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Inhibitory effect of *Schisandra chinensis* leaf polysaccharide against L5178Y lymphoma

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ABSTRACT

A water-soluble polysaccharide WSLSCP was extracted from leaves of *Schisandra chinensis* and purified by DEAE-cellulose and Sepharose CL-6B chromatography. In the present investigation, the physicochemical properties, antitumor and immunomodulating activities of WSLSCP were valuated. The results showed WSLSCP could significantly inhibit the growth of L5178Y lymphoma cells *in vivo*, and upgrade the secretion level of TNF-α in serum. Moreover, the survival rate of tumor-bearing mice treated with WSLSCP was significantly improved. WSLSCP could enhance phagocytic capability of macrophages *in vitro*, and remarkably promoted the secretion of NO and TNF-α of macrophages. These results suggested WSLSCP could significantly enhance functions of immune system, and the antitumor effects of WSLSCP were achieved by immunostimulating properties.

1. Introduction

Cancer has been a formidable health problem with increasing incidence. Despite advances in the understanding of the pathology and biology of the disease, as well as improved diagnostic imaging and staging studies, the overall 5-year survival rate remains still extremely low for many kinds of cancer (Sharma, Eltawil, Renfrew, Walsh, & Molinari, 2011). It is well known that many antitumor agents are immunosuppressive agent. Though, they can significantly repress tumor growth, they also play an adverse role to human immune system (Hoos et al., 2010). So, more and more research are focused on discovering and identifying new antitumor drugs for immunopharmacology and oncotherapy, which can potentialize host immune defense functions for inhibiting tumor growth and killing tumor cells (Chihara, 1992).

Immunomodulating polysaccharides from traditional Chinese medical plants have been considered as biological response modifier (BRM), which they can promote organism to quickly adapt to environmental change and biological stress (Leung, Liu, Koon, & Fung, 2006). In addition, medical plant polysaccharides stimulate humoral and cell-mediated immunity, and they also exhibit a wider range of immunostimulating activities (Liu et al., 2006; Sun et al., 2008; Sun, 2011). Moreover, many researches show that medical plant polysaccharides can effectively inhibit the growth of various kinds of tumor in experimental animals and increase the survival rate (Cao & Lin, 2006; Luo et al., 2009). Besides, most polysaccharides derived from medical plants are relatively nontoxic and can not cause side effects, which is a major problem associated with immunomodulatory bacterial polysaccharides and synthetic compounds. Thus, polysaccharides from traditional Chinese medical plants have been widely used in Asian countries as therapeutic agents for cancer due to their significant immunomodulatory and antitumor effects without toxicity (Li et al., 2010; Tan & Vanitha, 2004).

Schisandra chinensis (Turcz.) Baill distributes abundantly in the northeast region of China, Korea and Japan. The seeds and the fruits are the parts used in medicine. *S. chinensis* has been used in traditional Chinese medicine for thousands of years (Halstead, Lee, Khoo, Hennell, & Bensoussan, 2007; Wang et al., 2008). It is officially listed in the Chinese Pharmacopeia and indexed as a tonic and sedative. It is also listed in the 'Shen Nong Ben Cao Jing' book, year 1596 (2697 BC) as a superior drug that helps in coughs and prevents asthma. According to Chinese philosophy the drug has sour and warm

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properties. It: (a) enters the lung and kidney channels and the stomach meridians; (b) contains the leakage of lung 'Qi' and stops coughing (used for deficient lung and kidney patterns with cough and wheezing); (c) restrains the 'Essence' and stops diarrhoea (used for nocturnal emission, spermatorrhea, deficiency of the spleen and kidneys); (d) stops excessive sweating (used for deficient 'Yang' spontaneous sweating or deficient 'Yin' night sweat); (e) calms the spirit (used for forgetfulness and insomnia).

