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Antitumor and immunomodulatory activities of a polysaccharide from *Artemisia argyi*

Xiaoli Bao[a], Huihui Yuan[b,*], Chengzhong Wang[a], Jinjin Liu[a], Minbo Lan[a,c,*]

[a] Shanghai Key Laboratory of Functional Materials Chemistry, and Research Centre of Analysis and Test, East China University of Science and Technology, Shanghai 200237, P. R. China
[b] Institute of Applied Chemistry, East China University of Science and Technology, Shanghai 200237, P. R. China
[c] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, P. R. China

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ABSTRACT

A water-soluble polysaccharide (FAAP-02), composed of N-acetyl-D-glucosamine, glucose, mannose, galactose, rhamnose, arabinose, xylose and ribose, with an average molecular weight of 5169 Da, was isolated from *Artemisia argyi*. The antitumor and immunomodulatory activities of FAAP-02 were evaluated in Sarcoma 180 (S180) tumor-bearing mice by intraperitoneal administration. As a result, FAAP-02 significantly inhibited the growth of the S180 transplanted tumors and prolonged the survival time of the tumor-bearing mice. Moreover, FAAP-02 could obviously increase the thymus and spleen indices, the levels of serum Interleukin 2 (IL-2), Interleukin 6 (IL-6), Interleukin 12 (IL-12) and tumor necrosis factor-α (TNF-α), and the expression of CD4+ and CD8+ splenic T lymphocytes which were suppressed by the transplanted tumor or/and 5-fluorouracil (5-FU) in the mice. These results indicated that the antitumor activity of FAAP-02 might be associated with its immunostimulatory effects.

1. Introduction

Cancer is a universal health problem with high morbidity and mortality. Conventional cancer therapies, such as surgery, chemotherapy and radiotherapy, are showing various limitations because of poor prognosis and serious side effects (Wang et al., 2012a,b). Recently, growing attention has been focused on natural products in an attempt to search for novel effective anticancer agents with less toxic effects (Nobili et al., 2009; Reddy, Odhav, & Bhoola, 2003; Fan et al., 2012).

In past decades, polysaccharides isolated from natural sources, such as fungi, plants, algae and animals, have been widely studied owing to the versatile biological functions and low toxicity (Zong, Cao, & Wang, 2012; Liu, Sun, Liu, & Yu, 2012; Liu, Li, Yang, Zhang, & Cao, 2012). The bioactivities of natural polysaccharides, especially the immunostimulating and antitumor effects, have attracted considerable attention in the biomedicinal field since defending against tumors by increasing the human body's immunity has become popular today (Cai et al., 2012; Li, Wang, Zhao, & Luo, 2012; Ding, Zhu, & Gao, 2012). Polysaccharides, such as lentinan, schizophyllan and krestin, have already been used as immunoceuticals in clinical cancer therapies (Tang et al., 2012).

Artemisia argyi (Asteraceae) is a perennial herbaceous plant widely distributed in China, and has been used as a traditional herbal medicine for the treatment of microbial infections, inflammatory diseases, diarrhea, hepatitis, malaria, cancer, and circulatory disorders (Adams, Efferth, & Bauer, 2006; Cai, 2001; Khan et al., 2012). Phytochemical and pharmacological studies on this plant have revealed the presence of coumarins, glycosides, flavonoids, polyacetylenes, monoterpenes, sterols, triterpenes, sesquiterpene lactones and essential oils, some of which have shown various biological functions, such as antiulcer (Yoon, Chin, Yang, & Kim, 2011), antiallergic (Ji, Kim, Kim, Jeong, & Lee, 2010), antidiabetic (Adams, Garcia, & Garg, 2012), antioxidant (Ferreira, Luthria, Sasaki, & Heyerick, 2010), antimutagenic (Nakasugi, Nakashima, & Komai, 2000), antiproliferative, anti-inflammatory (Cai, 2001) and anticancer (Adams et al., 2006; Lee et al., 2002, 2005; Seo et al., 2003) activities. Although the antitumor activity of *A. argyi* has been evidenced, the relevant studies were mostly limited to small molecular compounds, and there was little information available about the antitumor activities of the polysaccharides from this plant. In current study, a water-soluble polysaccharide was isolated from *A. argyi* and its antitumor activity

* Corresponding authors. Tel.: +86 021 64253574; fax: +86 021 64252947.
E-mail addresses: yuanhuihui@ecust.edu.cn (H. Yuan), minbolan@ecust.edu.cn (M. Lan).

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was evaluated *in vivo*. In addition, the immunomodulatory effects of this polysaccharide were also assessed to analyze the underlying mechanisms of its antitumor activity.

2. Materials and methods

2.1. Materials

A. argyi cultivated in Hubei Province was purchased from Anguo Herbal Market (China). DEAE-Sepharose Fast Flow was obtained from GE Healthcare (Canada). ADS-7 macroporous absorption resin was provided by Nankai University (China). T-series dextrans, trifluoroacetic acid (TFA) and 5-fluorouracil (5-FU) were purchased from Sigma (USA). Mouse ELISA kits for Interleukin 2 (IL-2), Interleukin 6 (IL-6), Interleukin 12 (IL-12) and tumor necrosis factor-α (TNF-α) were supplied by 4A Biotech Co. Ltd. (China). Fluorescein isothiocyanate (FITC)-conjugated anti-Mouse CD4 and phycoerythrin (PE)-conjugated anti-Mouse CD8 monoclonal antibodies were provided by eBioscience (USA). All other chemicals and reagents used were of analytical grade.

