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Dipsacus asperoides polysaccharide induces apoptosis in osteosarcoma cells by modulating the PI3K/Akt pathway

Jun Chen[a,1], Dong Yao[b,c,*,1], Huixin Yuan[c], Shaojun Zhang[c], Jinhong Tian[c], Wenjing Guo[c], Weizhi Liang[c], Huiyuan Li[c], Yong Zhang[a,*]

[a] Institute of Osteosarcoma, Tangdu Hospital, The Fourth Military Medical University, Xi'an, 710038, China
[b] Department of Orthopaedic Surgery, Xijing Hospital, The Fourth Military Medical University, Xi'an, 710032, China
[c] Department of Orthopaedic Surgery, The Chinese People's Armed Police Force Shanxi Hospital, Taiyuan, China

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ABSTRACT

An alkaline extractable and water-soluble polysaccharide (ADAPW), with an average molecular weight of 16 kDa, was purified from the alkaline extraction of the roots of *Dipsacus asperoides*. Monosaccharide component analysis indicated that ADAPW was composed of glucose, rhamnose, arabinose and mannose in a molar ratio of 8.54:1.83:1.04:0.42. This study aimed to investigate the effect of ADAPW on the viability of human osteosarcoma cell line HOS cells, and explore the possible mechanisms. The results revealed that ADAPW inhibited the proliferation of HOS cells in a dose-dependent manner by inducing apoptosis. Furthermore, treatment with ADAPW caused a loss of mitochondrial membrane potential and accumulation of reactive oxygen species (ROS). In addition, Western blot analysis demonstrated that ADAPW down-regulated the protein expressions of PI3K and phosphorylated Akt (pAkt) in HOS cells. Taken together, induction of apoptosis on HOS cells by ADAPW was mainly associated with ROS production, mitochondrial dysfunction, and inhibition of PI3K/Akt signaling pathway. So this finding suggests that ADAPW may be potentially effective in cancer prevention against human osteosarcoma.

1. Introduction

Osteosarcoma is the most common primary malignant tumor of bone that usually develops during the period of rapid growth in adolescence, as a teenager matures into an adult (Anninga et al., 2011). The improvement in survival has been attributed to the use of intensive multi-agent chemotherapy given in combination with advanced surgery. Although modern multimodality treatment has significantly improved tumor resectability and the long-term outcome of these patients, 25–50% of patients with initially non-metastatic disease, subsequently develop metastases despite aggressive combination chemotherapy and surgery, and this remains the major cause of death from this condition (Link et al., 1986). In addition, most chemotherapeutics carry the risk of short-term and long-term toxic effects. Thus the search for a novel agent to treat osteosarcoma is currently of major interest to scientists.

Polysaccharides from natural sources are found to be effective, non-toxic substances with wide variety of biological activities, and have attracted lots of attention in the biochemical and medical areas (Ooi & Liu, 2000). So there is a promising research field for discovering and evaluating polysaccharides from various kinds of organism with bioactive properties.

Dipsacus asperoides C.Y. Cheng et T.M. Ai is a perennial herb widely distributed in the southwest of the People's Republic of China. The roots of *D. asperoides*, also named Xu Duan or Himalayan Teasel Root, were first recorded in Shennong Emperor's Classic of Materia Medica (Shennong Bencao Jing) and have been used in Traditional Chinese Medicine for hundreds of years as an antiosteoporosis, tonic and antiaging agent for the therapy of low back pain, traumatic hematoma, threatened abortion and bone fractures (Liu et al., 2009; Hung et al., 2006). In previous studies, several chemical constituents, particularly dipsacus saponins, iridoid, phenolics and alkaloids have been identified from the roots of *D. asperoides* (Jeong et al., 2008; Zhao & Shi, 2011). Recently, saponins isolated from the roots of this plant have been demonstrated to possess anticomplementary, antinociceptive, cytotoxic, osteoprotective, cardioprotective and inhibition of Alzheimer's disease activities, while phenolics possess neuroprotective and antioxidant effects (Zhao & Shi, 2011). However, there are fewer reports specifically concerning the purification and biological

* Corresponding authors. Tel.: +86 29 84777430; fax: +86 29 84777430.
E-mail addresses: dong_yao567@126.com (D. Yao), zhangyongtdgk@yeah.net (Y. Zhang).
[1] These authors contributed equally to this work.

