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Purification of a polysaccharide from *Boschniakia rossica* and its synergistic antitumor effect combined with 5-Fluorouracil

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ABSTRACT

Current study we purified a polysaccharide (BRP) from *Boschniakia rossica* and the antitumor effects of BRP alone or combined with 5-Fluorouracil (5-FU) was examined in S180 tumor bearing mice by intragastric administration. The high performance size-exclusion chromatography (HPGEC) analysis showed that BRP was a homogeneous polysaccharide and had a molecular weight of 2.2×10^4 Da. Total carbohydrate content in BRP was determined to be 96.9%, without the presence of protein and nucleic acid. BRP alone or combined with 5-FU could significantly inhibit Sarcoma-180 (S180) tumor growth and increase the spleen index in a dose-dependent manner. Meanwhile a synergistic effect was observed in boosting various immunity functions when the tumor bearing mice receiving BRP plus 5-FU administration, such as stimulating lymphocytes proliferation, increasing NK cell cytotoxicity, enhancing serum interleukin-2 (IL-2) and interferon-gamma (TNF-γ) secretion, as well as augmenting CD4+ and CD8+ spleen T lymphocytes subsets. The results showed that BRP combined with 5-FU presented synergistic effects on antitumor activity in tumor bearing mice. In conclusion, the combination of BRP may boost the suppressed immunity in tumor bearing mice subject to 5-FU chemotherapy, and could serve as a new, promising approach for cancer treatment.

1. Introduction

Chemotherapy is often the first choice and the main tool for treatment of many cancers. In particular, the chemotherapy agent5-FU, which has been used against cancer for about 40 years, is a suicide inhibitor and principally works through irreversible inhibition of thymidylate synthaseacts, resulting in disrupting the growth of cancer cells. It is typically administered with leucovorin (Conklin, 2000; Longley, Harkin, & Johnston, 2003). Although 5-FU is one of the most effective chemotherapeutic agents available for the treatment of cancer, continuous use of 5-FU is not always feasible because of cumulative toxicity, which particularly lead to myelosuppression, mucositis, dermatitis and diarrhea (Jung et al., 2002). Therefore, it is essential to develop new adjuvants to actually prevent the cancer patients from unwanted side-effects produced by chemotherapy without decreasing the therapy's efficacy against tumors (Ramakrishnan, Antonia, & Gabrilovich, 2008).

In recent decades, polysaccharides isolated from botanical sources (mushrooms, algae, lichens and higher plants) have also attracted a great deal of attention in the biomedical arena because of their relatively low toxicity and antitumor activities primarily through macrophage stimulation and modulation of the complement system (Chihara, 1992; Wang et al., 1997; Wasser, 2003). Consequently, modulation of innate immunity has a significant impact on the host's ability to respond rapidly and potently to a diverse array of pathogens (Beutler, 2004). Some polysaccharides have been used as immunodulatory agents, in combination with chemotherapeutic drugs, for cancer treatment (Miyazaki et al., 1994; Shi, Nie, Chen, Liu, & Tao, 2007; Zhou, Sheng, Yao, & Wang, 2005; Zhou, Sheng, Yao, & Wang, 2006).

Boschniakia rossica Fedtsch. et Flerov (Orobanchaceae), a parasitic plant often parasitizing on the root of plants of the genus Alnus species (Betulaceae), distributes widely in the Oriental or western North America. It is widely used in Chinese traditional medicine as a substitute for *Cistanchis Herba*, a famous staminal tonic agent. It is called an "anti senility herb" in China and is valued for its ability to tonify kidney and strengthen Yang. The pharmacognostical study had identified the existance of two major groups of compounds in *B. rossica*, namely phenylpropanoid glycoside and iridoid glucoside substances, displaying anti-inflammatory (Diaz et al., 2004), hepatoprotective (Quan, Piao, Xu, Li, & Yin, 2009; Quan, Yin, & Xu, 2011) and free-radical scavenging activities (Tsuda et al., 1994). So