2.2. Isolation of FAAP-02

About 300 g of the powder of dried *A. argyi* was steeped in 95% ethanol for 72 h to remove fats and lipids. Then the residue was dried and extracted with boiling water for 2 h. After centrifugation and concentration, the aqueous extract was deproteinated by trichloroacetic acid (TCA) followed by centrifugation. The supernatant was treated with 80% ethanol at 4 °C overnight. After filtration, the precipitate was washed with ethanol, acetone, ether and ethanol sequentially to yield the crude polysaccharide, 27.3 g. The crude polysaccharide was dissolved in distilled water, then applied to a column (4.0 cm × 40 cm) of ADS-7 macroporous absorption resin, eluting at a flow rate of 4.0 mL/min with distilled water until no reaction of anthrone–sulfonic acid (Roe, 1955). The eluent was concentrated and loaded to a column (2.5 cm × 60 cm) of DEAE-Sepharose Fast Flow pre-equilibrated with Tris–HCl buffer (pH 8.1), which was subsequently fractionated with 0, 0.1, 0.2, 0.5, 1.0 M NaCl aqueous solutions in turn at a flow rate of 2.0 mL/min, with collection of 10 mL for each tube. The presence of carbohydrate in each fraction was monitored using the anthrone–sulfonic acid assay (Roe, 1955). Relevant fractions were pooled, concentrated, dialyzed (cut-off Mw 3500 Da) and finally lyophilized. The fraction eluted with 0.2 M NaCl aqueous solution was FAAP-02, 0.99 g. Fig. 1 summarizes the isolation process of FAAP-02 from *A. argyi*.

2.3. Characterization of FAAP-02

The homogeneity and molecular weight distribution of FAAP-02 were determined by high performance gel permeation chromatography (HPGPC) on an Agilent 1100 HPLC system equipped with a TSK G3000 PWXL column (7.5 mm × 300 mm, column temperature: 30 °C) and a differential refractive index detector. The sample solution was eluted with Na_2SO_4 aqueous solution (5 g/L) at a flow rate of 0.5 mL/min. The standard T-series dextrans of different molecular weights (M_w = 1000, 5000, 12,000, 50,000 Da) had been chosen as the calibration standards to obtain a regression equation. The average molecular weight of FAAP-02 was calculated by the calibration equation.

Total carbohydrate content in FAAP-02 was determined by the anthrone–sulfonic acid colorimetric method using glucose as the standard sample (Roe, 1955). The uronic acid content in FAAP-02 was quantified by m-hydroxybiphenyl method, using galacturonic acid as standard (Filisetti-Cozzi & Carpita, 1991). The protein content in FAAP-02 was measured by Bradford's method (Bradford, 1976), with bovine serum albumin as the standard sample. The sulfate radical content in FAAP-02 was tested by barium chloride–gelatin assay, using potassium sulfate as the standard (Lloyd & Dodgson, 1961).

A simple and sensitive high performance liquid chromatographic method was applied to the simultaneous determination of nine kinds of monosaccharides (glucose, rhamnose, mannose, arabinose, galactose, xylose, ribose, galacturonic acid and N-acetyl-D-glucosamine) in FAAP-02 by pre-column derivatization with 1-phenyl-3-5-pyrazolone (PMP) as described previously with proper modification (Lv et al., 2009). In brief, FAAP-02 was hydrolyzed with TFA (3 M) for 8 h at 100 °C in a sealed ampoule. After cooling to room temperature, the reaction mixture was centrifuged and the supernatant was collected and lyophilized. The hydrolyzed sample or monosaccharide standard aqueous solution was mixed with aqueous NaOH (0.25 M) and PMP methanol solution (0.25 M) thoroughly. Then each mixture was incubated at 70 °C for 90 min. After cooled and neutralized with HCl (0.25 M), the resulting solution was extracted with chloroform to remove the excess reagents. The aqueous layer was filtered through a 0.45 μm membrane before detection. The analysis of PMP-labeled monosaccharides was performed on an Agilent 1260 HPLC system equipped with a ZORBAX Eclipse XDB-C_{18} (250 mm × 4.6 mm, id: 5 μm, column temperature: 30 °C). The wavelength of detection was 250 nm. Elution was carried out with a mixture of acetonitrile and phosphate buffer (0.06 M, pH 6.8) in a ratio of 17: 83 (v/v, %) at a flow rate of 1 mL/min.

The UV spectra of FAAP-02 were obtained with a UV–vis spectrophotometer (Thermo Scientific, USA). The FT-IR spectra of FAAP-02 were recorded on a Nicolet 6700 spectrophotometer (Thermo Scientific, USA) in the range of 400–4000 cm^{-1} using the KBr disk technique. The NMR spectra of FAAP-02 (50 mg/mL D_2O) were recorded on an Avance 500 NMR spectrometer (Bruker, Germany).

