Anti-tumor and Immunostimulatory Functions of Two Feruloyl Oligosaccharides Produced from Wheat Bran and Fermented by *Aureobasidium pullulans*

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Feruloyl oligosaccharide 1 (FO1) and feruloyl oligosaccharide 2 (FO2) were produced from wheat bran fermented by *Aureobasidium pullulans (A. pullulans)* through one- and two-stage temperature and pH controlling processes, respectively. Here the anti-tumor and immunostimulatory functions of FO1 and FO2 were further examined. Both FO1 and FO2 inhibited the growth of cancer cells but were non-toxic to normal cells *in vitro*. In S180 tumor-bearing mice, both FO1 and FO2 significantly inhibited the growth of transplanted tumors and promoted thymus, spleen indexes, interferon-γ, and interleukin-3 production. They also increased peripheral leukocyte count and bone-marrow cellularity. The biological activity of FOs prepared by different processes was further determined. Interestingly, FO2 possessed more potent anti-tumor and immunostimulatory effect than FO1 in a dose-dependent manner. At a dose of 250 mg/kg, the tumor inhibition rates for FO1 and FO2 were 22.42% and 44.85%, respectively. These antitumor properties of FOs may be mediated by their beneficial effects on immunity responses. These data suggest that FOs from wheat bran may be used as anti-tumor agents in the future.

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INTRODUCTION

Feruloyl oligosaccharides (FOs) have widespread presence in gramineous plants. FOs are formed by the carboxyl esterification of ferulic acid (FA) and sugar hydroxyls (Yuan 2006). They are water-soluble and heat-resistant due to their molecular structures, *i.e.*, ferulic acyl and hydrophilic oligosaccharides (Yuan 2006; Xie 2010). Previous research has focused on the preparation methods and the antioxidant activity of FOs (Yuan *et al*. 2006; Ou *et al*. 2007; Wang *et al*. 2010; 2011). For example, Xie (2010) and Ohta *et al.* (1994) reported that FOs exhibited a better antioxidant activity than ferulic acid and dietary fibers. Yuan *et al*. (2006) found that FOs had a strong ability to remove Fe^{2+}, H_2O_2, and hydroxyl-free radicals. In addition, research indicated FOs also inhibit the growth of *Staphylococcus aureus* (Yuan 2006), induce proliferation of *Bifidobacterium* (Yuan *et al*. 2005), inhibit non-enzymatic glycosylation of protein (Yuan

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2006), and stimulate immunity (Yuan 2006; Akhtar *et al*. 2012). Xie (2010) showed that FOs with a polymerization degree of 3 to 6 could significantly limit the growth of human colon cancer cell HCT-116 *in vitro*. However, to date, there has been no literature report that focused on the in vivo anti-tumor properties of FOs.

The biological functions of FOs are closely related to their purity and structure (Zhi *et al*. 2000; Yuan 2006; Xie 2010). Wheat bran (WB) is an important source of FOs (Yuan *et al*. 2005). Previous research by the authors showed that component purity or the composition of feruloyl arabinoxylans (FAXs), degree of polymerization or the number of xylose, and structure of FOs can be modulated by *A. pullulans*'s fermentation processes (Yu *et al*. 2013, 2014a). However, the manner in which the component purity and degree of polymerization of FOs affect their antitumor function *in vivo* is still unknown.

In previous work by the authors, two components (FO1 and FO2) were produced from wheat bran fermented by *A. pullulans via* one- and two-stage pH and temperature controlling processes, respectively (Yu *et al*. 2013, 2014a). In the current study, the WB FOs' anti-tumor and immunostimulatory functions were investigated *in vivo* and *in vitro*. Moreover, the effects of purity and degree of polymerization of FOs on the anti-tumor and immunostimulatory functions of the two FOs were determined. Because FOs can be produced easily and abundantly from *A. pullulans*-fermented WB, it is feasible to use FOs from WB as a potential anti-tumor component in functional foods. Therefore, this study provides both a theoretical basis and a practical method to develop FOs as a potential functional component of food.

EXPERIMENTAL

Materials

Raw materials and reagents

Commercial fresh WB was supplied by Qinda Flour Co. (Yancheng, Jiangsu, China) and stored at 4 °C until use. Fluorouracil injection (5-FU; batch number: 1111262; specifications: 10 mL, 0.25 g) was from Hengrui Medicine Co. (Lianyungang, Jiangsu, China). Media (RPMI-1640, IMDM, and F-12) were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS), 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT), ethylene diamine tetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), hematoxylin (H), and eosin (E) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cancer cell lines

Human lung adenocarcinoma cells A549, human gastric cancer cells BGC-823, and human hepatoma cells HepG2 were purchased from the Cell Bank and Tumor Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Sarcoma S180 cells were provided by Jiangsu Provincial Institute of Cancer Research (Nanjing, Jiangsu, China). Human normal liver cells L-02 and human normal stomach cells GES were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Animals

Five-week-old female ICR mice, grade clean with weight of 18 to 22 g, were purchased from the Comparative Medicine Centre of Yangzhou University (Yangzhou, Jiangsu, China; Certificate No. SCXK-2007-0001). They were housed for three days under pathogen-free conditions at 18 to 24 °C and a relative humidity of 70% on a 12 h light/12 h dark cycle. All experiments were carried out according to the governmental legislation of China for the use and care of laboratory animals and were approved by the Bioethics Committee of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (Beijing, China).