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far, however, there has been few research reported on the isolation and biological functions of polysaccharide from this plant. Zhang, Li, Li, Wei, and Zheng (1999) reported that the crude polysaccharides from *B. rossica* could stimulate the lymphocyte proliferation and IL-2 secretion in rat and the effect of different polysaccharide from *B. rossica* on macrophages activation was also evaluated by Liu et al. (2011). Furthermore, a homogenous polysaccharide was purified and identified from *B. rossica* (Wei, Zhang, Liang, & Tian, 2003). Subsequently Fei et al. (2008) testified *B. rossica* polysaccharide to be nontoxic and without mutagenic action by the test of acute toxicity, maximum tolerated dose, micronucleus of bone marrow in mice and sperm abnormality in mice, with the maximum tolerated dose was 30 g/kg. Based on the above preliminary investigation on the polysaccharide purification and their improving the immune function, as well as the safety for humans in clinic, the aim of this study was to investigate the synergistic antitumor effects of *B. rossica* polysaccharides combined with 5-FU in S180 tumor-bearing mice.

2. Materials and methods

2.1. Materials

B. rossica was purchased from a local drugstore of Xi'an city in China. Ovalbumin (OVA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ConcanavalinA (ConA) and Lipopolysaccharide (LPS), standard sugars, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5-Fluorouracil (5-FU) was purchased from Jiangsu Hengrui Co. (Lianyungang, China). RPMI 1640 medium was purchased from Gibco Invitrogen Co. (USA). Fetal calf serum (FCS) was provided by Hangzhou Sijiqing Corp. (Hangzhou China). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (L3T4, cloneH129.19) and phycoerythrin (PE)-conjugated rat antimouse CD8 (LY-2, clone 53-6.7) monoclonal antibody were from BD Biosciences Pharmingen (San Diego, CA, USA). All other chemicals were of grade AR.

2.2. Polysaccharide preparation and preliminary chemical analysis

After the extraction of fat, protein and low molecular weight carbohydrates, the residue of *B. rossica* was extracted with 1000 mL water for three times at 70–80 °C, and each time 3 h. The extraction was combined by centrifugation and concentrated till 200 mL under reduced pressure, then protein was removed with Sevag method (Staub, 1965), followed by dialyzing against distilled water. The concentrated dialysate was precipitated with three times of volume with 95% EtOH and kept at 4 °C for 4 h. After centrifugation, the resulting precipitate was washed with ethanol, acetone and ether in turn to yield crude polysaccharides.

The crude polysaccharide was applied to a DEAE-cellulose column (2.6 cm × 40 cm), eluted with water and two step gradient of sodium chloride (0.2 and 0.4 mol/L). Fractions (8 mL/each tube) were collected at a flow rate of 4 mL/min and monitored using the phenol–sulfuric acid method at 490 nm. Two fractions were obtained, with water-eluted fraction constituting 70% of the products. Further water-eluted fraction was purified on a Sephacryl S-200 HR column (1.6 cm × 100 cm), eluted with 0.15 mol/L NaCl at a flow rate of 2 mL/min. One major polysaccharide fraction named as BRP were separated.

Total carbohydrate content was determined by the anthrone–sulphuric acid method as D-glucose equivalents (Scott & Melvin, 1953). Protein was analyzed by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

2.3. Experimental animal and experimental design

Mouse sarcoma S180 cell lines was provided by Fourth Military Medical University and maintained by routine intraperitoneal passage. S180 cell lines, collected from ascites of the tumor-bearing mice, was harvested and washed three times with aseptic phosphate-buffered saline (PBS). The cells were pelleted by brief centrifugation at 3000 × *g*. The supernatant was aspirated, and the cells were resuspended in PBS at 1×10^7 cells/mL. Male ICR mice (6–8 weeks old, weighing 18–22 g) were used and inoculated subcutaneously (s.c.) into the armpit for 0.2 mL per mouse. Twenty-four hours after inoculation, the mice were randomly divided into 8 groups (10 mice each group), respectively. 25 h later, the mice inoculated with S180 cells were administered intragastrically with BRP in PBS at the doses of 50, 100 and 200 mg/kg once daily for 10 days or combined with 5-FU at a dose of 25 mg/kg. The positive control was given 5-FU. The same volume of PBS (pH 7.0) was oral administrated into the tumor-bearing mice as negative control. The tumor was allowed to grow on the mice for 10 days before it was removed from the mice and weighed. On day 11, the mice were sacrificed. Tumors and spleens were removed and weighed. The trunk blood collected and serum was separated by centrifugation at 720 g for 5 min and was kept at −20 °C for the estimation of cytokines levels later. The in vivo antitumor activity of polysaccharides was expressed as an inhibition ratio (*A*) calculated as: $A = [1 - Wt/Wc] \times 100\%$, where Wc is the average tumor weight of the control group, and Wt is the average tumor weight of the test group.