In the last decades, the pharmacology and chemistry of this drug has been extensively studied. *S. chinensis* has been used to treat various kinds of disorders such as antitumor, cardialgia, facial distortion, epilepsia, migraine headache, vertigo, tetanus, infantile convulsion and rheumatic arthralgia (Sovová, Opletal, Bártlová, Sajfrtová, & Křenková, 2007). Much evidence shows that the polysaccharides of *S. chinensis* are important active component. However, there is no literature focusing on the relationship between antitumor and immunostimulatory activities of polysaccharides from leaves of *S. chinensis*. In order to fully develop the wild resources and extend the potential use of *S. chinensis* in antitumor and immunomodulating biomedicine, the present study was carried out to investigate antitumor and immunomodulating effects of polysaccharide from leaves of *S. chinensis* through *in vivo* and *in vitro* models, and clarified the anti-tumor mechanism of *S. chinensis* leaf polysaccharide.

2. Materials and methods

2.1. Materials and chemicals

S. chinensis leaves were obtained from a local medical farm, and identified according to the identification standard of Pharmacopeia of the People's Republic of China.

T-series dextrans, DEAE-cellulose, standard sugars, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (ConA) and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Medium RPMI-1640 was purchased from Gibco Invitrogen Co. Fetal calf serum (FCS) was provided by Hangzhou Sijiqing Corp. Sepharose CL-6B was purchased from Amersham Pharmacia Co. (Sweden). Trifluoroacetic acid (TFA), EDTA–Na_2, sodium hydroxide, hydroxylamine, inositol, acetic anhydride, pyridine, methanol and acetic acid were from Beijing Chemicals and Reagents Co. (Beijing, China). All other chemical reagents used were analytical grade.

2.2. Isolation and purification of polysaccharide fractions

After the leaves of *S. chinensis* were cleaned and ground in an electric mill, the powders were extracted with distilled water. The whole extract was filtered, centrifuged and concentrated. The polysaccharides were precipitated with 3 volumes of ethanol at 4 °C overnight. The crude polysaccharide precipitate was collected by centrifugation, deproteinated by a combination of proteinase and Sevag method (Staub, 1965), and then obtained crude water-soluble *S. chinensis* polysaccharides (cWSLSCP).

The cWSLSCP solution was loaded onto DEAE-cellulose column, eluted successively with distilled water and 0.5 M NaCl. Fractions were collected, and monitored with the phenol–sulfuric acid method. One main fraction was collected, dialyzed, lyophilized, and were further fractioned on Sepharose CL-6B column, eluted with 0.15 M NaCl to yield a main fraction, codes as WSLSCP. WSLSCP was collected, dialyzed and lyophilized for further analysis.

2.3. Physicochemical properties and chemical compositions analysis

Molecular weight of WSLSCP was determined by high performance liquid chromatography (HPLC). WSLSCP were dissolved in distilled water, applied to Agilent HPLC system (Agilent Technologies, USA) equipped with a TSK-GEL G3000 PWXL column, eluted with 0.1 mol/L Na_2SO_4 solutions and detected by a RID-10A Refractive Index Detector. Dextran standards with different molecular weights (T-2000, T-70, T-40, T-20, and T-10) were to calibrated the column and establish a standard curve.

Total carbohydrate content of WSLSCP was determined by phenol–sulfuric acid colorimetric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Total uronic acid content was measured by *m*-hydroxydiphenyl method (Filisetti-Cozzi & Carpita, 1991). Protein content was quantified according to the Bradford's method (Bradford, 1976). Monosaccharide composition was analyzed according to the method of Lehrfeld (1985). Simply, WSLSCP was hydrolyzed with TFA, and then hydrolyzed product was reduced with KBH_4, followed by neutralization with acetic acid. After adding myo-inositol and Na_2CO_3, the residue was concentrated. The reduced products were added with pyridine–propylamine, and acetylated with pyridine–acetic anhydride. The acetylated products were analyzed by gas chromatography (GC), and identified and estimated with myo-inositol as the internal standard.