Methods

Preparation of FO1 and FO2

Dried WB was crushed into flour, passed through a 40-mesh sieve to remove starch, and dissolved in distilled water. The pH of the mixture was adjusted to 5.5 with the addition of 2% (v/v) sulfuric acid to a solution containing 60 g/L WB, 10 g/L xylan and 1 g/L peptone. The mixture was incubated at 50 °C for 2 h. The resulting WB solution was used as the fermentation medium for *A. pullulans* 2012 (Yu *et al*. 2014b). The FO1 and FO2 were prepared by one- and two-stage pH and temperature processes to control the WB fermentation by *A. pullulans* 2012, respectively (Yu *et al*. 2013, 2014a). A yield of 904 nmol of FO1 per liter of fermentation broth was obtained under optimal conditions of initial pH 6.0, inoculation quantity 4.50%, and fermentation temperature 29 °C. FO2 yield via the two-stage fermentations reached 1123 nmol/L after fermentation for 96 h by controlling the initial pH at 4.0 and temperature at 33 °C, and at 36 h of fermentation changing the pH to 6.0 and temperature to 29 °C. This process was 12 h shorter than one-stage fermentation for producing FO1. The structures of FO1 and FO2 are shown in Fig. 1. The FO1 structure was reported in Yu *et al*. (2014a). FO2 was characterized by gas chromatography (GC), infrared spectroscopy (IR), and electrospray ionization mass spectrometry (ESI-MS) (Yu *et al*. 2014a). It was demonstrated that FO2 contained feruloyl arabinosyl xylohexose (FAX6, MW1118), feruloyl arabinosyl xylopentose (FAX5, MW986), and feruloyl arabinosyl xylotetraose (FAX4, MW854).
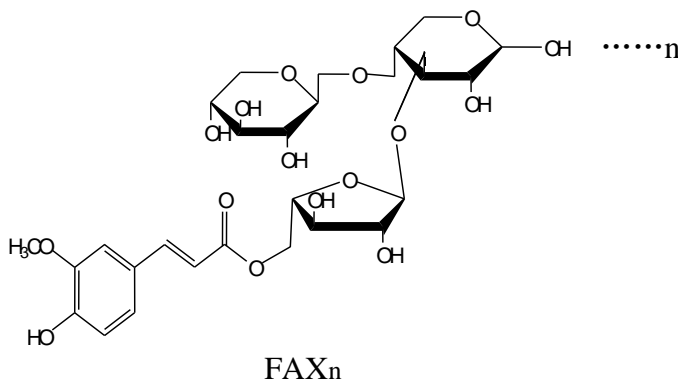

FAXn

Fig. 1. Molecular structures of feruloyl arabinoxylans (FAXs). FO1 consists of feruloyl arabinosyl xylopentose (FAX5), feruloyl arabinosyl xylotetraose (FAX4), feruloyl arabinosyl xylotriose (FAX3) and feruloyl arabinosyl xylobiose (FAX2). FO2 consists of feruloyl arabinosyl xylohexose (FAX6), FAX5 and FAX4.

Anti-tumor activity test for FOs in vitro

BGC-823 and HepG2 cells were cultured in RPMI-1640 medium with 10% FBS. A549 cells were cultured in F-12 nutrient mixture medium with 10% FBS, and L-02 and GES cells cultured in IMDM medium with 10% FBS. BGC-823, HepG2 and A549 cells in logarithmic phase were digested with 0.02% EDTA to count the living cells. They were cultured in 96-well flat-bottom plates with 100 μL 1x10^6 cells/mL suspension, incubated (in triplicate) in media containing 1000 μg/mL to 7.8 μg/mL of FO1 and FO2, at 37 °C in an atmosphere containing 95% ambient air and 5% CO_2. An equal volume of serum medium without added FOs was added to the cells as the control group. A final dose of 100 μg/mL 5-FU solution was added as the positive group. The medium, MTT, and DMSO together were used as the zero control. The absorbance value was measured by enzyme-labelling measuring instrument (TECAN Infinite M200, Austria) at 570 nm. The inhibition rate of the cells was calculated by the formula: (%) = (1-T/C)×100, where T and C are the average absorbance of the added FOs groups and control groups, respectively. BGC-823, HepG2 and A549 cells in logarithmic phase were digested with 0.02% EDTA to count the living cells. They were cultured in 6-well flat-bottom plates with 1x10^6 cells/mL suspension. Media containing 500, 1000, and 1500 μg/mL of FO1 and FO2 were introduced into the wells containing adherent cells that had been cultured for 12 to 24 h. The group without FOs was set up as the blank. After incubation at 37°C under 5% CO_2 for 24 h, the morphological change of cells was observed with an inverted microscope and then photographed at 20 × (Wang *et al*. 2009; Wang *et al*. 2011).