2.4. Determination of antitumor and immunomodulatory activities of FAAP-02

2.4.1. Animal treatment and experimental design

Kunming mice of clean grade (male, weighing 18–22 g) were provided by Shanghai SLAC Laboratory Animal Center (number of animal license: SCXK (Shanghai) 2012–0002). The animals were allowed free access to the standard diet and sterile water and maintained in a sterile and ventilated room under a controlled environmental condition (25 ± 1 °C, 50 ± 10% humidity, 12 h light/12 h dark cycle). All animal handling procedures were performed in accordance with the institutional guidelines for the use and care of laboratory animals established by the Ethical Committee for Animal Experiments of Shanghai SLAC Laboratory Animal Center.

Murine sarcoma 180 (S180) cells, obtained from the cell bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (China), were maintained as ascites in Kunming mice (6–8 weeks old) for one week. S180 cells, collected from the peritoneal cavity of the tumor-bearing mice, were washed with aseptic phosphate-buffered saline (PBS) and adjusted to a suspension with a concentration of 1×10^7 cells/mL in serum-free RPMI-1640 medium. The mice were implanted with 200 μL of cell suspension by subcutaneous injection to the fore right subaxillary. Seven days after the tumor inoculation, eight normal mice treated with only 0.9% normal saline (NS) served as the normal control (NC), and the tumor-bearing mice were randomly divided into six groups (eight mice in each group): tumor-bearing mice

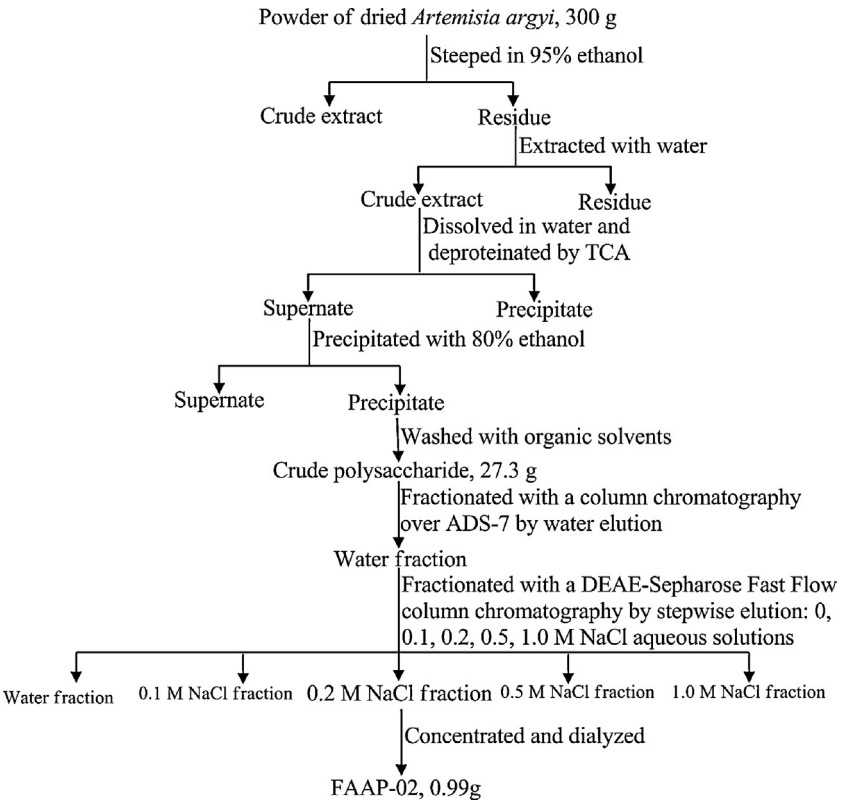

Fig. 1. Isolation scheme of FAAP-02 from *Artemisiae argyi*.

administrated intraperitoneally with 5-FU at the dose of 30 mg/kg/day (5-FU (30)); tumor-bearing mice administrated intraperitoneally with FAAP-02 at the respective dose of 50, 100 and 200 mg/kg/day (FAAP-02 (50), FAAP-02 (100), FAAP-02 (200)); tumor-bearing mice administrated intraperitoneally with FAAP-02 at the dose of 100 mg/kg/day combined with 5-FU at the dose of 30 mg/kg/day (FAAP-02 + 5-FU (100 + 30)); tumor-bearing mice administrated intraperitoneally with the same volume of NS (the model control, MC). All treatments were administrated once daily for seven consecutive days.

2.4.2. Tumor inhibitory rate, thymus and spleen indices

24 h after the last drug administration, four mice in each group were sacrificed. Tumor, thymus and spleen were dissected and weighed. Blood was collected by heart puncture, centrifuged at 1500 × *g* for 10 min, and the serum was collected for the determination of cytokine levels. The inhibition ratio of tumor was calculated as:

$$\text{Tumor inhibitory rate}(\%) = \left[\frac{A-B}{A}\right] \times 100$$

where *A* and *B* are the tumor weight of the model control and the treated groups, respectively.

Relative thymus weight was calculated as:

$$\text{Thymus index}(\text{mg/g}) = \frac{\text{weight of thymus}(\text{mg})}{\text{body weight}(\text{g})}$$

Relative spleen weight was calculated as:

$$\text{Spleen index}(\text{mg/g}) = \frac{\text{weight of spleen}(\text{mg})}{\text{body weight}(\text{g})}$$

2.4.3. Determination of survival time

The remaining four mice in each group were observed daily to record their death patterns due to tumor burden, and the percentage of life prolongation was calculated as:

$$\text{Life prolongation rate}(\%) = \left[\frac{T-C}{C}\right] \times 100$$

where *C* and *T* are the survival time of the model control and the treated groups, respectively.

