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Antitumor Activity of an Acidic Heteropolysaccharide Isolated from *Auricularia auricula*-Judae

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Research Article

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ABSTRACT

A water soluble acidic heteropolysaccharide named wAF was isolated from *Auricularia auricula-judae*. It composed of a backbone of α- (1→3)-linked *D*-mannopyranose residues. WAF fractions exhibited strong inhibition against Acinar cell carcinoma proliferation *in vitro* test. It significantly inhibited tumour growth in dose-dependent way in BALB/c mice. Moreover, the enhancement ratios of mice body weight for all wAF fractions were higher than that for 5-Fluorouracil, which is a chemotherapeutic anticancer agent. Fluorescence microscopy and tumour tissues cell morphology observation indicated that wAF3 fraction induced apoptosis of tumour cell. Immunohistochemistry for apoptosis-related proteins Bax and Bcl-2 expression revealed that wAF3 induced S-180 tumour cell apoptosis by up-regulation of Bax and down-regulation of Bcl-2. These findings confirmed that the wAF polysaccharide had good anti-tumour activity and could be considered as a potential antitumor agent.

INTRODUCTION

Cancer is a leading cause of death worldwide resulting from a mutation in the chromosomal DNA of a normal cell. Polysaccharides have attracted more and more attention recently because of their antitumor and immunomodulation effects [1]. Many results show that polysaccharides have a broad spectrum of biological effects, such as antibiotic, anti-mutant, anticoagulant and immunostimulant activities [2,3].

Acidic polysaccharide composed of α-1→3-D-mannopyranose as backbone is known to constitute the major part of water soluble fraction of *Auricularia auricula-judae*, an ear- shaped edible fungus [4,5]. In addition to the acidic character of the polysaccharide, the mannose rich structure might have beneficial effects as biomedical agents and natural drugs. Some mannose-binding proteins are C-type lectins, found in animal cell surface [6]. These lectins have potential functions in immune surveillance by recognizing high mannose structures present on pathogenic organisms such as bacteria and fungi [7]. Various polysaccharides isolated from *Pleurotus tuber regium* [8], *Lentinus Edodes* [1], *Poria cocos* [9,10], *Ganoderma tsugae* mycelium [11], *Auricularia auricula-judae* [12,13] have been studied, focusing on the effects of the structure and chain properties such as flexibility, or multiplex formations on the bioactivities.

In our previous work, the structure of a water soluble acidic heteropolysaccharide isolated from *Auricularia auricula-judae* was studied. Its chemical structure was determined to be composed of a backbone of α-(1→3)-linked *D*-mannopyranose residues with pendant side groups of β-D-xylose, β-D-glucose or β-D-glucuronic acid at position 06 or 02 **(Figure 1)** [14].

It's the first time to reveal conformation and structure of the most abundant polysaccharide contained in *Auricularia auricula-judae* [15]. However, its antitumor bioactivities have never been reported.

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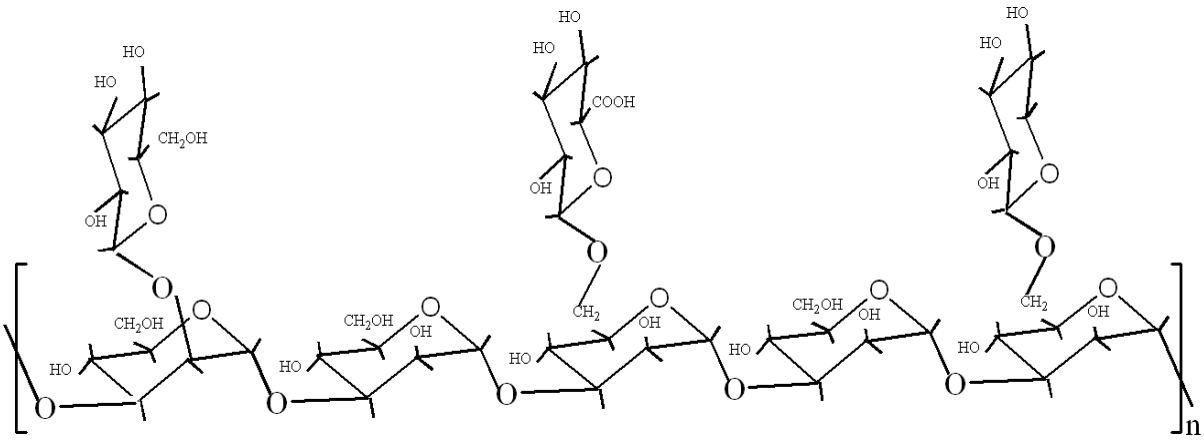

Figure 1. Molecular structure of polysaccharide wAF.