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activities of polysaccharides from the roots of *D. asperoides*. In the course for anti-proliferative activities from natural resources, an alkali extracted polysaccharide fraction from the roots of *D. asperoides* was found to show apoptosis-inducing activities on human osteosarcoma cell lines HOS. Therefore, the objective of the present study was to further purify and identify this polysaccharide and systematically evaluate the apoptotic effects of this component on HOS cells.

2. Materials and methods

2.1. Materials and reagents

D. asperoides was purchased from a herb market in Xi'an city of China.

Arabinose, fucose, galactose, glucose, mannose, rhamnose, xylose, glucuronic acid, trifluoroacetic acid (TFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma Chemical Co., St. Louis, MO, USA; DEAE Sepharose Fast Flow and Sephacryl S-300 HR were from Amersham Biosciences Co., Piscataway, NJ, USA; RPMI-1640 medium and fetal bovine serum (FBS) were from Gibco, Grand Island, NY, USA; all other chemicals and solvents used were of analytical grade.

2.2. Preparation of the polysaccharide from the roots of D. asperoides

The roots of *D. asperoides* were ground to pass through a 2 mm screen and the powder was refluxed twice with 95% ethanol at 75 °C under reflux for 3 h to deactivate the endogenous enzymes and remove some soluble materials, including free sugars, amino acids and some phenols. Then the residue was extracted with distilled water (5000 ml) at 75 °C for 3 times and 3 h for each time to enrich the water soluble polysaccharides. Then further extraction was conducted to residue with 5% alkali solution for 24 h for three times, and the whole mixture was kept overnight at 4 °C and filtered through linen cloth. The suspension was neutralized with hydrochloric acid (0.1 M) and filtered. The supernatant containing water-soluble polysaccharide was dialyzed, concentrated, ethanol precipitated and then dried to yield crude alkaline extracted polysaccharides (CADAP). Sevag reagent (1-butanol/chloroform, v/v = 1:4) (Staub, 1965) was used for the deproteination of CADAP and 30% H_2O_2 for decoloration, respectively. After CADAP was dialyzed against tap water and distilled water for 48 h, the resulting polysaccharide solution was concentrated, lyophilized and loaded on ion-change chromatography of DEAE Sepharose Fast Flow and gel filtration column chromatography of Sephacryl S-300 HR for the isolation of this preparation. Total sugar content of each tube was measured at 490 nm by Dubois' method, and protein absorption at 280 nm was recorded for each fraction.

2.3. Measurement of carbohydrate, uronic acid and protein contents

Total sugar and uronic acid contents of polysaccharide were quantified by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and m-hydroxydiphenyl analysis (Filisetti-Cozzi & Carpita, 1991) using D-glucose and D-glucuronic acid as standard, respectively. The protein content in the polysaccharides was measured by the Bradford's method (Bradford, 1976), with bovine serum albumin as the standard.

2.4. Analysis of monosaccharide compositions

Polysaccharide was also analyzed for monosaccharide by gas chromatography (GC). After hydrolysis with 2 M trifluoroacetic acid (TFA) and conversion of hydrolysate into alditol-acetates as previously described method (Jones & Albersheim, 1972; Oades, 1967), the resulting alditol-acetates were analyzed by GC on a Shimadzu GC-14C instrument (Japan) equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm) and flame-ionization detector (FID).