2.4. Splenocyte proliferation assay

The assay of splenic lymphocyte proliferation was implemented according to the method described by Shuai et al. (2010), with some modifications. In brief, spleens were aseptically collected from male ICR mice and miced in Hank's balanced salt solution by mincing with a pair of scissors and filtering through fine steel meshes to obtain homogeneous cell suspensions, followed by removing the erythrocytes with red blood cytolysate (Tris–NH_4Cl). After the splenocytes were collected by centrifugation (380 × *g* at 4 °C for 5 min), the pelleted cells were washed three times in PBS and resuspended in RPMI 1640 complete medium, which was supplemented with 100 IU/mL penicillin, 100 mg/L streptomycin and 10% FCS. The purity and viability of splenocytes as assessed by Trypan blue dye exclusion always exceeded 90%. One-tenth of milliliter aliquots of the cell suspension (2×10^6 cells/mL) were seeded into the wells of 96-well plates and incubated with RPMI1640 medium, concanavalin A (ConA; 5 μg/mL) or LPS (10 μg/mL) in a final volume of 200 μl at 37 °C in a humid atmosphere with 5% CO_2. After 44 hours' incubation, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for an additional 4 h. The plates were centrifuged (1400 × g, 5 min) and the untransformed MTT was carefully removed by pipetting. 200 μl of a DMSO working solution (182 μl with 8 μl 1 N HCl) was added to each well to dissolve the formazan crystals, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference after 15 min. All determinations were conducted in triplicate. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

2.5. Natural killer (NK) cell activity assays

The splenocytes prepared as described above was used as the effector cell. K562 cell line was used as target cells to measure NK cell activity. Briefly, splenocytes (1×10^6 cells/well) and K562 cells (2×10^4 cells/well) were seeded in 96-well U-bottom microtiter plate and co-cultured at 37 °C for 12 h in a CO_2 incubator. Finally 50 μl of MTT solution (2 mg/mL) was added to each well and the plate was incubated for another 4 h and subjected to MTT assay (Tu, Sun, & Ye, 2008). NK cell activity was calculated from the following formula: NK activity (%) = [ODT − (ODS − ODE)]/ODT × 100%, where ODT is optical density value of target cells control; ODS is optical density value of test samples; ODE is optical density value of effector cells control.

2.6. Determination of IL-2 and TNF-γ levels by the ELISA assay

The blood samples obtained from trunk of the S180- bearing mice or normal mice were prepared as previously described. After centrifugation at 720 g for 5 min, the serum samples were collected. The IL-2 and TNF-γ concentration in serum were measured using a murine enzyme-linked immunosorbent assay kit according to the manufacturer's instructions.

2.7. Flow cytometric assays

The method for flow cytomeric assay was conducted following procedures by Zaharoff, Rogers, Hance, Schlom, & Greiner (2007). Briefly, lymphoid cells (1×10^6) prepared from the spleen of male ICR mice were incubated with either FITC-conjugated rat anti-mouse CD4 mAbs or PE-conjugated rat anti-mouse CD8 mAbs for 1 h at 4 °C, and collected by centrifugation at $380 \times g$ for 15 min. Lymphoid cells were washed with PBS and centrifuged at $380 \times g$ for 5 min, and resuspended with 200 μl PBS for immediate flow cytometric analysis. The percentage of positively stained cells, determined over 10,000 events, was analyzed by flow cytometry (FACScan, Becton Dickinson, USA).

2.8. 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 and discussion

3.1. Preparation of BRP and its chemical properties

The water-soluble crude polysaccharides were obtained from *B. rossica* by defatting with ethanol, hot water extraction, ethanol precipitation, deprotein by Sevag method, dialysis against water and drying in vacuum. The yield of crude polysaccharide accounted for 9.3% of the dried material. The crude polysaccharides were fractionated by DEAE-cellulose anion-exchange chromatography to obtain two main fractions, which were selected based on total carbohydrate elution profile. Furthermore the water-eluted fraction was purified by Sephacryl S-200 HR gel-permeation chromatography into one major fraction, name as BRP, according to their molecular size. The HPGEC profile of BRP showed as a single and symmetrically sharp peak, indicating that its homogeneity. According the retention time and the molecular weight distribution curve of standard T-series dextrans, its molecular weight was estimated to be 2.2×10^4 Da (data not shown). Total carbohydrate content was determined to be 96.9% by the anthrone–sulphuric acid method. It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid.