In the present work, *in vivo* antitumor activities of the polysaccharide wAF against xenograft Sarcoma 180 tumor cells and *in vitro* inhibition ratio to the proliferation of Acinar cell carcinoma(ACC) [16] tumor cells were evaluated. The mechanism of the antitumor activities of the wAF was investigated by the morphological approaches (fluorescence microscopy with Hoechst stain) and immunohistochemical method, attempting to elucidate the antitumor mechanism.

EXPERIMENTAL

Materials

Auricularia auricula-judae was obtained from Fangxian (Hubei Province, China). Acinar cell carcinoma [16] was provided by School of Stomatology, Wuhan University (Wuhan, China). Sarcoma 180 (S-180) was supplied by Department of Pharmacy, Tongji Medical College Huazhong University of Science and Technology (Wuhan, China). Male BALB/c mice were got from The Animal Centre, Tongji Medical College Huazhong University of Science and Technology (Wuhan, China).

Isolation and Fractionation

wAF was isolated from fruit bodies of *Auricularia auricula-judae* according to our previously reported method [14]. To obtain samples with different molecular weights, WAF was dissolved in pure water and ultrasonicated at room temperature with an ultrasonic cleaner (MUS/1004) for different time such as 0 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 16 h corresponding to the samples coded as WAF, WAF1, WAF2, WAF3, WAF4, WAF5, WAF6, and WAF7. The WAF sample and the sonicated fractions were finally dried with freeze-dried.

In vitro antitumor test

MTT method was used for *in vitro* antitumor test. Briefly, Acinar cell carcinoma (ACC) tumor cells (1×10^5 cells/mL) were grown in Roswell Park Memorial Institute (EPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) under atmosphere of 5% carbon dioxide at 37°C for 72 h. The concentrations of samples were 5 and 50 μg/mL in 0.9 % aqueous NaCl. The number of living ACC tumor cells at the end of the 72 h incubation period was determined colorimetrically based on the tetrazolium salt MTT as described by Mosmann [17]. 5-fluorouracil (5-Fu) was treated as positive control.

Fluorescence microscopy and apoptotic assessment

ACC tumor cells were treated with the wAF3 sample, and were incubated simultaneously in RPMI 1640 medium containing 10% fetal bovine serum (FBS) under an atmosphere of 5% CO_2 at 37°C. The cells were stained by Hoechst 33342 (10 mg/mL, Sigma) for 15 min in the dark, followed by washing twice with phosphate-buffered saline (PBS). They were observed under inverted phase fluorescence microscope (Olympus DP70, Tokyo, Japan) equipped with a 40×objective and UV filter cube for apoptotic assessment. Quantitative analysis was performed by counting more than 500 cells in six random fields per sample. Each assay was repeated at least three times.

In vivo antitumor test

8-week-old male BALB/c mice weighting 20±1 g were inoculated sarcoma 180 (S-180) tumor cells. 24 h later, 5-Fu and WAF samples were dissolved in sterilized 0.9% aqueous NaCl solution and injected intraperitoneal once a day for 8 days. The same volume of NaCl solution was injected intraperitoneal into the control mice. After injection, the mice were sacrificed and the tumors were excised. The inhibition ratio (ξ) and enhancement ratio of body weight (f) were calculated as follows:

$$\xi = [(W_c - W_t) / W_c] \times 100\% \quad (1)$$

$$f = [(W_a - W_b) / W_b] \times 100\% \quad (2)$$

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Where W_c is the average tumor weight of the control group, W_t is the average tumor weight of the tested group; and W_b and W_a are the body weight of mice before and after the assay. Complete regression is indicated as the ratio of the number of tumor-free mice to the number of mice tested.

