)	Spleen index (mg/g)	Thymus index (mg/g)
Control (PBS)	—	124.3	2.18 ± 0.34	—	6.34 ± 0.84	1.78 ± 0.38
CTX	25	82.1	0.65 ± 0.09	65.56	4.32 ± 0.43*	1.53 ± 0.24
RRP-ws	25	110.7	1.36 ± 0.23	37.61	7.11 ± 0.55*	2.45 ± 0.27**
	50	116.2	1.12 ± 0.22	48.62	8.23 ± 0.77**	2.67 ± 0.31**
	100	128.8	0.85 ± 0.13	61.01	8.44 ± 0.80**	2.43 ± 0.43**

Each value is presented as mean ± SD ($n = 10$).
* $P < 0.05$ significantly different from the negative control.
** $P < 0.01$ significantly different from the negative control.

Table 3
Effects of the water-soluble polysaccharide from *R. rosea* on the number of peripheral blood T-lymphocyte subpopulations in S180 tumor-bearing mice.

Groups	Dose (mg/kg)	CD4+ ($\times 10^6$)	CD8+ ($\times 10^6$)	CD4+/CD8+
Control (PBS)	—	21.45 ± 3.54	26.32 ± 4.54	0.81
CTX	25	16.42 ± 0.87	6.85 ± 0.48**	2.40
RRP-ws	25	23.95 ± 2.34	10.36 ± 0.77**	2.31
	50	27.63 ± 3.11*	9.54 ± 0.81**	2.90
	100	29.65 ± 2.67**	7.43 ± 0.79**	3.99

Each value is presented as mean ± SD ($n = 10$).
* $P < 0.05$ significantly different from the negative control.
** $P < 0.01$ significantly different from the negative control.

Table 4
Effects of the water-soluble polysaccharide from *R. rosea* on the secretion of IL-2, TNF-α, and IFN-γ in S180 tumor-bearing mice.

Groups	Dose (mg/kg)	Concentration (pg/ml)		
		IL-2	TNF-α	IFN-γ
Control (PBS)	—	15.43 ± 1.43	75.34 ± 6.11	11.44 ± 1.77
CTX	25	7.24 ± 1.02**	34.55 ± 4.02**	5.21 ± 0.57**
RRP-ws	25	25.63 ± 2.23**	145.22 ± 12.67**	16.53 ± 1.20*
	50	43.24 ± 3.49**	220.46 ± 19.33**	20.14 ± 2.14**
	100	58.51 ± 3.88**	345.35 ± 32.70**	23.42 ± 2.79**

Each value is presented as mean ± SD ($n = 10$).
* $P < 0.05$ significantly different from the negative control.
** $P < 0.01$ significantly different from the negative control.

CD4+/CD8+ T lymphocyte in the control group was lower than that of RRP-ws or CTX-treated group. All the data suggested that RRP-ws might inhibit tumor-mediated immunological aberration and regulate immune response.

3.5. Effect of the polysaccharide on IL-2, TNF-α, and IFN-γ secretion from serum in mice

Since cytokines play a prominent role in the development of immune response (Fan & Luo, 2011), we investigated the effect of RRP-ws on production of IL-2, TNF-α, and IFN-γ in RRP-ws or CTX-treated S-180-bearing mice by an enzyme-linked immunosorbent assay according to the indication of the manufacturer. As presented in Table 4, the production of IL-2, TNF-α, and IFN-γ in serum from S-180-bearing mice was significantly decreased by CTX ($P < 0.01$), compared with that of model control group, which indicated CTX could suppress the secretion of the three cytokines. However, the concentrations of IL-2, TNF-α, and IFN-γ with different doses polysaccharide-treated had been markedly elevated in different degrees. Therefore, the results indicated that the levels of three cytokines were significantly increased compared with the control.

3.6. Effect of the polysaccharide on liver and kidney

In order to determine if RRP-ws has any side effect on liver and kidney, we conducted pathological examination of liver and kidney tissues. From the metallographs, organ tissues of both CTX and RRP-ws treatment groups did not appear obviously pathological change, while those from model control groups showed indications of inflammation and fibrosis, especially in the liver tissues where black spots and loose hepatocytes could be found (data not shown). These observations further indicated that RRP-ws could protect the vital internal organs of the animals and minimize the harmful side effects in mouse implanted with S-180 tumor.

4. Conclusion

In the present study, we isolated and characterized a polysaccharide fraction with antitumor effect from the rhizome and roots of *R. rosea*. This polysaccharide was a homogeneous heteroglycan, consisting of Glc, Gal, Man and Rha with a relative molar ratio of 4.2:2.4:1.6:1.0. RRP-ws contained 95.14% of total carbohydrate and 2.08% of protein. This fraction can not only directly kill tumor cells in vitro, but also can dramatically enhance the immune responses and protect the vital internal organs of S-180-bearing mice, such as heightening the spleen and thymus indexes, promoting secretion of IL-2, TNF-α, and IFN-γ, increasing the ratio of CD4+/CD8+ T lymphocyte in several stages. Based on our results we could suggest that the RRP-ws is a promising candidate as an immune modifier, useful for the treatment of immunosuppression in experimental tumors. However, further investigations are required to elicit the responsible mechanism by which RRP-ws take effect.

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