2.5. Homogeneity and molecular weight determination

The molecular weight of the polysaccharide was evaluated and determined by the gel permeation chromatography with a HPLC apparatus (HPGPC), which was performed on a SHIMADZU HPLC system fitted with one TSK-G3000 PW_{XL} columns (7.8 mm ID × 30.0 cm) and a SHIMADZU RID-10A detector. The detailed operation conditions were mobile phase: 0.7% (w/v) sodium sulfate; flow rate: 0.5 mL/min; column temperature: 35 °C; injection volume: 20 μL. The calibration curve for molecular weight determination was made using a series of Dextran standards.

2.6. Cell proliferation assay

The human osteosarcoma cell line HOS cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells (5×10^3 cells) were seeded in a 96-well plate with medium supplemented with 10% FBS and incubated for 24 h. HOS cells were then added with various concentrations of the polysaccharide (25, 50, 100, 200 and 400 μg/ml) for 24 h and washed with phosphate-buffered saline (PBS). The viability of cells was determined by the MTT assay. Briefly, at appropriated time intervals, 20 μl of a 5 mg/ml MTT solution was added to each well. After 2 h incubation, the medium was carefully aspirated and the purple formazan crystals were solubilized with 200 μL DMSO. The resulting intracellular purple formazan was quantified with a spectrophotometer by absorbance at 540 nm. Cell growth as percent viability was calculated by comparing the absorbance of treated verses untreated cells.

2.7. Measurement of apoptosis by Annexin V-FITC/PI staining

Cells were seeded in 6-well plate at a density of 2×10^6 cells/well and exposed to the polysaccharide at various concentrations (100, 200 and 400 μg/ml) for 24 h, and then were collected and washed twice with cold PBS. 100 μl of cell suspension was transferred to a 5 ml culture tube and incubated with 10 μl of Annexin V-FITC in the dark at room temperature for 10 min, and then added 10 μl PI in the dark 4 °C for 10 min. The apoptotic cells were analyzed with FACScan flow cytometry (BD FACSCalibur).

2.8. Analysis of intracellular reactive oxygen species (ROS)

The intracellular generation of ROS was measured using the oxidation-sensitive fluorescein substrate 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Huang et al., 2006), which easily diffuses into the cell and is then deacetylated by cellular esterases to the more hydrophilic, nonfluorescent DCFH. ROS generation in the cell oxidizes DCFH to the fluorescent 2′,7′- dichlorofluorescein (DCF). Therefore DCFH-DA was used as a probe for intracellular ROS formation (Lebel & Bondy, 1990). After HOS cells were cultured with ADAPW at three concentrations of 100, 200 and 400 μg/ml in 96-well culture plates for 24 h, 10 mM DCFH-DA was transferred to each well for 20 min further incubation at 37 °C. Then cells were washed with PBS twice and resuspended in Hanks solution for measurement of the intracellular oxidative stress. Fluorescence intensity was measured using a fluorescence plate reader CytoFluor 2350 (Millipore, Bedford, MA,

USA) with the excitation wavelength at 485 nm and the emission wavelength at 535 nm, respectively. The amount of intracellular ROS was calculated from a standard curve derived from DCF. Protein concentration was measured by the Bradford method (Bradford, 1976).

2.9. Evaluation of mitochondrial membrane potential ($\Delta\psi m$)

Mitochondrial membrane potential was evaluated as described by Hsu et al. (2009), with some modification. After incubating cells in the presence of ADAPW (100, 200 and 400 μg/ml) for 24 h, 25 μM JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was added to the cells and further incubated at 37 °C for 30 min. Cells were washed twice with PBS, and fluorescence was monitored with the fluorescence plate reader at wavelengths of 490 nm (excitation)/540 nm (emission) and 540 nm (excitation)/590 nm (emission) pairs. Changes in the ratio between the measurement at test wavelengths of 590 nm (red) and 540 nm (green) fluorescence intensities are indicative of changes in the mitochondrial membrane potential.