Immunohistochemistry for apoptosis-related proteins

The Immunohistochemistry test were carried out according to our previously reported method [13].

Pretreatment of tumor tissue for immunostaining

S-180 tumors tissue were excised from mice and cut into small pieces. It was fixed with formalin for 36 h. Fixed-tumors were immersed in a series from 70%, 80%, 90%, 95% to 100% of graded ethanol solution for 15-20 min. The ethanol in dehydrated-tumors was replaced by methyl benzoate by passing through successive changes of methyl benzoate. Subsequently, the tumors were embedded in the paraffin to keep for next investigation. 4 mm serial sections were cut from the pretreated tumor blocks and mounted on poly-L-lysine (PLL)-coated microscope slides, and then were dried at 60°C for 1 h for immunostaining.

Immunostain of Bax and Bcl-2

Immunohistochemistry of Bax and Bcl-2 test were performed by the peroxidase-labeled streptavidin biotin method with microwave antigen retrieval [18-20]. Paraffin in the sections was removed by xylene. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 for 10 min at 37°C, followed by washing for four times with PBS. Antigen retrieval was carried out in a microwave oven. Briefly, the sections of tumor tissue were heated to 95°C and maintained in 0.01 mol/L citrate buffer (pH 6.0) for 10 min. It was followed by rinsing in warm tap water. And then the sections were treated with normal goat serum for 10 min at 37°C to reduce nonspecific staining. Subsequently, the rabbit antimouse Bax and Bcl-2 polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, US) as primary antibodies were added in optimal dilution and the sections were incubated overnight in a humidified chamber at 4°C. This step and each of the following were succeeded by washing for four times in PBS for 5 min. The sections were incubated in a humidified chamber with biotinylated goat antirabbit IgG as secondary antibodies for 10 min at 37°C, followed by exposure to streptavidin horseradish-peroxidase for 10 min at 37°C. The color was developed using a solution containing 0.05% diaminobenzidine (DAB) as chromogen. After visualization of horseradish-peroxidase activity by color reaction with DAB, the sections were weakly counterstained with hematoxylin, mounted and examined using light microscope equipped with a 40× objective. To confirm immunospecificity, negative controls consisted of sections in which the primary antibody was omitted and replaced by buffer or a non-immune IgG. Formalin-fixed, paraffin-embedded sections of normal human lymph node served as positive controls. Both the number of immune reactive cells and the total number of cells (at least 500 cells) were determined to calculate percentage of Bax and Bcl-2 positive cells by visual inspection of six different fields per section.

Statistical analysis

Student's t-test was used to evaluate the differences between the controls and tested groups. Significant difference between two groups was defined as $p < 0.05$.

RESULTS AND DISCUSSION

***In vitro* and *in vivo* results**

The *In vitro* inhibition ratio to the proliferation of ACC tumor cells by wAF fractions were shown in **Figure 2.**

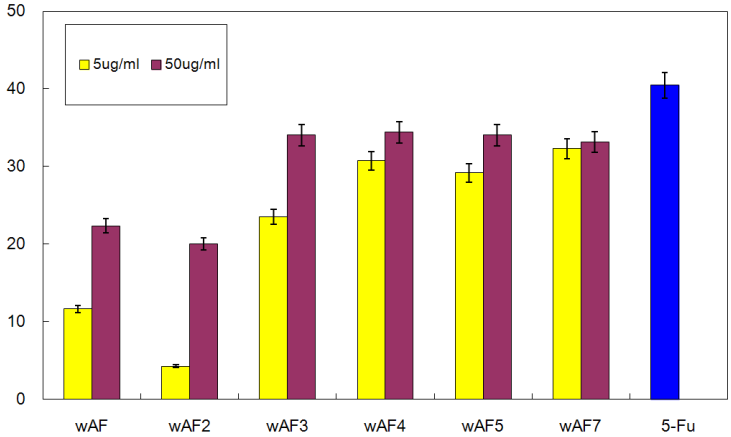

Figure 2. The *in vitro* inhibition ratio to the proliferation of ACC by different molecular weight fractions.