3.2. Effect of BRP and/or 5-FU on tumor growth and spleen index in S180 tumor-bearing mice

The anti-tumor effect of BRP was tested on S180-bearing mouse model at the dose of 50, 100 and 200 mg/kg in vivo, combined with or without 5-FU. As shown in Table 1, 5-FU administration to S180-tumer bearing mice could effectively inhibit the growth of tumor, whereas spleen index was much lower than that of control group. Moreover single RBR oral administration could also decrease the tumor weight, especially at the dose of 200 mg/kg, which was comparable to 5-FU treatment. Simultaneously the relative spleen weights of the mice were obviously enhanced by BRP treatment than those of the control group in a dose-dependent manner, which was well in agreement with the antitumor activity. Interestingly, a synergistic antitumor effect of BRP was observed when it was combined with 5-FU to treat tumor bearing mice. On the one hand, the decreased spleen index induced by 5-FU was rivaled by BRP to a high level. On the other hand, the inhibition rates of the mixtures were higher than those of BRP or 5-FU treatment alone. Taken together with these data, the results implied that 5-FU's damage to the immunological function in mice was overcome by the combined usage with BRP.

3.3. Effect of BRP and/or 5-FU on splenocyte proliferation in S180 tumor-bearing mice

To evaluate the in vivo immunostimulation effect of BRP and/or 5-FU, the proliferation of splenocytes from treated mice or control were compared. As data showed in Fig. 1, Con A- and LPS-induced splenocyte proliferation in the S180-bearing mice was significantly enhanced by BRP at the doses of 50, 100 and 200 mg/kg ($^{**}P < .01$) as compared to those of control group. In contrary, Con A- and LPS-stimulated splenocyte proliferations in the 5-FU-treated group were significantly lower than those of the control control ($^{*}P < .05$).

Table 1
Inhibiting effects of BRP and/or 5-FU on tumor growth and spleen index in S180 tumor-bearing mice. The values are presented as means ± S.D. based on 10 mice in each group.

Group	Dose (mg/kg/day)	Spleen index (mg/g)	Tumor weight (g)	Tumor inhibitory rate (%)
PBS	0.2 mL	7.45 ± 0.45	1.81 ± 0.11	
5-FU	25	5.03 ± 0.36[a]	1.03 ± 0.10[a]	43.1
BRP	50	9.05 ± 0.64[a]	1.32 ± 0.12	27.1
	100	12.84 ± 0.74[b]	1.28 ± 0.11	29.3
	200	14.39 ± 0.93[b]	1.07 ± 0.95[a]	40.9
BRP + 5-FU	50 + 25	8.38 ± 0.58[a]	0.97 ± 0.74[a]	46.7
	100 + 25	10.34 ± 0.82[a]	0.82 ± 0.83[b]	55.0
	200 + 25	11.05 ± 0.77[b]	0.65 ± 0.52[b]	64.1

[a] Significant differences from the negative control were designated as $P < .05$.
[b] Significant differences from the negative control were designated as $P < .01$.

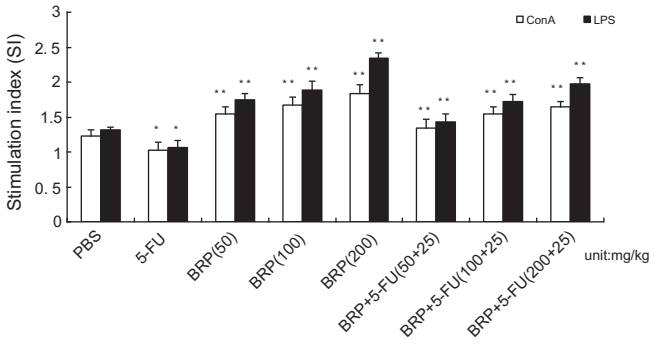

Fig. 1. Effect of BRP and/or 5-FU on splenocyte proliferation in S180 tumor-bearing mice. Significant differences from the negative control were designated as $^{*}P < .05$, $^{**}P < .01$.