2.10. Western blotting assay

When pretreatment with ADAPW (100, 200 and 400 μg/ml) for 24 h, HOS cells were collected and homogenized in 200 μl NP-40 lysis buffer (30 mM Tris–Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% NP-40). Cell lysate was centrifuged (12,000 × *g* for 15 min at 4 °C) and protein content was quantified. Equal amounts of protein were denatured and separated by 10% SDS-PAGE, then transferred onto nitrocellulose membrane. Nonspecific binding was blocked with 5% bovine serum albumin dissolved in TBST at room temperature for 1 h. Subsequently, the membranes were then washed three times and incubated with individual primary antibodies of PI3K and Akt at 4 °C over night. The membranes were incubated with the horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature followed by three washings. The signals were detected with a chemiluminescence ECL reagent and quantified by densitometry using a gel visualizer (Alpha Innotech, CA, USA).

2.11. Statistical analysis

All data were expressed as the mean ± SD of the indicated number of experiments. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by a Student's *t*-test, and *P*-values less than 0.05 were considered significant.

3. Results and discussion

3.1. Isolation and purification of ADAPW

The crude polysaccharide (CADAP) was obtained from the alkaline extraction of the roots of *D. asperoides* with a yield of 5.2% and fractioned on DEAE Sepharose Fast Flow using water as eluant, yielding only one homogeneous fraction (CADAPW). For more purification, CADAPW was again fractionized through Sephacryl S-300 HR in aqueous medium to give a colorless polysaccharide (ADAPW). A single elution peak appeared for ADAPW in Sephacryl S-300 HR (Fig. 1) and was detected by the phenol-sulfuric acid assay.

3.2. Physicochemical properties of ADAPW

Average molecular weight, total sugar content, protein content, uronic acid content and monosaccharide compositions of the

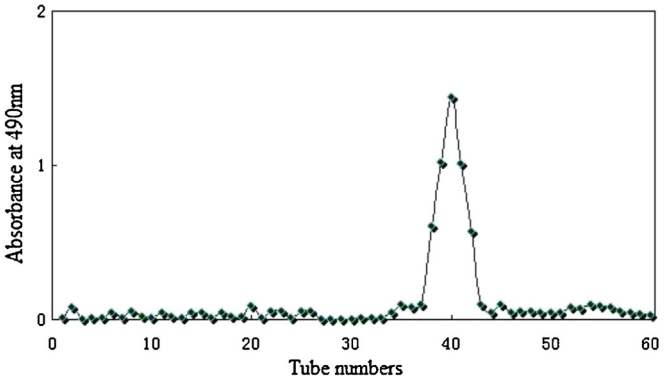

Fig. 1. Profile of ADAPW in Sephacryl S-300 HR column chromatography.

Table 1
Components of monosaccharide and properties of ADAPW from the roots of *D. asperoides*.

Properties	Data
Molecular weight (kDa)	16
Carbohydrate (wt%)	93.4
Protein	–
Uronic acid	–
Monosaccharide component (mol)	
Glucose	8.54
Rhamnose	1.83
Arabinose	1.04
Mannose	0.42

polysaccharide were determined and shown in Table 1. The homogeneity of ADAPW was estimated by HPGPC in which it showed one symmetrical peak. The average molecular weight of ADAPW was estimated to be 16 kDa by comparing with the Dextran standards of different molecular weight. According to retention time of the alditol acetate derivatives in GC, ADAPW consisted of four different monosaccharides, including glucose, rhamnose, arabinose and mannose and the molar ratio of 8.54:1.83:1.04:0.42.