Solid tumor development and drug administration

The ICR mice were inoculated with solid tumors according to the portable tumor approach (Xu *et al*. 2009; Cao *et al*. 2011). Each mouse was inoculated in the right thigh of the lower limb with 0.2 mL of S180 cells and weighed after 24 h. They were randomized into five groups, with eight mice in each group. Dose and administration of physiological saline for normal control (mice without tumor cells inoculated) and model control (mice inoculated with tumor cells) was 0.4 mL/20 g of body weight/d administered orally. The positive control was 5-FU intraperitoneally administrated once every other day at 0.4 mL per 20 g of body weight (five times in total). The treatment dose, dissolved in sterile, 0.5% (w/v) CMC-Na, was administered orally at 50, 100, and 250 mg/kg of body weight/d for the FO1 and FO2 groups. The first oral dose was administered after 24 h (d_1) of the inoculation of tumors and repeated once daily for a total of 10 times.

Effect of FOs on tumor growth, weight gaining rate, thymus and spleen indexes, bone-marrow cell count, and peripheral white blood cell count (WBC)

Mice were weighed and sacrificed by cervical dislocation on Day 11. Solid tumors, thymus, and spleen were each collected and weighed under aseptic manipulation, and were stored in an ice bath. The inhibition rate of tumor growth (%) was calculated according to the formula: (%) = (1-T/C)×100, where T and C are the average tumor weight (g) of the added FOs treatment groups and the model control groups, respectively. The weight gaining rate was calculated as (%) = [(Z-I)/I]×100, where I and Z are the initial and final weight of the mice, respectively. The thymus and spleen indexes were expressed as the thymus or spleen weight (mg) relative to its body weight (g). Bone marrow was collected from the left femur bones by flushing thoroughly with Hank's Balanced Salt Solution (HBSS) using a 1-mL syringe equipped with a 28-gauge needle.

The cells were collected in a sterile tube and diluted to 10 mL in HBSS. Bone marrow cell concentration was determined by an Innovatis CASY Model TT cell counter (Roche Innovatis AG; Bielefeld, Germany) (Cao *et al*. 2011). The blood samples were harvested by enucleating eyes of mice on Day 11, and mixed with 2.5 mg/mL EDTA. The peripheral white blood cell count (WBC) were determined using a Sysmex XE-2100 hematology analyzer (Sysmex Corporation; Kobe, Japan).

Histological tumor observation

Tumors of the mice were fixed in a 10% neutral buffered formalin solution. After the tumors were processed and embedded in paraffin, 4-mm sections were prepared and stained with hematoxylin and eosin (H&E) for microscopic observation.

Effect of FOs on helper T (Th) cytokines analysis

The concentration of cytokines including IFN-γ and IL-3 was detected by specific enzyme-linked immunosorbent assay (Diaclone, Rat IFN-γ ELISpot Kit; France). The amount of IFN-γ and IL-3 was measured by spectrophotometry at 450 nm using an enzyme-labelling measuring instrument (TECAN Infinite M200, Austria).

Statistical analysis

Experimental data were expressed as the mean ± SD. All the data were at least triplicated. The SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for ANOVA statistical analysis. When $P < 0.05$, the difference was considered to be statistically significant.

RESULTS AND DISCUSSION

Anti-tumor Property of FOs *in vitro*

Both FO1 and FO2 showed a significant ($p < 0.05$) inhibitory property against human liver cells HepG2 (Fig. 2a), human gastric cancer cells BGC-823 (Fig. 2b), and human lung adenocarcinoma cells A549 (Fig. 2c) *in vitro*. The tumor inhibition rate increased with increased FO dose (Figs. 2a, 2b, and 2c). When the concentration of FOs reached 1000 μg/mL, tumor cell growth was inhibited by approximately 50%. The inhibition effect of 5-FU, positive control, against cancer cell proliferation was good, but its toxic impact on normal cells was also notable. When normal cells were administered FO1 and FO2, only the group dosed with 1000 μg/mL FO2 showed inhibitory effect on cell proliferation. Specifically, at 1000 μg/mL FO2 the inhibition rate against the human normal liver cell L-02 was 10.9 ± 0.6% (Fig. 2d), and the human normal stomach cell GES was 14.3 ± 0.4% (Fig. 2e). FO dose below 500 μg/mL did not show inhibitory effect on cell growth. By contrast, they even promoted the normal cell proliferation. This result indicates that FO1 and FO2 not only have an inhibitory effect on tumor-cell proliferation, but also are not toxic to normal cells. The toxic difference between tumor cells and normal cells by FOs may be caused by the induction of certain signaling molecules inside tumor cells.

