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Studies on immunoregulatory and anti-tumor activities of a polysaccharide from *Salvia miltiorrhiza* Bunge

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ABSTRACT

In this study, we purified and characterized a polysaccharide (SMP-W1) from *Salvia miltiorrhiza* and investigated its anticancer and immunoregulatory potential in vitro and in vivo. The monosaccharide composition, protein content, uronic acid content, total carbohydrate content, viscosity and molecular weight of SMP-W1 were analyzed. In vitro, SMP-W1 had an antiproliferative effect on hepatocellular carcinoma H22 cells, especially at the high concentration of 400 μg/ml. Simultaneously the polysaccharide SMP-W1 significantly inhibited tumor growth and increased serum superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities in rats, as well as the secretion of TNF-α. In addition, the body weight, spleen index and thymus index in tumor-bearing mice were significantly improved by SMP-W1 treatment. Taken together, these results indicated that SMP-W1 possessed strong in vivo and in vitro anti-tumor activity and improves the immune response in tumor-bearing mice. Therefore, it could be developed as an anti-tumor agent with immunomodulatory activity.

1. Introduction

Human hepatoma is one of the most frequent cancers in the world and is known to be a type of tumor highly resistant to available chemotherapeutic agents (Llovet et al., 1999). Multidisciplinary scientific investigations are making the best efforts to combat this disease, but the sure-shot, perfect cure is yet to be brought into world medicine. The great majority of chemical compounds, which have been identified as cytotoxic to cancer cells, are also toxic to normal cells (Borchers, Stern, Hackman, Keen, & Gershwin, 1999). In addition, treatment is difficult if the cancer has spread beyond the liver. Therefore, high prevalence and high death rate have spurred the researcher to find novel antitumor substances with little toxicity to host to treat or relieve liver cancer.

Nowadays, traditional herbal medicine and complementary and alternative medicine (CAM) are becoming increasingly popular among cancer patients in the developed countries. Herbal medicines usually contain multiple bioactive components with specific biological activities and are also used as alternative therapeutic or preventive regimens for individuals with cancer (Eisenberg et al., 1998). Natural products are excellent sources of lead compounds to develop new medicaments for the treatment of diseases. This is particularly evident in the treatment of cancers in which more than 60% of drugs are of natural origin (Newman, Cragg, & Snader, 2003). Particularly, numerous polysaccharides isolated from plants, fungi, algae and animals are relatively nontoxic and do not cause significant side effects, which are ideal candidates for oncotherapy with improving immunity potential (Schepetkin & Quinn, 2006). Hence, finding a new polysaccharide from natural source with anticancer and immunopotentiation activities would be a new tool for cancer therapy.

Salvia miltiorrhiza has been used as a traditional medicine in China for the treatment of coronary artery disease and cerebrovascular diseases with minimal side effects (Zhou, Zuo, & Chow, 2005). Investigation of this herb for other pharmacological applications, particularly its antitumor potential, began in the early 1990s (Wu, Chang, & Chen, 1991). Most recently, the extract of *S. miltiorrhiza* and its tanshinones have been recently shown to possess broad-range growth inhibitory and cytocidal activities against various cancer cell lines such as prostate, lung, breast, leukemia, glioma, stomach, nasopharynx, and liver (Gong et al., 2011; Zhang et al., 2012). However, there is no report published about the effect of

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S. miltiorrhiza polysaccharides on liver cancer. Therefore the objectives of this study are to elucidate the isolation and characterization of the biological polysaccharide from *S. miltiorrhiza* and evaluate its antitumor and immunomodulatory activities on the immune response in tumor bearing mice by using both in vitro and in vivo assays.