3.3. Effect of ADAPW on HOS cell proliferation

To define the optimal concentration at which ADAPW was toxic to tumor cells, we treated HOS cells with various concentrations of ADAPW (25, 50, 100, 200 and 400 μg/ml) for 24 h and measured cell growth by an MTT assay. As shown in Fig. 2, pretreatment with ADAPW at all concentrations significantly inhibited cell

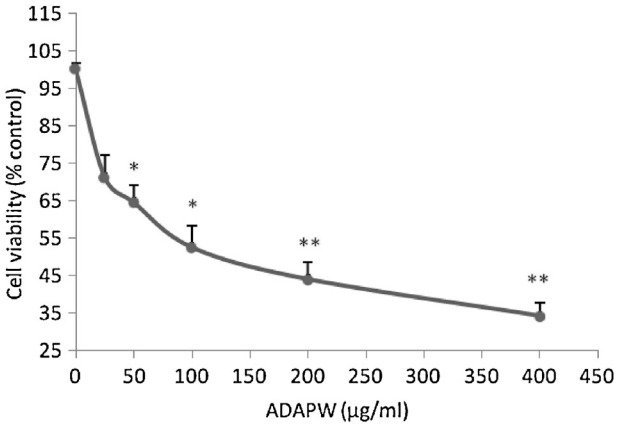

Fig. 2. Cell viability of HOS cells after treatment with different concentrations of. ADAPW. The data represent mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

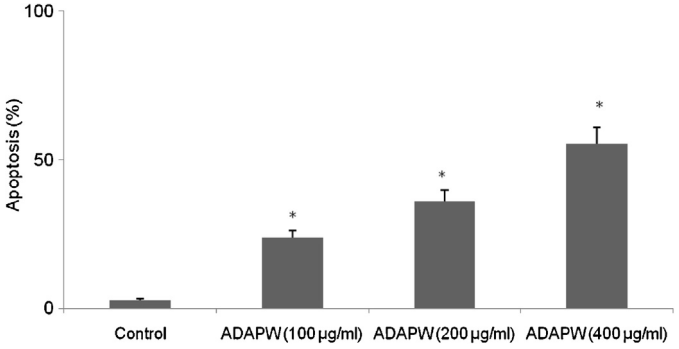

Fig. 3. ADAPW-induced apoptosis in HOS cells using annexinV-FITC/PI. The data represent mean ± SD of three independent experiments. $^{*}P < 0.05$ vs. control.

proliferation in a dose–response manner. Especially at the concentration of 400 μg/ml, the cell survival rate was less than 35% as compared to untreated cells ($^{**}P < 0.01$). To facilitate the study, ADAPW at three concentrations of 100, 200 and 400 μg/mL was chose in the following assays.

3.4. Apoptosis induced by ADAPW

For improvement of MTT result and also characterizing the type of cell death involved in our experiments, FITC-Annexin V/PI double staining was performed.

Fig. 3 showed that pretreatment with 100, 200 and 400 μg/ml of ADAPW significantly increased the number of apoptotic cells from 2.7% in untreated cells to 23.7%, 35.8% and 55.3%, respectively. These results suggest that the induction of apoptosis may be a major mechanism of the ADAPW-caused inhibition on the viability of HOS cells.

3.5. Effect of ADAPW on intracellular ROS in HOS cells

Since many anticancer drugs and DNA damage-causing agents turn on the apoptotic pathway through ROS generation (Kulms & Schwarz, 2002; Kulms, Zeise, Poppelmann, & Schwarz, 2002; Nomura, Imai, Koumura, Arai, & Nakagawa, 1999), the possibility that ROS increase are key steps for ADAPW-induced apoptosis was assessed. ROS production can be detected by flow cytometric analysis using DCFH-DA as a fluorescence probe for estimating ROS generation. As shown in Fig. 4, the tumor cells exposed to various concentrations of ADAPW (100, 200 and 400 μg/ml) showed significant increase of intracellular ROS as compared with untreated cells ($P < 0.05$ and $P < 0.01$).