Morphologically, it was shown that cancer cells without FOs, the control, had vigorous growth, clear cell outlines, compact connections, and attached growth (data not shown). When treated with FO1 and FO2, cancer cells failed to maintain the intercellular contacts and became rounded. Smaller cell sizes, indicative of nonviable cells, resulted

from an increased FOs dose. When the dose of FO1 and FO2 reached 500 μg/mL, attachment of the cancer cells decreased and the cells floated and became rounded, indicating that the cells had died. At 1500 μg/mL, the inhibitory effect of FOs was even more obvious. Hence, these data suggest FO1 and FO2 can inhibit the activities of human liver cell HepG2, human gastric cancer cell BGC-823 and human lung adenocarcinoma A549 cancer cells *in vitro.*
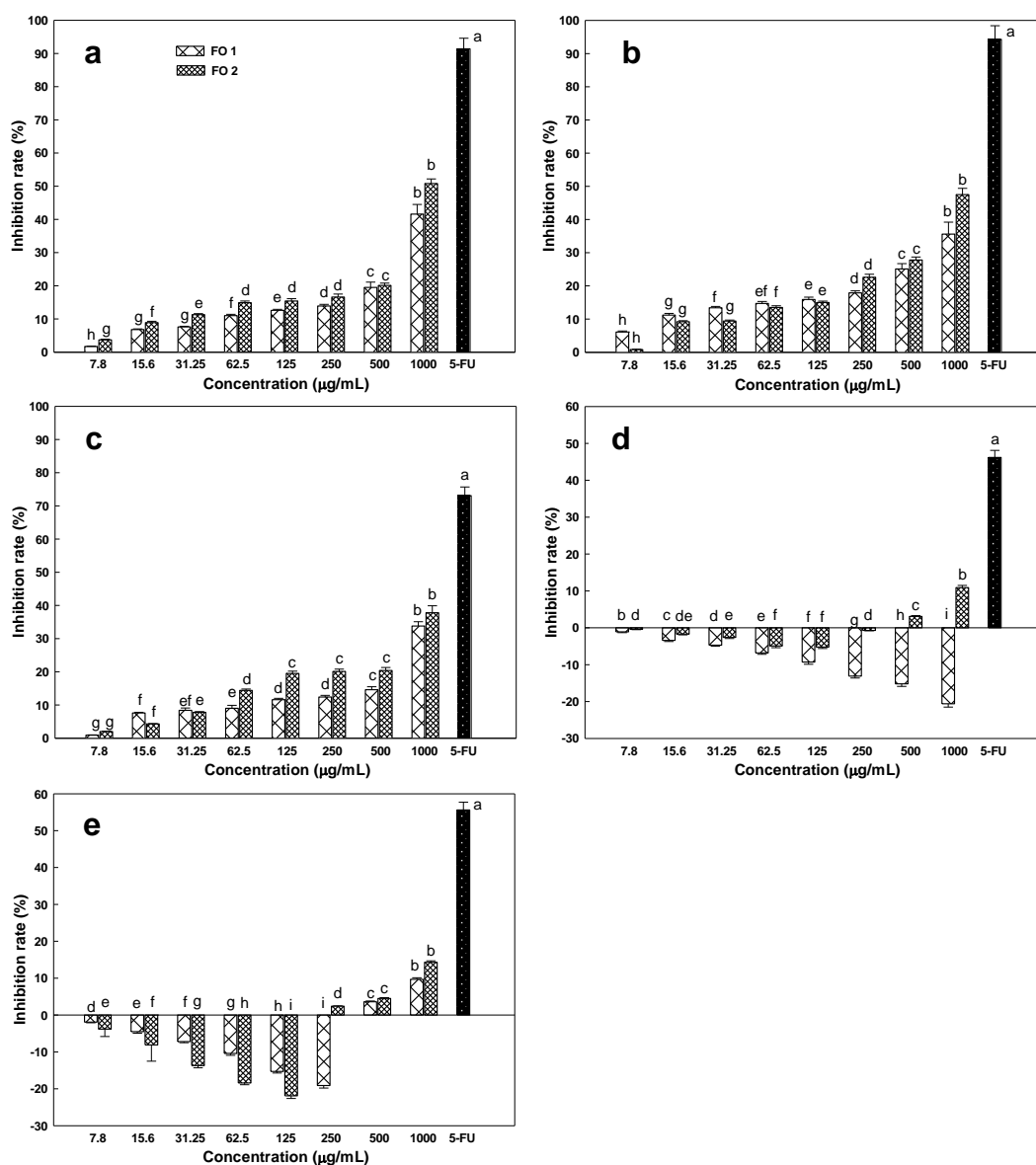