2.4.4. Determination of serum cytokine levels by the ELISA assay

The levels of IL-2, IL-6, IL-12 and TNF-α in the serum of the mice from each group were determined using commercial mouse ELISA kits according to the manufacture's protocols.

2.4.5. Evaluation of splenic T lymphocyte subpopulations by flow cytometry

Spleens were aseptically excised from the sacrificed mice and mashed with a Teflon pestle in serum-free RPMI-1640 medium. After filtering through a sieve mesh, erythrocytes in the cell mixture were lysed with RBC lysis buffer. The remaining cells were washed with PBS and resuspended to a final density of 1×10^{10} cells/L in RPMI-1640 medium supplemented with 10% FBS. Then 100 μL of splenocytes were incubated with either fluorescein isothiocyanate (FITC)-conjugated anti-Mouse CD4 or phycoerythrin (PE)-conjugated anti-Mouse CD8 monoclonal antibody in dark for 30 min at 4 °C. After washed with PBS, the lymphocytes were fixed in 2% paraformaldehyde and analyzed on a flow cytometry (10,000 cells per run).

2.5. Statistical analysis

Data were analyzed using the SPSS software package. The results were expressed as the means ± standard deviations. Student's *t*

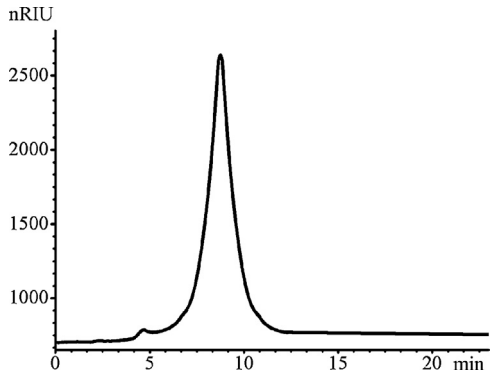

Fig. 2. HPGPC profiles of FAAP-02.

test was used to evaluate the significance of differences between groups. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Isolation and characterization of FAAP-02

The crude polysaccharide was obtained from the dried leaves of *Artemisiae argyi* by degreasing with ethanol, hot-water extraction, ethanol precipitation, deproteinization by TCA, rinsing with organic solvents and desiccation under vacuum and its yield was 9.1% of raw material. After purification by the ADS-7 macroporous absorption resin, a fraction was obtained from the water elution, which was further fractioned by the DEAE-Sepharose Fast Flow chromatography to yield a polysaccharide, named as FAAP-02, with a yield of 0.33% of the dried *Artemisiae argyi* leaves. FAAP-02 appeared as a white powder, which was soluble in water, but insoluble in ordinary organic solvents, such as ethanol, diethyl ether, acetone, and chloroform. In the HPGPC profile (Fig. 2), FAAP-02 showed a single symmetrical sharp peak, which indicated it was homogeneous. The average molecular weight of FAAP-02 was determined to be 5169 Da according to its retention time and the calibration curve of the standard dextrans.

The total carbohydrate content and the uronic acid content in FAAP-02 were determined to be 76.2% and 6.9%, respectively. No sulfate radical was determined by barium chloride–gelatin assay. The negative response in the Bradford's test and the lack of absorption at either 280 or 260 nm in the UV–vis spectra indicated that FAAP-02 contained no protein or nucleic acid (Li et al., 2012). The analysis of monosaccharide composition in FAAP-02 (Fig. 3) revealed that FAAP-02 was mainly composed of N-D-glucosamine, glucose, mannose, galactose, rhamnose, arabinose, xylose and ribose in a molar ratio of 11.13:8.09:6.86:3.82:3.12:2.74:1.80:1.00. The contents (mass fraction) of these monosaccharides in FAAP-02

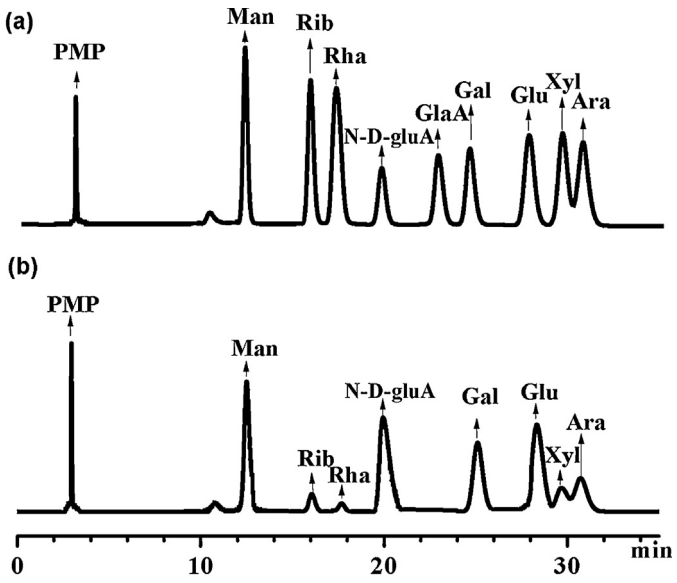

Fig. 3. HPLC of monosaccharides (as their PMP derivatives). (a) The mixture of standard monosaccharides. (b) FAAP-02; Man, mannose; Rib, ribose; Rha, rhamnose; GalA, galacturonic acid; Gal, galactose; Glu, glucose; Xyl, xylose; N-D-gluA, N-acetyl-D-glucosamine; Ara, arabinose.