2.4. Evaluation of in vivo antitumor activity in lymphoma ascites model

The *in vivo* tumor growth inhibition assay was carried out with a L5178Y lymphoma-bearing mice model. Under sterile condition, 0.5 ml of L5178Y lymphoma cell suspension (2×10^6 cells/ml) was inoculated i.p. to each mouse at day 0. The inoculated mice were divided into five groups (10 mice in each group) including negative control group, positive control group and three treatment groups. After 3 days of tumor inoculation, different doses (100, 200 and 400 mg/kg body weight) of WSLSCP were administrated to each treatment group for 10 days and the negative and positive control mice received PBS and 5-fluorouracil (5-FU, 50 mg/kg), respectively. After treatment, all mice were sacrificed and the total accumulated ascites fluid volume and tumor cells in the peritoneal cavity of each animal were harvested and total ascites cell count was determined. Tumor necrosis factor-alpha (TNF-α) in sera collected from the tumor-bearing mice was measured using murine enzyme-linked immunosorbent assay (ELISA) kit.

2.5. Effect of WSLSCP on survival rates of lymphoma-bearing mice

Mice were transplanted i.p. with L5178Y lymphoma suspension (2×10^6 cells/mouse) with 10 in each group. The effect of i.p. administration of WSLSCP on survival and progression of tumor on L5178Y lymphoma-bearing mice was investigated. After 3 days of tumor transplantation, WSLSCP was administrated to lymphoma-bearing mice through i.p. at a dose of 400 mg/kg body weight once a day for consecutive 10 days, and survival rate was recorded till two months later. Median survival time (MST) and increase in life span (ILS) were calculated accordingly to the mortality data within the observation period. The ILS was calculated by the following equation:

$$\text{ILS value} = \frac{\text{MST of the treated group}}{\text{MST of the control group} - 1)} \times 100\%$$

The ILS value of greater than 25% is considered for significantly improving survival rate.

2.6. Activation of peritoneal macrophage

Male BALB/c mice was injected intraperitoneally with 2 ml of sterile thioglycollate medium for consecutive 3 days, then the resident macrophages were harvested by peritoneal lavage and centrifugation. Then peritoneal macrophages were cultured in complete RPMI 1640 medium in 96-well plate for 2 h. Non-adherent cells were removed by washing the plate with PBS. Macrophages were cultured with different concentrations of WSLSCP (100, 200 and 400 μg/ml) for 24 h, LPS (20 μg/ml) was used as positive control. 0.075% aseptic neutral red solution 100 μl was added, and then cultured for another 1 h. Then each well was washed, and added 150 μl mixtures of ethanol and acetic acid (1:1, v/v) to lysate cell. The mixtures were evaluated at 550 nm in an ELIAS reader.

2.7. In vitro nitric oxide and TNF-α secretion assay

Peritoneal macrophages (2×10^6 cells/ml) prepared as described above were cultured in complete RPMI 1640 media in 48-well plate with WSLSCP at different concentrations (100, 200 and 400 μg/ml) and LPS (20 μg/ml). The cells were cultured for 24 h. Production of nitric oxide was estimated by measuring nitrite levels in cell supernatant with Greiss reagent (1% sulfanilamide in 2.5% phosphoric acid, 0.1% napthylethyldiamine dihydrochloride in 2.5% phosphoric acid). Absorbance was read at 540 nm vs. $NaNO_2$ standard curve.

For TNF-α secretion assay, peritoneal macrophages (2×10^6 cells/ml) were cultured in 48-well plates. After incubated with WSLSCP at different concentrations (100, 200 and 400 μg/ml) for 24 h, TNF-α secretion was measured using murine enzyme-linked immunosorbent assay (ELISA) kit.