Obviously, all the wAF fractions exhibited strong inhibition against cell growth. Obvious dose-response relationship of the wAF fractions *in vitro* inhibition of ACC cells proliferation was observed. The inhibition of 50 ug/mL dose was higher than 5 ug/mL. Interestingly, the wAF3, wAF4 and wAF5 with intermediate M_w showed considerably stronger inhibition of ACC cell. The samples of wAF3 (29.4×10^4), wAF4 (12.8×10^4) and wAF5 (11.2×10^4) at concentration of 50 ug/mL had a high inhibition ratio of

34.0%, 34.4% and 34.1%, respectively. It was comparable to 5-Fu (40.5%). However, it is well known that 5-Fu is toxic whereas polysaccharides from natural products are relatively safer. In general, relative high molecular weight glucan appear to be more effective antitumor activity than those of low molecular weight [21]. But medical properties of some mushroom polysaccharides like (1→3)-*β*-glucuronoxylomannans are not strongly dependent on molecular weights. Their hydrolyzed fractions containing glucuronoxylomannans with molecular weights from 53 to 1000 KDa are as effective as those fractions, with higher molecular weights [22].

To evaluate the *in vivo* antitumor activity of wAF fractions, the sample solutions were injected intraperitoneal (i.p. 20 mg/kg and 60 mg/kg) to BALB/c mice once daily for 8 days after S-180 tumor cells inoculation for 24 h. The results of the *in vivo* anti-tumor activities of the wAF samples were summarized in **Table 1,** in which also lists that of 5-Fu, a chemotherapeutic anticancer agent as a positive control. The results showed that wAF fractions significantly inhibited tumor growth, but all the doses show certain inhibition ratios against tumor-cell growth. The tumor weight of the control group was 0.73 g, while the tumor weight of mice treated with low and high concentration of wAF3 were reduced to average weighs 0.20 g and 0.48 g, respectively. Compared with the other dose, the dose 20 mg/kg exhibited relatively higher antitumor activities. The tumor weight of mice treated with a low, or high dose wAF fractions were comparable with that of 5-Fu group. Moreover, the enhancement ratios of body weight for all the doses weigh significantly higher than that for 5-Fu, indicating that the wAF samples had much lower toxicities than 5-Fu, which killed normal cells as well as cancer cells **(Table 1).**

Table 1. Antitumor activity of the wAF fractions against Sarcoma 180 solid tumor grown in BALB/c mice.

Sample	$M_w \times 10^{-4}$	Dose (mg/kg×days)	Inhibition ratio (%)	Enhancement ratio weight (%)
control	—	20 × 8	—	41.6
5-Fu	—	20×10	70.3*	-10.0*
wAF	64.7	20×10	31.7*	38.1
		60×10	50.3*	37.4
wAF2	51.0	20×10	39.5*	42.4
		60×10	33.4*	36.8
wAF3	29.4	20×10	71.9*	31.0
		60×10	32.9*	41.9
wAF4	12.8	20×10	35.1*	50.0
		60×10	21.8*	32.1
wAF5	11.2	20×10	35.0*	42.1
		60×10	53.6*	50.6
wAF7	5.9	20×10	31.2*	43.8
		60×10	22.0*	47.1

Acidic polysaccharide composed of a-1→3-D-mannopyranose of water soluble fraction of Auricularia auricula-judae. The mannose rich structure might have beneficial effects as biomedical agents and natural drugs. It may have potential functions in immune surveillance by recognizing high mannose structures present on cell apoptosis.

Fluorescence microscopy and apoptotic assessment

Fluorescence microscopy was utilized to observe apoptosis by changes in cellular morphology, including cell shrinkage, condensation and fragmentation of nuclei, all of which are indicative of apoptosis. **Figure 3** showed nuclear morphology of various type of ACC cell death with Hoechst staining following exposure to the wAF3 for 24 h at concentration of 0.5 mg/mL. Hoechst stains the nuclei of cells with a broken or an intact cell membrane. Round nuclei, blue fragment nuclei or shrinkage cells corresponded to viable and apoptotic cells. As illustrated in **Figure 3.**