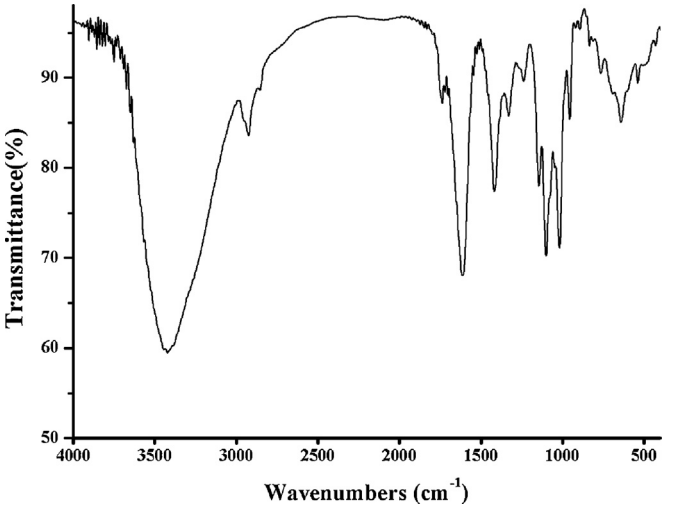

Fig. 4. FT-IR spectra of FAAP-02.

were 32.50% for N-D-glucosamine, 19.24% for glucose, 16.31% for mannose, 9.10% for galactose, 6.76% for rhamnose, 5.42% for arabinose, 3.88% for xylose and 1.93% for ribose, respectively.

In the FT-IR spectra (Fig. 4), FAAP-02 displayed a broad O—H stretching vibration band at around 3425 cm^{-1} and a weak C—H

Table 1
Tumor inhibition and life prolongation effects of FAAP-02 in S180 tumor-bearing mice.

Group	Dosage (mg/kg/day)	Tumor inhibitory rate (%)	Life prolongation rate (%)
5-FU	30	46.22 ± 1.19	34.39 ± 3.60
FAAP-02	50	28.37 ± 1.35	14.82 ± 1.72
	100	30.58 ± 0.76	52.53 ± 8.23
	200	38.58 ± 2.53	55.67 ± 7.29
FAAP-02 + 5-FU	100 + 30	57.44 ± 4.12##	71.71 ± 3.60##

S180 tumor-bearing mice were administrated intraperitoneally with FAAP-02 (50, 100, 200 mg/kg/day), 5-FU (30 mg/kg/day), FAAP-02 (100 mg/kg/day) in combination with 5-FU (30 mg/kg/day) for seven consecutive days, once daily. Model control group received the same volume of normal saline. Day 8, Tumor inhibitory rate (%) was determined as: [(the tumor weight of the model control – the tumor weight of the treated groups)/the tumor weight of the model control] × 100. Life prolongation rate (%) was determined as: [(the survival time of the treated groups – the survival time of the model control)/the survival time of the model control] × 100. Each value is presented as means ± S.D. ($n = 4$).

Significant differences from the 5-FU group were designated as $P < 0.05$.

Significant differences from the 5-FU group were designated as $P < 0.01$.