3. Results and discussion

3.1. Isolation and purification of polysaccharides from Schisandra chinensis

The crude water-soluble polysaccharide was extracted from *S. chinensis* with hot water and ethanol precipitation, and the yield of the crude polysaccharide was 8.1%. After deproteinated by a combination of proteinase and Sevag method, the crude polysaccharide sample (cWSLSCP) was loaded onto the DEAE-cellulose column eluted by de-ionized water and 0.5 M NaCl, one main fraction was further purified by Sepharose CL-6B column eluted with 0.15 M NaCl, and the main fraction (WSLSCP) was separated for further analysis of physicochemical properties and antitumor and immunomodulating activities.

3.2. Physicochemical properties and chemical compositions

The total sugar, protein, uronic acid contents, molecular weight and monosaccharides composition of the polysaccharide fractions are summarized in Table 1. WSLSCP appeared as a white powder, and it had a negative response to Bradford test. In addition, no absorption was detected by the UV spectrum at either 280 or 260 nm, which indicated the absence of protein and nucleic acid. HPLC profile demonstrated that WSLSCP had a single and symmetrically sharp peak revealing that WSLSCP was a homogeneous polysaccharide with the average molecular weight of 127 kDa. Results from phenol–sulfuric acid assay showed that WSLSCP contained 96.9% carbohydrate, and contained 12.2% uronic acid evaluated by *m*-hydroxydiphenyl colorimetric method and GC analysis. According to GC analysis, WSLSCP was composed of mannose, glucose, glucuronic acid with a relative molar ratio of 5.6:3.3:1.

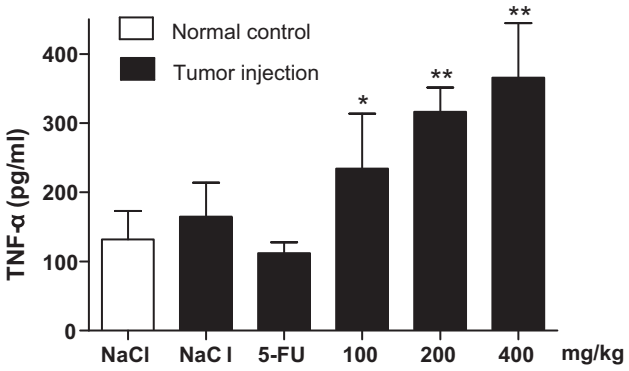

Fig. 1. Concentration of TNF-α in serum of tumor-bearing mice treated with WSLSCP. Values are mean ± SD; $^*p < 0.05$, $^{**}p < 0.01$ vs. model control group.

3.3. In vivo anti-tumor activity in lymphoma ascites model

The antitumor effect of WSLSCP was evaluated *in vivo* in lymphoma ascites mice model. The inhibitory effect of WSLSCP was exhibited in terms of the total number of cells and volume of ascites in mice treated with or without sample. As shown in Table 2, the WSLSCP treated group showed a significant decrease in ascites volume and tumor cell number compared to control group. The tumor inhibitory rates of WSLSCP were 23.8%, 52.2% and 72.3%, respectively, at the doses of 100, 200 and 400 mg/kg body weight. While the frequently used chemotherapy drug, 5-fluorouracil (5-FU), exhibited an inhibitory rate of 67.9%, slightly lower than of WSLSCP at the dose of 400 mg/kg body weight, which indicated WSLSCP possessed excellent antitumor activity. Moreover, the level of TNF-α in serum of WSACP treated mice was raised in a dose-dependent manner (Fig. 1).

3.4. Effects on survival rate of lymphoma tumor-bearing mice

To determine the survival effect of WSLSCP on lymphoma-bearing, mice were treated with 400 mg/kg body weight for 10 days starting from 3 days after inoculation of tumor cells and left until death. When compared against the control group, the WSLSCP-treated group showed a significant higher survival rate. All the animals ($n = 10$) in controls developed tumor and died within 8–23 days whereas the WSLSCP-treated group with restricted tumor growth died in with in 11–55 days. The ILS value was 112.9% far greater than 25%, which showed significantly improving survival rate by WSLSCP (Table 3).