Fig. 2. Inhibitory effects of FOs on (a) human hepatoma cells HepG2, (b) human gastric cancer cells BGC-823, (c) human lung adenocarcinoma cells A549, (d) human normal liver cells L-02, and (e) human normal stomach cells GES. Both FO1 and FO2 dose ranged from 7.8 μg/mL to 1000 μg/mL. The positive control, 5-FU, was used at 100 μg/mL. Different letters within the same treatment indicate a statistical difference ($P < 0.05$).

Anti-tumor Function of FOs *in vivo*

All three doses (50, 100, and 250 mg/kg body weight) of FO2 had significant ($P <$ 0.05) inhibitory effect against solid tumor formation in S180 tumor-bearing mice, with inhibitory rates of 29.39%, 34.85%, and 44.85%, respectively (Table 1). Treatment with the positive control (5-FU) showed the highest tumor inhibitory effect (62.12%), with a tumor weight significantly ($P <$ 0.05) lower than that of FO1 at the same dose. But the body weight of mice decreased. Collectively, FO2 showed a more efficient anti-tumor activity than FO1. In addition, no toxic symptoms were observed in treatments with FOs (Table 1).

Table 1. Inhibitory Effects of FOs on Sarcoma S180 in Mice

Group	Dose (mg/kg)	Weight gaining rate (%)	Tumor weight (g)	Tumor inhibiting rates (%)
Blank control	—	19.63±1.46 [b]	—	—
Model control	—	16.47±0.91[b]	1.10±0.18 [a]	—
FO1	50	26.03±1.92 [a]	0.97±0.18 [ab]	11.67±0.16 [d]
	100	24.98±3.28 [a]	0.92±0.09 [abc]	16.36±0.08 [cd]
	250	25.12±3.70 [a]	0.85±0.07 [bc]	22.42±0.06 [cd]
FO2	50	25.34±2.76 [a]	0.78±0.11 [bcd]	29.39±0.10 [bcd]
	100	18.93±1.78 [b]	0.72±0.13 [cd]	34.85±0.11 [bc]
	250	18.26±1.28 [b]	0.61±0.08 [de]	44.85±0.07 [ab]
5-FU	30	5.95±1.72 [c]	0.42±0.08 [e]	62.12 ±0.07 [a]
Different letters in the same column indicate a statistical difference ($P <$ 0.05)				

Figure 3 displays the histological morphology of stained tumor tissue. The model control (Fig. 3a) was a large hyperchromatic tumor group, with vigorous tumor growth. The positive control of 5-FU (Fig. 3b) had a number of white cavities with holes suggesting tumor cells apoptosis. However, there was no red dye structure in the 5-FU treatment (Fig. 3b). FO1 group at a dose of 50 mg/kg (Fig. $3c_1$) appeared to contain some net-like eosinophilic areas and a few white cavities, but had no red-stained structure. Apoptotic cells have a distinctive morphological appearance, such as shrinking and chromatin condensation. The cells with white cavities were identified as apoptotic cell bodies with holes. The presence of apoptotic cells under FO1 treatment were less frequently found than the positive control. At a FO1 dose of 100 mg/kg (Fig. $3d_1$), tumor cells showed the mass of necrosis and white cavities but no red dye structure. The rate of tumor cell apoptosis in FO1 at 100 mg/kg group was higher than FO1 at 50 mg/kg, but less than the positive control. When FO1 dose increased to 250 mg/kg (Fig. $3e_1$), a number of eosinophilic areas and white cavities showed, indicating the mass necrosis of tumor cells mixed with disintegration, and degeneration of nucleoli. In contrast, for FO2, at a dose of 50 mg/kg (Fig. $3c_2$), a large tumor group developed in the middle of cells, with eosinophilic areas and a few white cavities on the edge, perhaps caused by inflammation. Thus, a few apoptotic cells were found and some of them were phagocytized by other tumor cells. Increasing FO2 dose to 100 mg/kg (Fig. $3d_2$) caused only a little tumor group, but a number of white cavities, indicating tumor cell disintegration, and degeneration of nucleoli. When FO2 was further increased to 250 mg/kg (Fig. $3e_2$), a large area of red dye and a few white cavities were observed,