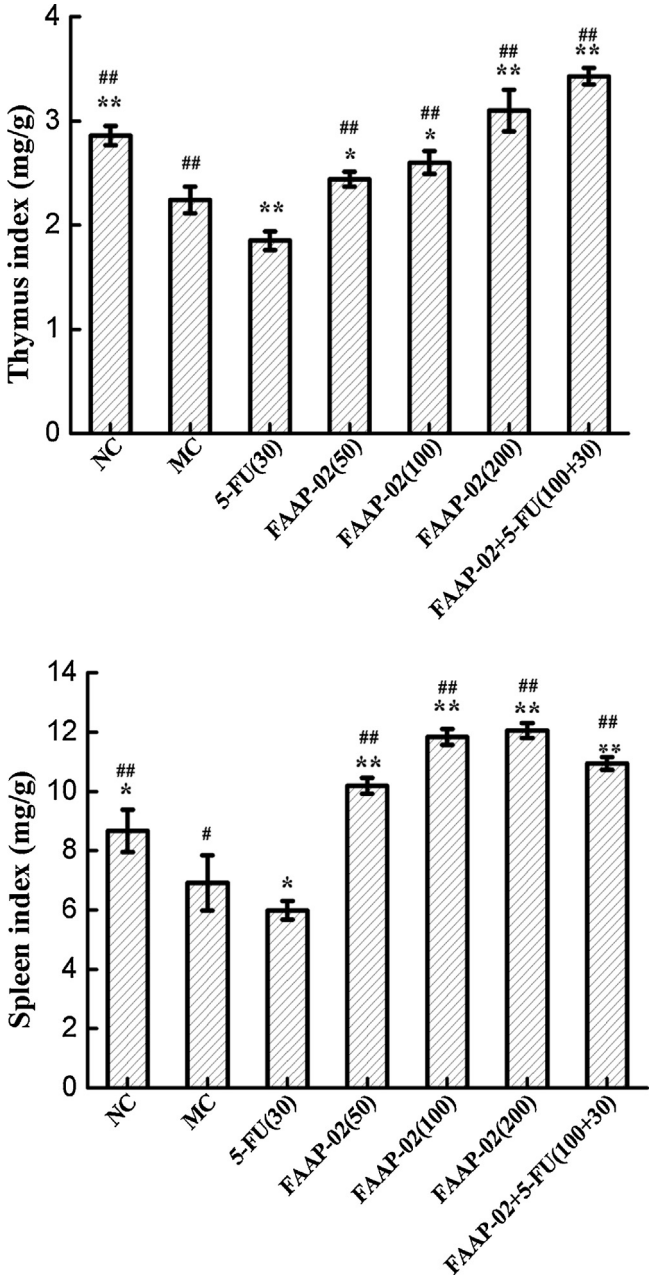

Fig. 5. Effects of FAAP-02 on thymus and spleen indices in S180 tumor-bearing mice. S180 tumor-bearing mice were administrated intraperitoneally with FAAP-02 (50, 100, 200 mg/kg/day), 5-FU (30 mg/kg/day), FAAP-02 (100 mg/kg/day) in combination with 5-FU (30 mg/kg/day) for seven consecutive days, once daily. Model control and normal control groups received the same volume of normal saline. Day 8, thymus index was calculated as: the weight of thymus (mg)/body weight (g); spleen index was calculated as: the weight of spleen (mg)/body weight (g). Each value is presented as means ± S.D. ($n = 4$). NC, the normal control; MC, the model control. Significant differences from the model control were designated as $^{*}P < 0.05$, $^{**}P < 0.01$. Significant differences from the 5-FU group were designated as $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

stretching vibration band at 2927 cm^{-1}, which were distinctive absorption of polysaccharides. The absorption near 1750 cm^{-1} was attributed to the stretching vibration of C=O in the protonated carboxylic acid, while the band toward 1620 cm^{-1} was related to the COO^{-} deprotonated carbonyl group (Yang et al., 2009). The bands appeared at 1745 and 1618 cm^{-1} resulted from the presence of uronic acid. FAAP-02 also presented distinct absorption at 1081 cm^{-1} and 1030 cm^{-1}, which characterize galactopyranose in the backbone and arabinofuranose units in side branches, respectively (He, Yang, Jiao, Tian, & Zhao, 2012). Specifically, the absorption bands at 840 cm^{-1} and 890 cm^{-1} confirmed the co-existence of α- and β-configurations (Liu, Sun, et al., 2012; Liu, Li, et al., 2012).

The 1D ^{1}H NMR spectra of FAAP-02 showed several signals in the anomeric proton region (4.50–5.00 ppm). The chemical shifts at 4.93 ppm, 4.88 ppm, 4.59 ppm and 4.57 ppm, which were smaller than 5.00 ppm, were assigned to β-anomeric protons, while the signals at 5.29 ppm, 5.13 ppm, 5.06 ppm and 5.03 ppm were assigned to α-anomeric protons (Liu, Sun, et al., 2012; Liu, Li, et al., 2012; Masuda et al., 2009; Pan et al., 2012; Zha, Luo, Luo, & Jiang, 2007; Zou, Zhang, Yao, Niu, & Gao, 2010). Presence of both α- and β-glycosidic bonds was identical with the results from the FT-IR spectra.

3.2. In vivo antitumor activity of FAAP-02

S180 tumor-bearing mice were used to evaluate the antitumor activity of FAAP-02 *in vivo*. As shown in Table 1, FAAP-02 inhibited the growth of the transplanted tumor dose-dependently, with the inhibitory rates of 28.37%, 30.58%, and 38.58% at the respective dose of 50, 100, 200 mg/kg/day. Simultaneously, FAAP-02 could significantly prolong the survival time of the mice, showing the life prolongation ratios of 14.82%, 52.53%, 55.67% at the respective dose of 50, 100, 200 mg/kg/day. As expected, 5-FU, a frequently used chemotherapeutic drug, showed a higher tumor inhibition rate of 46.22%. Nonetheless, the survival time of the mice treated with 5-FU was shorter than that of the FAAP-02-treated mice (at the doses of 100 and 200 mg/kg/day). In addition, either FAAP-02 or 5-FU alone showed much lower tumor inhibitory rate and life prolongation rate than the combination use of FAAP-02 and 5-FU. These results indicated that FAAP-02 exhibited significant antitumor activity *in vivo*, and a synergistic antitumor effect of FAAP-02 was also observed when it was combined with 5-FU to treat the mice.

3.3. Immune response of FAAP-02

The immune system has the capacity either to prevent tumor development and restrain established tumors, or to promote tumorigenesis, tumor progression and metastasis, which depends on the balance between the pro- and anti-tumor mediators of both innate and adaptive immune (Byun et al., 2010). Cancer arises more easily in a background of immunodeficiency (Hadden, 2003). Recently, enhancing the human body's immune functions in defending against tumors has become more and more popular (Li et al., 2012; Xu et al., 2009). Many polysaccharides have been proven to be potent antitumor and immunostimulating agents, and their antitumor activity might be achieved by improving immunity (Bai et al., 2012; Bohn & BeMiller, 1995; Han et al., 2001; Leung, Liu, Koon, & Fung, 2006; Liu et al., 2006; Park, Lai, & Kim, 2004). In light of this, the potential immunostimulating effects of FAAP-02 were further assessed here.