2. Materials and methods

2.1. Materials and chemicals

The crude polysaccharides from *S. miltiorrhiza* were purchased from Shaanxi Lixin Biotechnology Co. (China). Arabinose, fucose, galactose, glucose, mannose, rhamnose, xylose, glucuronic acid, trifluoroacetic acid (TFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DEAE-cellulose and Sephadex G-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA), fetal bovine serum (FBS) and RPMI-1640 media were purchased from Gibco/Invitrogen (Grand Island, NY, USA). All other reagents were all of analytical grade.

2.2. Extraction and purification of the polysaccharide

The crude polysaccharides solution (dissolved in distilled water) was deproteinated by the Sevage method (Staub, 1965), combined with papain. After extensively dialysis (molecular weight cut-off 3000 Da), concentration and centrifugation, the supernatant was added with 3 volumes of 95% EtOH to precipitate the crude polysaccharides (named cSMP, 25.7 g). Then cSMP (5 g) was subjected to a DEAE-cellulose column (1.6 cm × 60 cm, Cl^{-1} form) and eluted stepwise with 0, 0.3, 0.6 and 1.0 M NaCl to give four sub-fractions (cSMP-W1, cSMP-W2, cSMP-W3 and cSMP-W4). The eluate was monitored by the phenol–sulfuric acid method. The 0 M NaCl elution was concentrated, lyophilized and purified on a Sephadex G-100 column (2.6 cm × 100 cm). The resulting purified polysaccharide, named SMP-W1, was obtained by the above processes and the yield rate of SMP-W1 was 8.26% (0.413 g) of cSMP.

2.3. Analytical methods

The carbohydrate content was determined spectrophotometrically by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), using Glc as standard. Total uronic acid content was determined by photometry with m-hydroxybiphenyl at 523 nm (Blumenkrantz & Asboe-Hansen, 1973), using GalA as standard. The protein content of protein-bound polysaccharide was measured (Lowry, Rosebrough, Farr, & Randall, 1951), using BSA as standard.

2.4. Characterization of the polysaccharide

2.4.1. Molecular weight (Mw) determination of the polysaccharide

The homogeneity and molecular weight of polysaccharides were estimated by high performance gel permeation chromatography (HPGPC) method (Yamamoto, Nunome, Yamauchi, Kato, & Sone, 1995), using a HPLC (Agilent-1200) equipped with a TSK-gel G4000PWxl column (7.8 mm × 300 mm, column temperature 30 °C) and Refractive Index Detector (RID, detecting temperature 40 °C). A sample solution (20 μL) was injected and run with purified water at 0.6 ml/min as mobile phase. The standard curve was established using T-series Dextran as the standards (T-10, T-40, T-70, T-500 and T-2000) (Zhu et al., 2011).

2.4.2. Monosaccharide analysis of the polysaccharide

The polysaccharide sample (5 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA, 2 ml) and converted to their alditol acetates as previously described (Li, Xie, Su, Ye, & Jia, 2012; Sun et al., 2008). The monosaccharide standards including rhamnose, arabinose, fucose, xylose, mannose, glucose and galactose were acetylated in the same way. Finally, the acetylated samples were analyzed by gas chromatography (GC) using an Agilent 6890N instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector (FID) and a HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 μm). The temperature of the column was kept at 160 °C for 10 min and then increased to 280 °C at the rate of 5 °C/min. The rate of N_2 carrier gas was 1.2 ml/min.

2.4.3. Intrinsic viscosity of the polysaccharide

The viscosity of polysaccharides was measured in 0.2 M NaCl at 25 °C by an Ubbelohde capillary viscometer (internal diameter size 0.8 mm). The flow time of the solvent was always higher that 120 s, the kinetic energy correction was negligible. Huggins and Kraemer equations were used to estimate intrinsic viscosity (Huggins, 1942; Kraemer, 1938).

2.5. Cell lines and culture

Mouse hepatocellular carcinoma cells H22 were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). The cells were cultured in RPMI 1640 medium containing 10% FBS, 1% glutamine (200 mmo1/L), penicillin (100 IU/ml) and streptomycin (100 mg/L) in a humidified 5% CO_2 atmosphere at 37 °C before use.