indicating tumor cell apoptosis. In general, at 250 mg/kg, FO2 was more effective in inhibiting tumor growth than FO1.
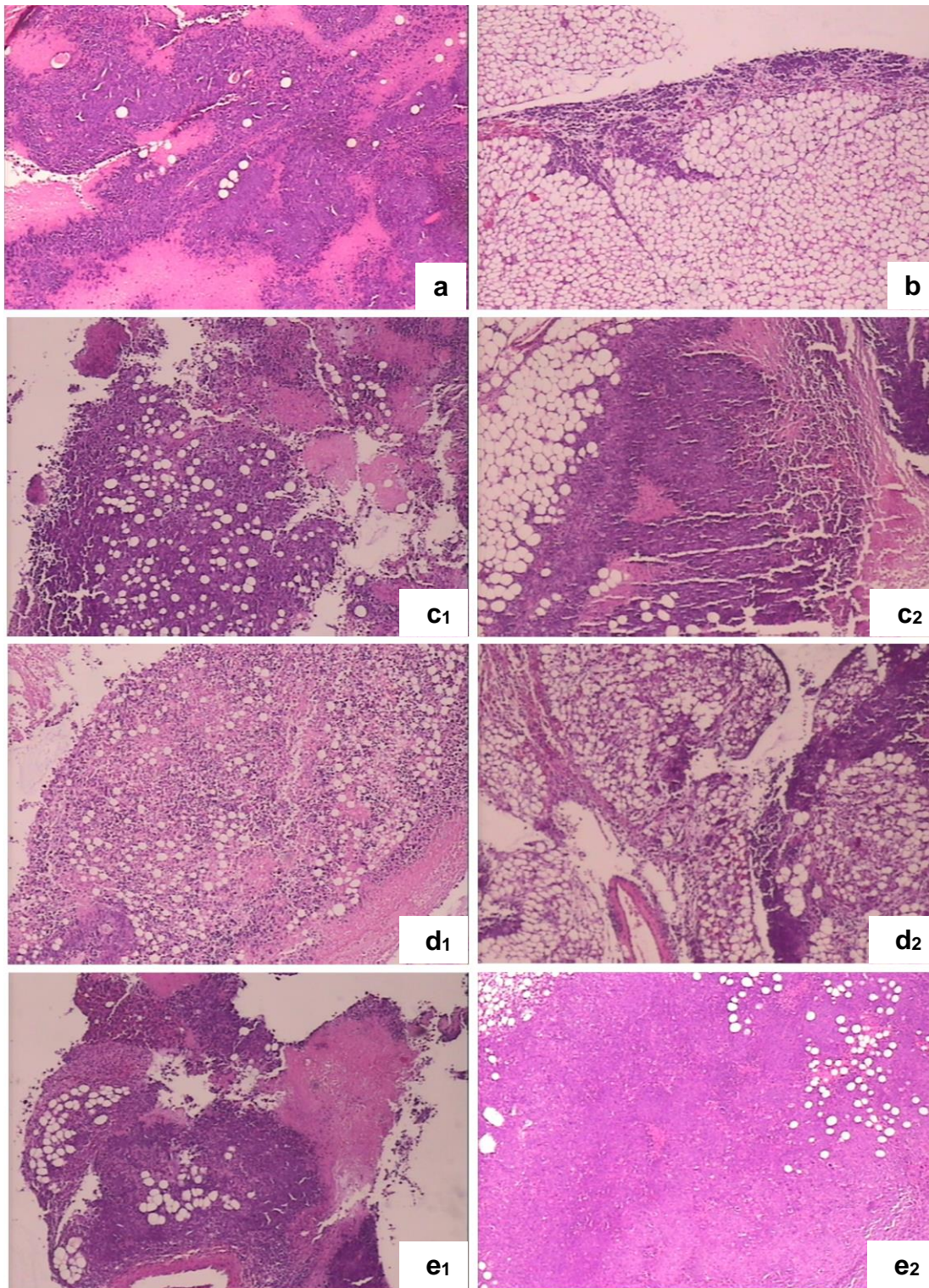

Fig. 3. Histological morphology of tumor tissue. (a) Model control, (b) Positive control, (c$_1$) FO1, 50 mg/kg, (d$_1$) FO1, 100 mg/kg (e$_1$) FO1, 250 mg/kg, (c$_2$) FO2, 50 mg/kg, (d$_2$) FO2, 100 mg/kg, and (e$_2$) FO2, 250 mg/kg

Immunity Functions of FOs in S180 Tumor-bearing Mice

The immunopotentiation property of FO1 and FO2 on S180 tumor bearing mice was supported by the fact that spleen indexes of mice bearing sarcoma were higher than those of blank control group, except for the 5-FU group (Table 2). Spleen indexes of FO2 at 250 mg/kg were significantly lower ($p < 0.05$) than those of FO1, indicating that FO2 was more efficient than FO1 in improving immunity function of the mice bearing sarcoma S180.