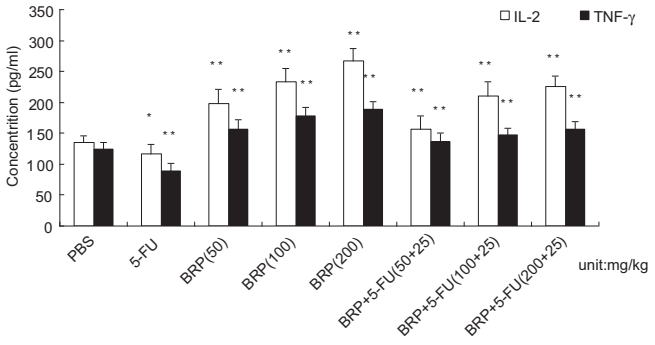

Fig. 3. Effect of BRP and/or 5-FU on IL-2 and TNF-γ levels in S180 tumor-bearing mice. Significant differences from the negative control were designated as $^{*}P < .05$, $^{**}P < .01$.

When 5-FU was mixed with BRP, the inhibition effect of 5-FU alone on splenocyte proliferation was reversed by BRP due to attenuating damage of 5-FU to lymphocytes.

3.4. Effect of BRP and/or 5-FU on NK cell activity in S180 tumor-bearing mice

As we know, NK cells in part play central role in the defense against tumors and viruses (Boon, Cerottini, Van den Eynde, van der Bruggen, & Van Pel, 1994; Moretta, Bottino, Cantoni, Mingari, & Moretta, 2001). Therefore the effect of BRP and/or 5-FU on NK cell activity was evaluated in tumor-bearing mice and the results was show in Fig. 2. BRP alone or combined with 5-FU administration could significantly increase the activity of NK cells in the S180-bearing mice at three doses ($^{**}P < .01$), especially at the dose of 200 mg/kg. However the killing activity to K 562 cell mediated by NK cells was prominently depressed compared to that of control group ($^{**}P < .01$), when only 5-FU was given to tumor bearing mice.

3.5. Effect of BRP and/or 5-FU on IL-2 and TNF-γ levels in S180 tumor-bearing mice

IL-2 has many immunopotentiating effects and exhibit high cytotoxic activity against autologous tumour cells (Ehrke, 2003; Wang et al., 2007). IFN-γ is an important immunoregulatory molecule, which induces the generation of T cells and macrophages activation, and regulates Th1 and Th2 cells (Ding, Zhu, & Gao, 2012). Since cytokines play a prominent role in the development of immune response, thus we examined the effects of BRP and/or 5-FU administration on the cytokines level in sera of tumor bearing mice. From the results of Fig. 3, the concentrations of IL-2 and TNF-γ in the group with 5-FU-treated were significant lower than control group, which indicated 5-FU could not promote the secretion of two cytokines. However, the concentrations of IL-2 and TNF-γ in mice treated with BRP (50, 100 and 200 mg/kg) have different degrees increase, especially at the dose of 200 mg/kg, as compared to those of control group ($^{**}P < .01$). When BRP was mixed with 5-FU, the levels of IL-2 and TNF-γ in tumor bearing mice were improved greatly.

3.6. Effect of BRP and/or 5-FU on CD4+ and CD8+ spleen T lymphocytes subsets in S180 tumor-bearing mice

T cells could differentiate into two different subsets according to their specific membrane molecule, that is CD4+ and CD8+ T lymphocytes, which play different roles in immunomodulation. It is well known that Th cells and Cytotoxic T lymphocytes (CTLs) responses are closely related with the enhancement of CD4+ and CD8+ T lymphocytes, respectively (Vendrell et al., 2011). Thus the effect of BRP and/or 5-FU on CD4+ and CD8+ spleen T lymphocytes subsets was discussed here. As seen in Fig. 4, the percentage of CD4+ and CD8+ T lymphocytes in tumor bearing mice were increased remarkably by BRP ($^{**}P < .01$) as compared with control group. On the contrary, the mice treated with 5-FU administration alone displayed a much lower value of CD4+ and CD8+ T lymphocytes as compared to control group ($^{**}P < .01$). More importantly, BRP plus 5-FU administration to tumor bearing mice could significantly augment the proportion of CD4+ and CD8+ T lymphocytes in tumor bearing mice when compared to control group ($^{**}P < .01$). These results suggested that BRP could serve as an ideal adjuvant to reverse the decreased proportions of both CD4+ and CD8+ T lymphocytes induced by 5-FU, resulting in secreting cytokines to mediate immunologic enhancement.