2.6. Tumor growth inhibition assay

The inhibition effect of SMP-W1 on the growth of H22 cells was determined by MTT-based colorimetric method (Mosmann, 1983), which is based on the ability of mitochondrial enzyme, succinate dehydrogenase to cleave MTT to the blue compound formazan. Briefly, H22 cells suspended in RPMI-1640 medium at a density of 1×10^5 cells/ml was pipetted into a 96-well plate (50 μl/well) and inoculated at 37 °C in a humidified 5% CO_2 incubator for 24 h. Then, 50 μl of test sample with different concentrations (0, 50, 100, 200 and 400 μg/ml in fresh growth medium) was added into each well separately. After incubation for 48 h at 37 °C, the culture medium was removed and 100 μl of MTT reagent (diluted in culture medium, 0.5 mg/ml) was added to each well, and the plate was further incubated for another 4 h. Then, the supernatant was removed carefully and DMSO (100 μl) was added into each well for the dissolution of formazan crystals. Absorbance of the colored solution at 570 nm was measured on a 96-well microplate reader (Bio-Rad, Tokyo, Japan). All experiments were performed in triplicate and the inhibitory rate was calculated as follows:

$$\text{Inhibition rate } (\%) = \left(1 - \frac{A1}{A0}\right) \times 100$$

where A1 and A0 are the absorbance of test sample and control, respectively.

2.7. Animals and experimental design

Male BALB/c mice (5–7 weeks old), obtained from the Experimental Animal Center of the Fourth Military Medical University, were kept in our animal facility for at least 1 week before use. All the animals were housed with a 12/12-hour light–dark cycle at room temperature and allowed free access to standard rodent food and water during the experiments. All animal (used in this experiment) handling procedures were performed in strict compliance with the

Table 1
Chemical composition and properties of SMP-W1 from *S. miltiorrhiza*.

Sample	TA (%)	Protein (%)	UA (%)	V (cm^3/g)	Mw (Da)	Monosaccharide composition				
						Man	Rha	Ara	Glc	Gal
SMP-W1	96.9	n	0.12	34.53	6.9×10^5	2.14	2.35	1.27	0.99	1.11

n, not detected; Man, mannose; Rha, rhamnose; Ara, arabinose; Glc, glucose; Gal, galactose; UA, uronic acid; TA, total carbohydrate; *V*, viscosity; Mw, molecular weight.

PR China legislation the use and care of laboratory animals, with the guidelines established by Institute for Experimental Animals of Fourth Military Medical University, and were approved by the College Committee for Animal Experiments.

Under sterile condition, 0.2 ml of H22 cell suspension (1×10^6 cells/ml) were washed three times with sterilized PBS and subcutaneously inoculated into the right armpits of BALB/c mice on day 0. Starting 24 h after tumor inoculation, different doses of SMP-W1 (50, 100 and 200 mg/kg) were administered intragastrically each day for 10 consecutive days. 0.2 ml of physiologic saline (P.S.) and 5-FU (25 mg/kg) were the negative and positive control, which were treated by the same method as the polysaccharide sample. Another 10 mice received an equal volume of P.S. served as normal control group and was fed on standard pellet rat diet.

Twenty-four hours after last drug administration, mice were weighed and sacrificed by cervical dislocation. Tumors, spleens and thymus were excised and weighted, respectively. The tumor inhibitory rate was calculated as $((A - B)/A) \times 100\%$, where A and B were the average tumor weights of the model group and the tested group, respectively. Thymus index was expressed as the thymus weight relative to body weight. Spleen index was expressed as the spleen weight relative to body weight.