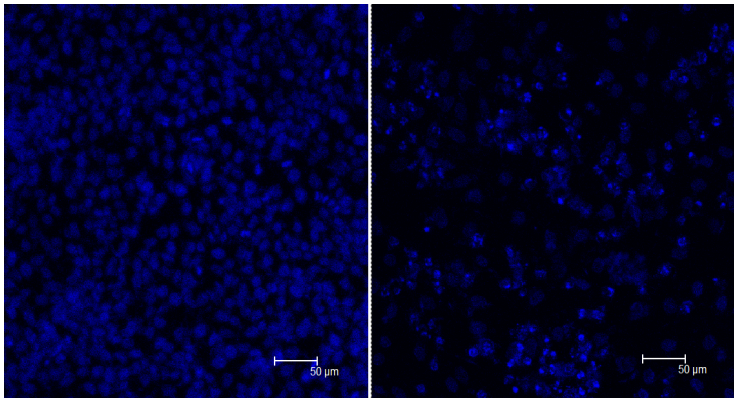

Figure 3. Nuclear morphology of the various type of cell death visualized by fluorescence microscopy with Hoechst stain following exposure to the control group (left), wAF3-treated concentration of 0.5 mg/mL (right)

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The control group was mainly living cells, while the wAF3 treated group had many apoptotic cells with fragmented nuclei. The obvious inhibition against tumor cells indicated that the wAF3 sample could induce apoptosis in ACC cells. Morphological changes can provide the most reliable criteria for recognizing apoptotic process. The changes in cellular morphology, including cell shrinkage, condensation and fragmentation of nuclei, all of which are indicative of ACC cell apoptosis after treatment of wAF3.

Effect of wAF on tumor tissues cell morphology

Figure 4 showed histological section of tumor tissues in control group and wAF3, wAF4 and wAF5 treated mice. Compared with the tumor tissues of the control group mice, the tumor tissues from wAF samples treated mice showed a massive necrosis such as nucleus atrophy, disintegrating and structure less red staining region. Meanwhile, chromatin condensation, cell shrinkage, plasma membrane blabbing and the formation of membrane-enclosed apoptotic bodies were observed in the tumor sections of wAF fractions treated mice, indicating many tumor cells were undergoing apoptosis.

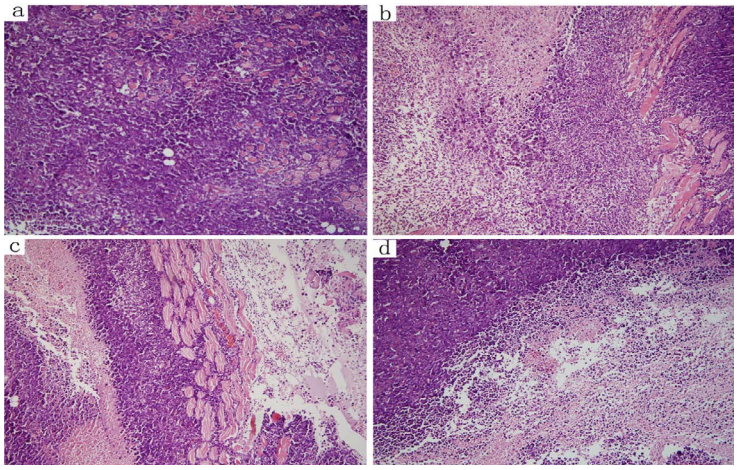

Figure 4. Histological sections of tumor tissues in control group (a), wAF3-treated mice (b), wAF4-treated mice (c) and wAF5-treated mice (d).Immunohistochemistry for apoptosis-related proteins Bax and Bcl-2.

The expression of apoptosis-related proteins Bcl-2 and Bax in S-180 tumor treated by intraperitoneal injection of wAF3 aqueous solution into BALB/c mice were investigated **(Figure 5).**