All the thymus indexes of mice bearing sarcoma S180 either with or without FO treatment were lower than that of the blank control group. Thymus indexes increased with FO doses (Table 2). Thymus indexes of FO2 at 100 and 250 mg/kg did not show significant differences from blank control group, but were significantly higher ($P < 0.05$) than those of FO1 at the same dose. Thus, FO2 had more effects on immunity adjustment than FO1. Thymus index of positive control, 5-FU group, was significantly lower ($P < 0.05$) than that of other groups, suggesting the thymus was atrophied.

Table 2 shows that IFN-γ and IL-3 contents of mice bearing sarcoma S180 fed with FO1 and FO2 were higher than those of model control group, especially when the dose reached 100 mg/kg, indicating that both FO1 and FO2 improve the immunity function of mice bearing sarcoma S180. When the dose of FO2 reached 250 mg/kg, IFN-γ and IL-3 contents were significantly higher than those of positive control 5-FU and FO1 feeding group. It was therefore concluded that FO2 was more efficient in modulating immunity function for the mice than FO1.

Table 2. Effects of FOs on the Immunity Functions of the Tumor-bearing Mice

Group	Dose (mg /kg)	Spleen index (mg/g)	Thymus index (mg/g)	IFN-γ concentration (pg/mL)	IL-3 concentration (pg/mL)
Blank control	—	7.70±1.09 [bc]	6.50±0.42 [a]	51.42 ±8.60 [a]	37.23±8.51 [def]
Model control	—	9.50±0.84 [ab]	4.30±0.56 [c]	1.06±7.67 [d]	26.60±8.51 [f]
FO1	50	11.34±1.89 [a]	4.37±0.68 [c]	9.57±6.38 [cd]	32.98±12.77 [ef]
	100	10.87±1.20 [a]	4.54±0.50 [c]	20.92±4.43 [bc]	47.87±6.38 [de]
	250	10.53±1.23 [a]	4.62±0.92 [bc]	24.47±4.26 [bc]	64.89±8.51 [bc]
FO2	50	10.82±1.29 [a]	4.92±0.53 [bc]	15.25±7.47 [bcd]	35.11±12.77 [def]
	100	9.14±0.65 [ab]	5.69±0.67 [ab]	26.60±8.51 [b]	52.13±8.51 [cd]
	250	7.92±0.79 [bc]	6.35±0.56 [a]	45.74±12.77 [a]	92.55±8.51 [a]
5-FU	30	6.30±1.72 [c]	1.70±0.33 [d]	26.60±6.38 [b]	73.40±8.51 [b]
Different letters in the same column indicate a statistical difference ($P < 0.05$)					

Effect of FOs on WBC Count and Bone-marrow Cellularity in S180 Tumor-bearing Mice

As shown in Table 3, the bone-marrow cell concentration in S180 tumor-bearing mice increased significantly at 250 mg/kg of both FO1 and FO2 treatment compared with the model control group ($P < 0.05$). The peripheral WBC cell concentration in S180 tumor-bearing mice also increased by 250 mg/kg of FO1, 100 and 250 mg/kg of FO2, but decreased significantly by 5-FU administration compared with model control group ($p <$

0.05). The effects of FO1 and FO2 on WBC count and bone-marrow cellularity in S180 tumor-bearing mice were not significantly different ($p > 0.05$).

Table 3. Effects of FOs on Peripheral White Blood Cell (WBC) Count and Bone-marrow Cell Count in S180 Tumor-bearing Mice

Group	Dose (mg/kg)	Femoral bone-marrow cell count ($\times 10^7$/mL)	WBC count ($\times 10^9$ cells/L)
Model control	—	4.37±0.99 [cd]	5.94±1.28 [c]
FO1	50	4.91±1.01 [bcd]	6.23±1.22 [c]
	100	5.73±1.53 [abc]	7.69±1.05 [abc]
	250	6.97±1.75 [ab]	8.65±0.81 [ab]
FO2	50	5.06±1.02 [abcd]	6.74±1.31 [bc]
	100	6.21±0.98 [abc]	8.27±1.01 [ab]
	250	7.32±1.13 [a]	9.18±1.03 [a]
5-FU	30	2.87±0.83 [d]	2.61±0.71 [d]
Different letters in the same column indicate a statistical difference ($P < 0.05$)			

In the present study, FO1 and FO2 not only inhibited the growth of human liver cells HepG2, human gastric cancer cells BGC-823, and human lung adenocarcinoma A549 cancer cells *in vitro*, but also inhibited tumor development and improved immunity function in the mice bearing sarcoma S180 *in vivo*. Moreover, pathologically they changed the histopathological profile of the tumor mass compared with model control group. Since FOs had no effect on normal human liver cells L-02 and stomach cells GES, it can be presumed that the anti-tumor activity of FOs was not due to cytotoxicity, but its inhibitory effect on cancer cell proliferation and immunostimulatory properties.