3.5. Activation on peritoneal macrophages

Peritoneal macrophages were incubated with WSLSCP and LPS. The results showed that WSLSCP could significantly enhance the peritoneal macrophages phagocytosis compared with negative control group at the concentration of 200 and 400 μg/ml ($p < 0.01$, Fig. 2). In addition, WSLSCP could induce the production of NO and TNF-α of stimulated macrophages as shown in Table 4. The production of NO increased significantly with increasing concentrations from 50 to 400 μg/ml. When the dose of WSLSCP reached 400 μg/ml, the amount of NO produced by macrophages a little exceeded that of LPS. TNF-α was measured by sandwich ELISA. When the doses were 200 and 400 μg/ml, the level of TNF-α was significantly increased compared with negative control level ($p < 0.01$).

Table 1
Physicochemical properties and chemical compositions of WSLSCP.

Sample	Mw (Da)	Total sugar (%)	Protein (%)	Uronic acid (%)	Molar ratios		
					Man	Glc	GlcUA
WSLSCP	127,000	96.9	nd	12.2	5.6	3.3	1

Table 2
In vivo antitumor activity on L5178Y lymphoma-bearing mice model.

Group	Dose (mg/kg)	Total tumor cell count ($\times 10^7$)	Packed cell volume (ml)	Inhibition rate (%)
Control		16.4 ± 2.17	3.7 ± 0.44	
WSLSCP	100	12.5 ± 2.21*	2.8 ± 0.47*	23.8
	200	7.84 ± 1.18**	1.5 ± 0.33**	52.2
	400	4.55 ± 0.62**	0.9 ± 0.25**	72.3
5-FU	50	5.27 ± 0.73**	1.1 ± 0.27**	67.9

Values are expressed as mean ± SD ($n = 10$).
* $p < 0.05$.
** $p < 0.01$ vs. control.

Table 3
Effect of WSLSCP on survival rates of L5178Y lymphoma-bearing mice.

Group	MST (days)	AST (days)	ILS (%)	p value (vs. control)
Control	15.5	15.6	–	1
WSLSCP (400 mg/kg body wt.)	33.0	26.5	112.9	<0.001

Table 4
In vitro NO and TNF-α produced by macrophages stimulated with WSLSCP.

Group	NO (μM)	TNF-α (pg/ml)
Negative control	21.3 ± 4.3	165.4 ± 21.6
LPS	63.5 ± 15.4**	438.6 ± 52.3**
WSLSCP		
50 μg/ml	27.6 ± 4.8	199.7 ± 42.5
100 μg/ml	37.3 ± 8.4**	247.9 ± 56.1*
200 μg/ml	56.8 ± 16.3**	361.7 ± 75.3**
400 μg/ml	69.8 ± 15.9**	466.3 ± 73.8**

Values are expressed as mean ± SD.
* $p < 0.05$.
** $p < 0.01$ vs. negative control.

4. Discussion and conclusion

More and more polysaccharides from traditional Chinese medical plants have shown effective antitumor activities by attacking the cancer cell directly or enhancing the host's immune function, and some of them have been applied for clinical treatment for cancer in Asia countries, especially in China (Chang, 2002). *S. chinensis*, as a frequently used Chinese folk medicine, has been used for cancer treatment for a long time. However, the specific active chemical compositions and the possible mechanism have been still unclear. Much *in vivo* and *in vitro* evidence demonstrated that active polysaccharides isolated from medical plants could stimulate both humoral and cell-mediated immunity; they also exhibit a wider range of immunostimulating and antitumor activities compared to other chemical compounds (Kim et al., 1996). Therefore, in the present investigation, a water-soluble polysaccharide WSLSCP was extracted from the leaves of *S. chinensis* by hot-water extraction and ethanol precipitation, and further isolated and purified by DEAE-cellulose and Sepharose CL-6B column, and then the antitumor and immunomodulatory activities of WSLSCP were evaluated by *in vivo* and *in vitro* models.