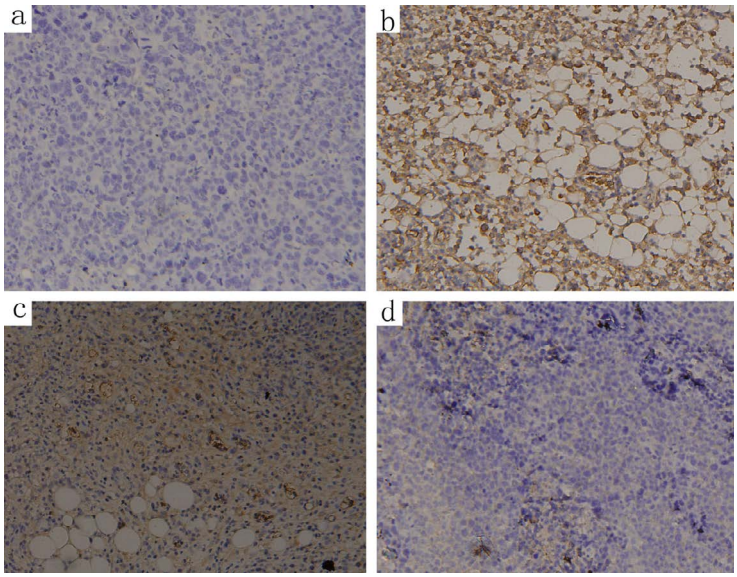

Figure 5. Bax immunostain in S-180 tumour of the untreated control mice (a), and wAF3-treated mice (b). Bcl-2 immunostain for the untreated control mice (c) and wAF3-treated mice (d).

Showed the apoptosis-related proteins Bax and Bcl-2 immunostain in S-180 tumor of the untreated control mice and wAF3 treated mice. The results revealed a significant difference in expression of the proteins in control and wAF3 groups. In the control group, Bax was not detected, whereas Bcl-2 immunostain was significantly strong in S-180 tumor. Compared with the control group, the mice treated with wAF3 exhibited a significant decreased expression of Bcl-2 and increased expression of Bax. The uniquely high Bax expression with low Bcl-2 immunostain in the wAF3 group indicated that the wAF3 polysaccharide induced apoptosis of S-180 tumor cells probably by up-regulating expression of Bax to counteract the effect of Bcl-2. Furthermore, a high expression level of Bax accompanied by a low Bcl-2 immunoreactivity could sustain a low Bcl-2: Bax ratio in favor of apoptosis, or alternatively, it could effectively antagonize the anti-apoptotic levels of the Bcl-2. The immunohistochemical results indicated that wAF3 induced apoptosis in xenograft S-180 tumor cell by up-regulating Bax and down-regulating Bcl-2.Apoptosis of cancer

cells normally relates to the expression of many oncogenes and anti-oncogenes [23]. Bcl-2 family act as a rheostat in regulating programmed cell death and are considered as targets of anticancer therapy [24]. The ratio of death antagonists (Bcl-2, Bcl-xL) to agonists (Bax, Bad, Bid) determines whether a cell will respond to an apoptotic stimulus. Down-regulation of the death suppressor Bcl-2 could inhibit tumor growth via promoting programmed cell death [25]. It has been proven that Bax promotes apoptosis whereas Bcl-2 suppresses apoptosis [26]. Bax resides in an inactive state in the cytosol of many cells. In response to death stimuli, Bax protein undergoes conformational changes that expose membrane-targeting domains, resulting in its translocation to mitochondrial membranes, where Bax inserts and causes release of cytochrome c to activate caspase-3 and other apoptogenic proteins [27,28]. When Bax predominates, apoptosis is accelerating [29]. Our results showed that the proportions of Bcl-2/Bax of wAF3 treated groups were significantly lower than that of the control group, suggesting thatwAF3 could also regulate and control the expression of Bcl-2 and Bax to induce apoptosis. However, the related mechanism of action has not been well understood and needs further study.

CONCLUSION

A water soluble acidic heteropolysaccharide named wAF was isolated from *Auricularia auricula-judae*. The *in vivo* and *in vitro* evaluation showed that wAF fractions exhibited strong inhibition against tumor cell growth in a dose-dependent way. Fluorescence microscopy and tumor tissues cell morphology observation indicated that wAF fractions induced apoptosis of tumor cell. Further analysis of the tumor inhibition mechanism revealed that the expression of Bax increased, while the expression of Bcl-2 decreased dramatically in S-180 tumor tissue section after. The immunohistochemical results indicated that the wAF polysaccharide could induce apoptosis in S-180 tumor cell by up-regulating Bax and down-regulating Bcl-2. These findings confirmed that the wAF could be considered as a potential antitumor agent.

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