2.8. Anti-oxidant enzyme activities and tumor necrosis factor-a (TNF-α) levels in serum of mice

Serum taken from the mice were immediately separated by centrifugation at 3000 rpm at room temperature for 10 min and then stored at −70 °C until assayed for various biochemical Parameters, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and tumor necrosis factor-alpha (TNF-α). The superoxide dismutase (SOD) activity was determined using Marklund and Marklund's method (Marklund & Marklund, 1974). The catalase (CAT) activity was measured using Abei's method (Abei, 1984). The glutathione peroxidase (GSH-Px) activity was measured using Paglia and Valentine's method (Paglia & Valentine, 1967). The concentration of TNF-α was measured using an enzyme-linked immunosorbent assay according to the indication of the manufacturer. The absorbance was read at 450 nm on an automatic ELISA plate reader.

2.9. Statistical analyses

All data are expressed as mean ± S.D. Significances were analyzed by one-way analysis of variance combined with Student's *t*-test. $p < 0.05$ was considered significant and $p < 0.01$ was considered highly significant. All statistical analyses were performed by using commercially available statistical software.

3. Results and discussion

3.1. Isolation and purification of SMP-W1

The crude polysaccharide extract obtained from *S. miltiorrhiza* was separated into one neutral fraction (cSMP-W1) and three acidic fractions (cSMP-W2, cSMP-W3 and cSMP-W4) by using anion-exchange chromatography on DEAE cellulose column. Furthermore cSMP-W1 was investigated using gel filtration chromatography with a Sephadex G-100 column, resulting in one polysaccharide fraction, namely SMP-W1. The following analysis was focused on the main fraction SMP-W1.

3.2. Composition and physical property of SMP-W1

Monosaccharide composition, molecular weight, the viscosity, total sugar, protein and uronic acid were summarized in Table 1. Chemical composition analysis indicated that SMP-W1 contained 96.9% total sugar and a minor amount of uronic acid (0.12%). 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. SMP-W1 was eluted as a single and symmetrically sharp peak on HPGPC, and its apparent molecular weights was estimated to be 6.9×10^5 Da. Accordingly, SMP-W1 had a viscosity of 34.53 cm^3/g, which indicated that it had a moderate flexible chain conformation (Jin, Zhang, Zhang, Chen, & Cheung, 2003). Additionally, SMP-W1 appeared as a single band on the cellulose acetate membrane electrophoresis (data not shown). These results revealed that SMP-W1 might be homogeneous polysaccharide based on the molecular weight and charge distribution. According to retention time of the alditol acetate derivatives in GC, SMP-W1 consisted of five different monosaccharides, including mannose, rhamnose, arabinose, glucose and galactose, in the molar ratio of 2.14:2.35:1.27:0.99:1.11.

3.3. Effects of SMP-W1 on tumor cells growth in vitro

H22 hepatocellular carcinoma cells were incubated up to 48 h in the presence or absence of SMP-W1 at concentration of 50, 100, 200 and 400 μg/ml, and measured by MTT method. The results showed that the polysaccharide SMP-W1 had significantly toxicity effects on H22 hepatocellular carcinoma cell line. As presented in Fig. 1, the cell viability was markedly decreased 48 h after exposure to SMP-W1 in a dose-dependent manner. Upon a 48 h exposure to SMP-W1 (200 and 400 μg/ml), the inhibitory rate of SMP-W1 on H22 cells was above 43%. Thus, SMP-W1 seems to be capable of exerting inhibition effect on H22 cell proliferation.

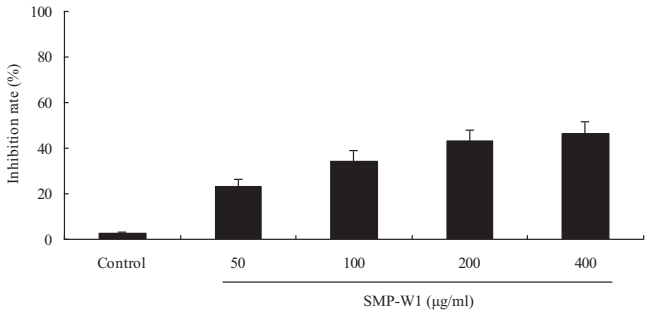

Fig. 1. Inhibition of proliferation of H22 cancer cells by different concentrations of SMP-W1 from *S. miltiorrhiza*. The values are presented as mean ± S.D. ($n = 3$).