Thymus and spleen are major immune organs. The effects of FAAP-02 on the thymus and spleen indices in S180 tumor-bearing mice are shown in Fig. 5. Compared to the normal control, the thymus and spleen indices of the tumor-bearing mice were significantly decreased ($P < 0.05$), suggesting that the thymus and spleen of the mice were probably damaged and diminished due to the tumor transplantation (Fan et al., 2012). Intraperitoneal administration of FAAP-02 could obviously increase the thymus and spleen indices in a dose-dependent manner, while the 5-FU administration significantly decreased these indices as compared with the model control ($P < 0.05$). These results indicated that the innate immunity of the tumor-bearing mice was activated by FAAP-02, and the immune organs were protected well from the grievous damage,

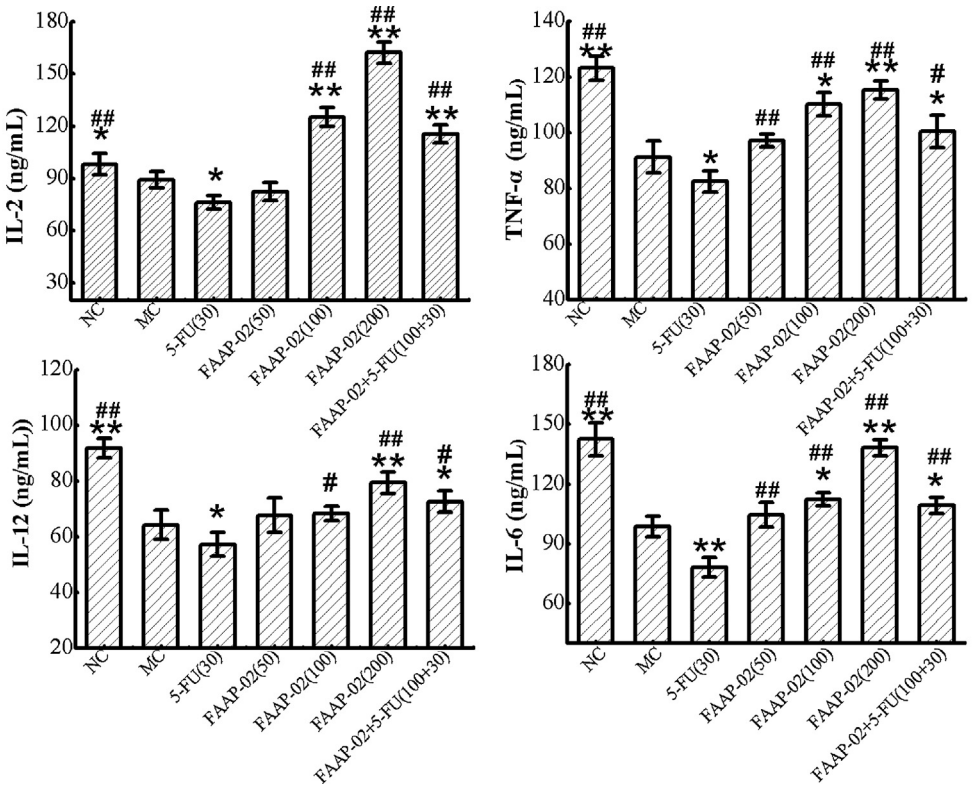

Fig. 6. Effects of FAAP-02 on cytokine production in serum of S180 tumor-bearing mice. S180 tumor-bearing mice were administrated intraperitoneally with FAAP-02 (50, 100, 200 mg/kg/day), 5-FU (30 mg/kg/day), FAAP-02 (100 mg/kg/day) in combination with 5-FU (30 mg/kg/day) for seven consecutive days, once daily. Model control and normal control groups received the same volume of normal saline. Day 8, the concentrations of IL-2, IL-6, IL-12 and TNF-α in the serum of the mice were determined by ELISA kits. Each value is presented as means ± S.D. ($n = 4$). NC: the normal control; MC: the model control. Significant differences from the model control were designated as $^{*}P < 0.05$, $^{**}P < 0.01$. Significant differences from the 5-FU group were designated as $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

whereas 5-FU with a high tumor inhibition rate further damaged the immunity of the mice (Wang et al., 2012a,b). Interestingly, after the administration of the mixture of FAAP-02 with 5-FU, the thymus and spleen indices were increased greatly as compared with either the FAAP-02-treated group (at the dose of 100 mg/kg/day) or the 5-FU-treated group. These results implied that FAAP-02 could restore the thymus and spleen indices decreased by 5-FU to some extent.

IL-2, IL-6, IL-12 and TNF-α play a prominent role in both immunoregulation and antitumor mechanisms, and have been extensively tested *in vitro*, *in vivo*, as well as in clinical practice for the immunotherapy of malignant diseases (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006; Byun et al., 2010; Hiraki et al., 2002; Kimura & Yamaguchi, 1995; Naugler & Karin, 2008; Neurath & Finotto, 2011). In this study, the effects of FAAP-02 on the secretion of serum IL-2, IL-6, IL-12 and TNF-α in S180 tumor-bearing mice were investigated by using ELISA kits. As shown in Fig. 6, the serum levels of IL-2, IL-6, IL-12 and TNF-α in the mice were significantly decreased owing to the transplanted tumor ($P < 0.05$). FAAP-02 significantly augmented the secretion of serum IL-2, IL-6, IL-12 and TNF-α as compared with the model control, especially at the doses of 100 mg/kg/day and 200 mg/kg/day ($P < 0.05$). On the contrary, the levels of serum IL-2, IL-6, IL-12 and TNF-α in the 5-FU-treated group were significantly lower than those of the model control ($P < 0.05$).