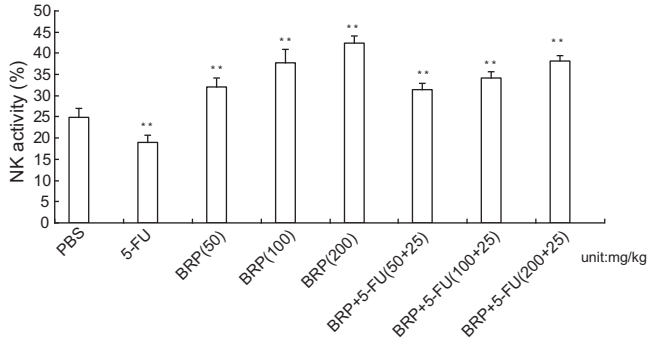

Fig. 2. Effect of BRP and/or 5-FU on NK cell activity in S180 tumor-bearing mice. Significant differences from the negative control were designated as $^{**}P < .01$.

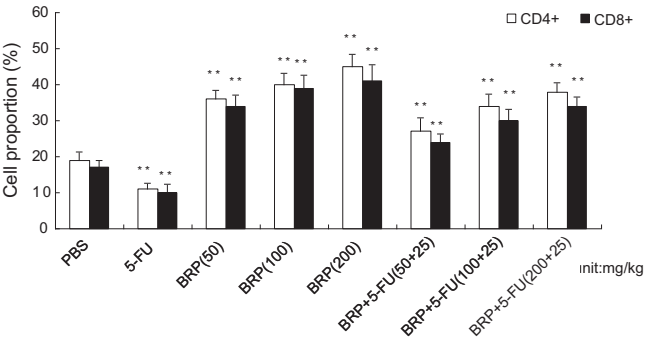

Fig. 4. Effect of BRP and/or 5-FU on CD4+ and CD8+ spleen T lymphocytes subsets in S180 tumor-bearing mice. Significant differences from the negative control were designated as $^{**}P < .01$.

4. Conclusion

There is great hope offered by new therapies targeted to biological abnormalities in cancer. Most chemotherapeutic drugs are immunosuppressive because they kill many immune cells in addition to tumor cells. In fact, the majority of the curable cancers today require combination chemotherapy (Goldspiel, 1997). Also, a recently established principle of chemotherapy for cancer is that drugs used in combination are more effective than drugs used individually. An alternative rationale for using combinations of anticancer agents is the potential additive or synergistic activities they could engender.

Although *B. rossica* is a popular folk medicine and has attracted great attention due to its role as a famous staminal tonic agent, there is little information available about its polysaccharide component, let alone its combined therapeutical antitumor potential with 5-FU. This is the first report providing in vivo evidences of the adjuvant antitumor potential of the bioactive polysaccharide from *B. rossica* combined with 5-FU. In the present study, we purified a homogenous polysaccharide with a molecular weight of 2.2×10^4 Da from *B. rossica* and tested the adjuvant antitumor activities of BRP in combination with 5-FU in S180 tumor bearing mice. The results demonstrated that BRP combination with 5-FU could increase the tumor inhibitory rate and spleen index, and improve the splenocyte proliferation, NK cell activity, IL-2 and TNF-γ secretion, as well as CD4+ and CD8+ spleen T lymphocytes subsets in S180 tumor bearing mice. The mixture of BRP and 5-FU produced a synergistic effect on antitumor activity and the mechanism whereby BRP take effect would be as a result of its counteracting the side effect of the synthetic anticancer drug, as well as being achieved by enhancing the immune system functions in tumor bearing mice. Thus, observed in vivo findings have revealed an excellent therapeutic prospective for BRP as a novel adjuvant in the treatment of cancer with a chemotherapeutic agent 5-FU. In addition, the antitumor potency might be caused and influenced by some fine structures, such as glycosidic linkages, the length of the backbones and hair region, the number of branched points, the molecular size and steric configuration. Therefore an in-depth research on the fine structure of BPR should be needed in the future.

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