Table 2
Effects of SMP-W1 from *S. miltiorrhiza* on body weight, tumor growth, spleen index and thymus index in mice.

Groups	Increase of body weight (g)	Spleen index (mg/g)	Thymus index (mg/g)	Weight of tumor (g)	Inhibitory rate of tumor (%)
Normal control	5.02 ± 0.64	10.23 ± 1.14	5.43 ± 0.76	–	–
Negative control	4.53 ± 0.37	9.94 ± 0.87	5.36 ± 0.84	2.56 ± 0.23	–
5-FU (25 mg/kg)	3.54 ± 0.54[a]	5.58 ± 0.71[b]	3.32 ± 0.77[a]	1.01 ± 0.12[c]	60.5
SMP-W1 (50 mg/kg)	4.97 ± 0.33	13.13 ± 1.11[b]	5.39 ± 1.12	1.34 ± 0.18[c]	47.7
SMP-W1 (100 mg/kg)	5.35 ± 0.61[a]	15.74 ± 1.44[c]	6.11 ± 1.33	1.23 ± 0.15[c]	52.0
SMP-W1 (200 mg/kg)	6.16 ± 0.75[b]	17.43 ± 1.68[c]	6.57 ± 1.53[a]	1.15 ± 0.15[c]	55.1

The values were expressed as mean ± S.D. ($n = 10$).
[a] $p < 0.05$, compared with the model control.
[b] $p < 0.01$, compared with the model control.
[c] $p < 0.001$, compared with the model control.

3.4. Effects of SMP-W1 on tumor growth, body weight, spleen and thymus indexes in vivo

To investigate the anticancer activity of SMP-W1 in vivo, murine hepatoma H22 bearing mice were fed orally with SMP-W1 for 10 days. At the end of experiment, tumors, spleens and thymus were excised and weighted from mice to calculate the tumor inhibitory rate, spleen and thymus indexes, respectively. The body weight was balanced before and after experiment. As seen in Table 2, tumor weights were effectively reduced by the administration of polysaccharides in a dose dependent manner compared to the negative control group ($p < 0.001$). Especially at the high dose of 200 mg/kg, SMP-W1 exerted a maximum antitumor effect with the tumor inhibitory rate of 55.1%, which was near to the 5-FU administration. The standard reference drug 5-FU had high antitumor effect, but considerably decreased the body weight, as well as spleen and thymus indexes in H22-bearing mice compared with negative control group ($p < 0.05$ or $p < 0.01$). Simultaneously the appetite, activity and coat luster of each polysaccharide treated groups were clearly better than the negative and positive control groups. Moreover, the SMP-W1 treatment exhibited a stronger effect on the thymus index, spleen index and mice body weight than 5-FU. These results indicated that SMP-W1 has significant inhibitory effect on H22 tumor growth in BLAB/c mice, comparable to 5-FU, and showed no side-effect on body weight and immune organ.

3.5. Effects of SMP-W1 on TNF-α production

To further elucidate immunological effect of SMP-W1, TNF-α level in the serum of mice were determined by ELISA as presented in Fig. 2. It is well known that TNF-α plays a pivotal role in host defense and can induce the expression of a number of other immunoregulatory and inflammatory mediators to eliminate tumor cell (Baugh & Bucala, 2001). In this study, SMP-W1 could significantly increase the secretion of TNF-α in H22-bearing mice at all doses ($p < 0.01$), compared with model control group. However, the TNF-α concentrations were significantly decreased in 5-FU-treated group, which was in agreement with its adverse effect on immune system. It suggested that SMP-W1 may indirectly play the role of anti-tumors activity through the releases of effective molecules TNF-α.