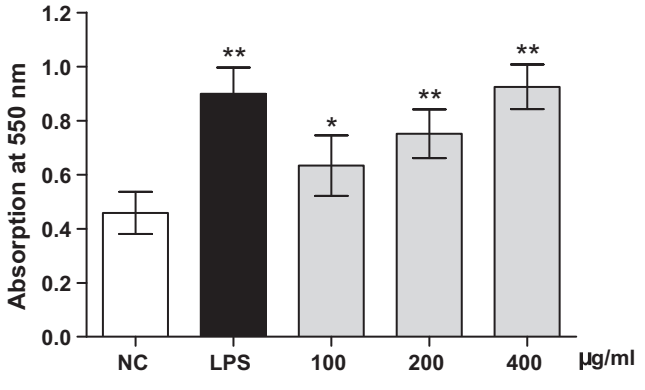

Fig. 2. Effects of WSLSCP on phagocytosis activity of macrophage. Values are means ± SD; *$p < 0.05$, **$p < 0.01$ vs. negative control.

The results showed that WSLSCP could effectively inhibit the proliferation of L5178Y lymphoma *in vivo*, and the tumor inhibitory rate reached 72.3% at the highest concentration. In addition, the level of TNF-α in serum was markedly increased in WSLSCP-treated groups compared to negative control group. Moreover, WSLSCP showed significantly improving survival rate on lymphoma-bearing mice.

Most polysaccharides have no significant direct cytotoxicity, therefore, the enhancement of host immune defense system has been considered as a possible mechanism for inhibiting tumor growth and attacking tumor cells. The immunologic enhancement may begin with activating effector cells. Macrophages are major population of immune cells in the host defense system, which act against invading pathogen. Macrophages are derived from precursors in the bone narrow via monocytes of peripheral blood and constitute the mononuclear phagocyte system (Moore & Tabas, 2011; Ueha, Shand, & Matsushima, 2011). They are indispensable for keeping homeostasis, and play an essential and pivotal role in host defense against any type of invading cells including cancer cells. Macrophages protect the host by phagocytosis, presenting antigens to lymphocytes and releasing diverse cytokines that regulate the activity of other cells. Activated macrophages can effectively remove tumor cells by producing NO and TNF-α (Singh & Sodhi, 1991). Therefore, NO and TNF-α have been identified as the major effector molecules involved in the destruction of tumor cells by activated macrophages. In order to confirm whether the polysaccharide WSLSCP could activate macrophages, we first detected the phagocytosis by the up-take of neutral red by

WSLSCP-treated macrophages. As the increasing concentration of WSLSCP, the phagocytosis was significantly increased. The effects treated with WSLSCP were much better compared with those observed with the negative control and LPS control. Furthermore, WSLSCP could significantly increase the NO production and TNF-α secretion of macrophages compared with negative control at the doses of 100–400 μg/ml. Thus, the polysaccharide may indirectly play the role of anti-tumors activity through the releases of effecter molecules such as TNF-α, NO produced by macrophages.

Based on the results, we have confirmed that the polysaccharide WSLSCP isolated from leaves of *S. chinensis* had significant antitumor effects and the mechanism of antitumor activity was achieved by enhancing functions of immune system, such as promoting of enhancing the peritoneal macrophages phagocytosis, and increasing the releases of effecter molecules (such as TNF-α and NO) produced by macrophages. WSLSCP could be explored as a novel potential antitumor agent with immunomodulatory activity for the functional food and pharmaceutical purpose. This study also provided evidences to support the therapeutic effects of this traditional medicine for treatment of cancer in China. Besides, the detail structural characteristic and structure–function relationships involved in immunomodulation need further to be elucidated.

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