The normal immunity function can be compromised by the development and formation of tumors (Williams *et al*. 1996; Miao *et al*. 2013; Pan *et al*. 2013). The important immunity organs, spleen, and thymus, participate in modulating immunity function through their production of lymphocytes and macrophages (Williams *et al*. 1996). Tumors usually change the weight of immunity organs, while immunity promoters can restore the immunity organ weight to normal levels (Zheng 1999). Here, in the current study, the effect of FO1 and FO2 prepared by different processes on thymus and spleen index of mice bearing sarcoma was studied comparatively. For the chemotherapy drug 5-FU, the S180 tumor inhibition rate was higher than that of FO1 or FO2, but the mice lost weight, and the thymus and spleen index of the mice were significantly reduced. Thus while 5-FU could inhibit the growth of tumor cells, it simultaneously inhibited the growth of normal cells. The effects of 5-FU *in vitro* also showed the same results. For FO1 and FO2, they not only inhibited cancer cell growth in the mice bearing sarcoma S180, but simultaneously increased thymus and spleen indexes for the mice. Therefore, FO1 and FO2 might function as antitumor agents indirectly by improving the host's immunity function.

As important antineoplastic constituent of organisms, cytokine IFN-γ and IL-3 caused a change of cell behavior to enhance cell ability for killing tumor cells (Liu *et al*. 2003). In this study, FO1 and FO2 acted as immunity accelerators, which stimulated and recovered low immunity function of the mice bearing sarcoma S180 to a normal level, and effectively reversed the low level of IFN-γ and IL-3 due to tumor growth. These

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results indicate that FOs' anti-tumor effects may be mediated through improving the production of cytokine in the bodies.

The inhibition effect of FO2 on tumor was higher than that of FO1 at the same dose. Previous research showed that there is a close relationship among the biological activity of oligosaccharides and their compositions, degrees of polymerization, and structures (Katapodis *et al*. 2003; Chen and Yan 2005). For example, strong antioxidant activity was found in agar oligosaccharide with a polymerization degree of 6 (Chen and Yan 2005). The effects of xylo-oligosaccharides on proliferation of lactic acid bacteria and *Bifidobacterium* are different because of different polymerization degrees (Moura *et al*. 2007). For FOs, at a polymerization degree of 3 to 4, they were capable of inhibiting the growth of *Staphylococcus aureus* (Yuan 2006). Xie (2010) indicated that FOs with polymerization degrees of 3 to 6 could significantly inhibit the growth of the colon cancer cell line HCT-116. To date, data have revealed that polymerization degree of 2 to 8 is beneficial to the improvement of biological activity of oligosaccharides. In the present research, polymerization degree of FOs ranged from 2 to 6. For FOs, the inhibitory effect on tumor growth *in vivo* and *in vitro* was related with their purity (numbers of different compositions) as well, except polymerization degree. The purity and average polymerization degree of FO2 were higher than that of FO1. Although both of them had a significant inhibitory effect on tumor growth *in vivo* and *in vitro*, FO2 was more efficient than FO1. The current data imply that the different inhibitory function of FOs against cancer cells is related to the specific structure that FOs contain.

Peripheral WBC count and bone-marrow cellularity are two frequently studied clinical parameters that accurately reflect chemotherapeutic injury (Cao *et al*. 2011). In the present study, treatments with FOs increased peripheral WBC counts and bone marrow cellularity in S180 tumor-bearing mice. The current data further confirmed that FOs could stimulate the hemopoietic system and proliferation of stem cells. Oral administration of FOs also had an immunostimulatory effect, suggesting that the immunostimulatory activity of FOs might be caused by their metabolites. In the future, more detailed studies are required to further explore how FOs contribute to health and the biomolecular mechanisms underlying their antitumorous and immunostimulatory functions.

CONCLUSIONS

1. In this study, WB was fermented by *A. pullulans* through one-stage and two-stage controlling temperature and pH process, which produced FO1 and FO2, respectively. Results showed that both FO1 and FO2 significantly inhibited the *in vitro* growth of cancer cells and transplanted tumors in mice without toxicity to normal cells.

2. FO treatments increased the levels of IFN-γ, serum IL-3, WBC, and bone-marrow cellularity in tumor-bearing mice. At the same dose, FO2 exhibited more efficient antitumor functions than FO1.

3. Structure of FOs affects their immunity responses and functions. The antitumor activity of FOs might be mediated through an improvement in the immune response.

ACKNOWLEDGMENTS

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