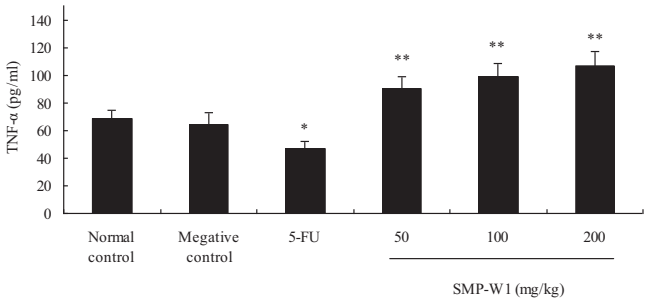

Fig. 2. Effects of SMP-W1 from *S. miltiorrhiza* on serum TNF-α concentration in mice. The values are presented as mean ± S.D. ($n = 10$). *$p < 0.05$, compared with the model control. **$p < 0.01$, compared with the model control.

Table 3
Effects of SMP-W1 from *S. miltiorrhiza* on anti-oxidant enzymes activities in mice.

Groups	SOD	CAT	GSH-Px
Normal control	204.3 ± 15.6	23.4 ± 1.32	17.76 ± 1.21
Negative control	113.8 ± 11.2[a]	11.43 ± 0.87[a]	7.93 ± 0.58[a]
5-FU (25 mg/kg)	109.5 ± 13.1[a]	12.50 ± 0.93[a]	8.95 ± 0.84[a]
SMP-W1 (50 mg/kg)	192.5 ± 13.8[b]	18.95 ± 1.31[b]	15.64 ± 1.06[b]
SMP-W1 (100 mg/kg)	197.9 ± 14.3[b]	22.30 ± 1.26[b]	17.32 ± 1.21[b]
SMP-W1 (200 mg/kg)	220.3 ± 19.3[c]	26.31 ± 1.47[b]	18.98 ± 1.19[b]

The values were expressed as mean ± S.D. ($n = 10$).
[a] $p < 0.01$, compared with normal control.
[b] $p < 0.01$, compared with negative control.
[c] $p < 0.001$, compared with negative control.

3.6. Effects of SMP-W1 on anti-oxidant enzymes activities

SOD, CAT and GSH-Px are the major antioxidant enzymes, which protect the tissue against oxidative damage in patient suffered from cancer. The enhancing effect of the polysaccharide SMP-W1 on serum antioxidant enzymes activities in rats is presented in Table 3. Serum SOD, CAT and GSH-Px activities in model control group were significantly decreased in compared with the normal control group ($p < 0.01$). The administration of SMP-W1 dose dependently increased the activities of these antioxidant enzymes in blood. Moreover there was no significant difference in serum anti-oxidant enzymes activities between 5-FU and the negative groups.

4. Conclusion

In this investigation, we have succeeded in isolating and characterizing the polysaccharide SMP-W1 from *S. miltiorrhiza*, and the in vivo and in vitro anti-tumor activity of the polysaccharide was evaluated for the first time. SMP-W1 could effectively prevent the H22 tumor growth both in vitro and in vivo. Neither inhibitory action on immune organs nor a loss of body weight was observed in the experimental animals during the treatment with SMP-W1. Meanwhile, the appetite, activity and coat luster of each animal in SMP-W1-treated groups were better than the tumor-bearing controls. Furthermore, SMP-W1 significantly increased the concentration of TNF-α in serum of H22-bearing mice. The augment of TNF-α production may contribute to part of the therapeutic effects of SMP-W1 on tumor. Biological investigations indicated that SMP-W1 not only displayed promising antitumor activity, but also elevated antioxidase activities in H22-bearing mice, such as SOD, CAT and GSH-Px. These improvements extensively provide a scientific basis for developing the polysaccharide SMP-W1 as a safe antitumor agent for patients.

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