Table 2
Effects of FAAP-2 on CD4+ and CD8+ splenic T lymphocyte subsets in S180 tumor-bearing mice.

Group	Dosage (mg/kg/day)	Cell proportion (%)	
		CD4+	CD8+
NC	–	$25.00 \pm 1.80^{\#\#}$	$12.50 \pm 0.46^{\#\#}$
MC	–	22.17 ± 1.86	11.73 ± 0.23
5-FU	30	$15.33 \pm 1.42^{**}$	$8.20 \pm 0.30^{**}$
FAAP-02	50	$24.23 \pm 1.36^{\#\#}$	$11.57 \pm 0.35^{\#\#}$
	100	$28.93 \pm 0.67^{**,\#\#}$	$12.90 \pm 0.20^{**,\#\#}$
	200	$29.87 \pm 1.16^{**,\#\#}$	$13.17 \pm 0.35^{**,\#\#}$
FAAP-02 + 5-FU	100 + 30	$26.17 \pm 0.95^{*,\#\#}$	$12.13 \pm 0.45^{\#\#}$

S180 tumor-bearing mice were administrated intraperitoneally with FAAP-02 (50, 100, 200 mg/kg/day), 5-FU (30 mg/kg/day), FAAP-02 (100 mg/kg/day) in combination with 5-FU (30 mg/kg/day) for seven consecutive days, once daily. Model control and normal control groups received the same volume of normal saline. Day 8, the proportions of CD4+ and CD8+ splenic T lymphocytes were examined by the fluorescence-activated cell-sorting (FACS) method. Each value is presented as means ± S.D. ($n = 4$). NC, the normal control; MC, the model control.

* Significant differences from the model control were designated as $P < 0.05$.
** Significant differences from the model control were designated as $P < 0.01$.
$^{\#}$ Significant differences from the 5-FU group were designated as $P < 0.05$.
$^{\#\#}$ Significant differences from the 5-FU group were designated as $P < 0.01$.

However, when FAAP-02 was mixed with 5-FU, the serum levels of these cytokines were increased greatly. These results demonstrated that FAAP-02 could be potent inducers of IL-2, IL-6, IL-12 and TNF-α, and the mixture of FAAP-02 with 5-FU could restore these cytokine levels reduced by 5-FU to a certain degree.

CD4+ and CD8+ T lymphocytes are the respective primary helper and effector cells in adaptive immune response, and many immunotherapy strategies are aimed at stimulating these cells to facilitate destruction of tumor cells and long-term immune memory against recurrence of primary disease or outgrowth of metastases (Suzanne, 2008). The effects of FAAP-02 on the change of immune response with respect to the expression of CD4+ and CD8+ splenic T lymphocytes were also examined here by the fluorescence-activated cell-sorting (FACS) method. As shown in Table 2, the proportions of CD4+ and CD8+ T lymphocytes in the tumor-bearing mice were increased by FAAP-02 dose-dependently, but only displayed significant difference at the doses of 100 mg/kg/day and 200 mg/kg/day compared with the model control ($P < 0.01$). On the contrary, the mice treated with 5-FU displayed much lower percentages of CD4+ and CD8+ T lymphocytes when compared with the model control ($P < 0.01$). However, the mixture of FAAP-02 with 5-FU administrated to the tumor-bearing mice significantly increased the proportions of CD4+ and CD8+ T lymphocytes relative to the 5-FU-treated group ($P < 0.01$). These results indicated that FAAP-02 could enhance the expression of CD4+ and CD8+ T lymphocytes, and the combination of FAAP-02 and 5-FU could reverse the decrease of CD4+ and CD8+ T lymphocytes induced by 5-FU.

All these results revealed that administration of FAAP-02 could alleviate both tumor- and 5-FU-induced immunological dysfunction by improving the host immune response. Immunosuppression is considered as one of the mechanisms by which immunogenic tumors are enabled to escape from host immune surveillance, grow progressively, and metastasize. Tumors can evade destruction through inhibiting immune response directly. Therefore, enhancement of the host immune responses in tumor-bearing host could restore the dynamic balance between the tumor cells and the immune response, and consequently exert potential antitumor effects (Li et al., 2011). Thus, the antitumor activity of FAAP-02 might be achieved by improving the host immune response. In present study, the combination of FAAP-02 and 5-FU exhibited a synergistic antitumor effect *in vivo* and the mechanism by which FAAP-02 took effect would be in consequence of its antagonizing the side effects of the synthetic chemotherapy drug, as well as being achieved by enhancing the immune functions in the tumor-bearing mice.

4. Conclusion

In conclusion, FAAP-02, the novel heteropolysaccharide with a molecular weight of 5169 Da from *A. argyi*, not only significantly inhibited the growth of tumor with or without 5-FU *in vivo*, but also obviously restored the immunity of the mice which was suppressed by the transplanted tumor or/and 5-FU. These results suggested that FAAP-02 had clear antitumor and immunomodulatory activities *in vivo*, and immumostimulating effects might be one of the underlying mechanisms of its antitumor activity. However, further investigation will be needed to clarify the relationship between the antitumor and immunomodulatory activities.

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