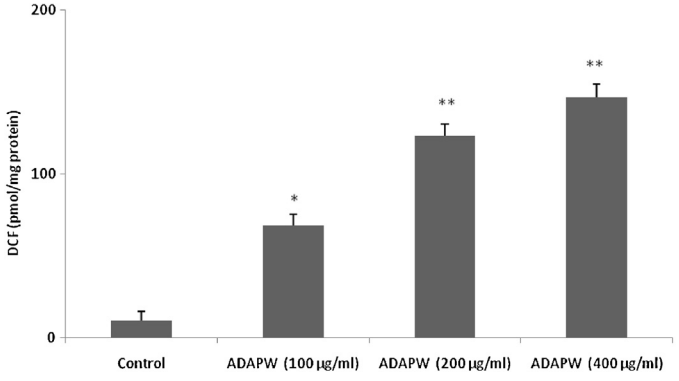

Fig. 4. Quantification of intracellular ROS was detected by flow cytometry using DCFH-DA. The data represent mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

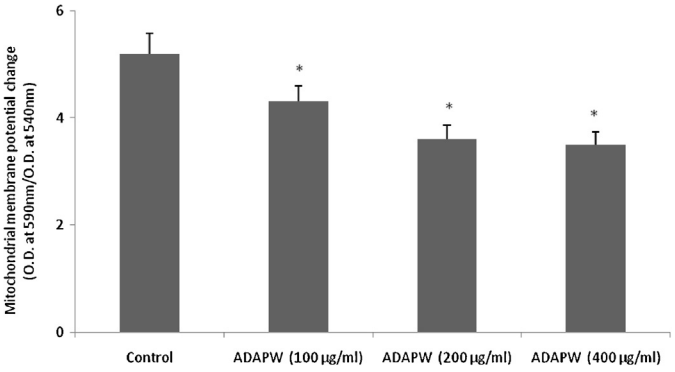

Fig. 5. Effects of ADAPW on mitochondrial membrane potential ($\Delta\psi m$) in HOS cells. The data represent mean ± SD of three independent experiments. $^{*}P < 0.05$ vs. control.

3.6. Effect of ADAPW on the change of mitochondrial membrane potential ($\Delta\psi m$) in HOS cells

The increase of intracellular ROS may impair a variety of intra- and extra-mitochondrial membrane transport system such as distribution of mitochondrial ion-transport system and decrease of mitochondrial membrane potential which is one of the earliest intracellular events that occur following the induction of apoptosis (Qi et al., 2010). The effect of ADAPW on mitochondrial membrane potential was examined by the JC-1 method. As presented in Fig. 5, treating with ADAPW (100, 200 and 400 μg/ml) for 24 h evidently decreased mitochondrial membrane potential of the tumor cells as compared with non-treated cells ($P < 0.05$).

3.7. Effects of ADAPW on PI3K/Akt pathway

The PI3K/Akt pathway is recognized to play a key role in regulating cell proliferation and apoptosis (Pan, Lin, Lin, & Chen, 2007), and Akt is a major downstream target of PI3K (Kauffmann-Zeh et al., 1997). PI3K converts phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-triphosphate, which stimulates phosphoinositide-dependent kinase 1. Therefore, we investigated the effects of ADAPW on PI3K/Akt pathway in HOS cells. The results showed that incubation of HOS cells with ADAPW (100, 200 and

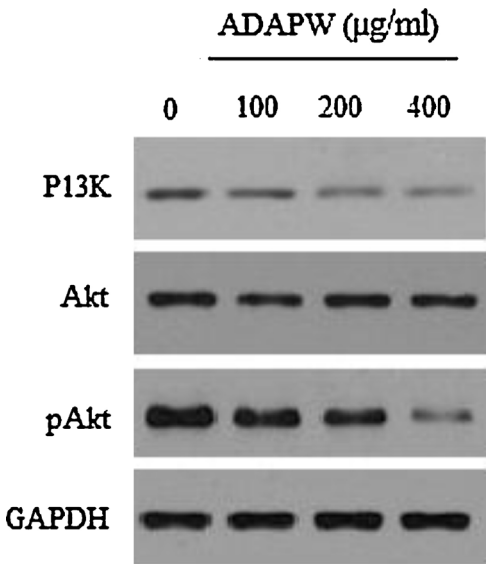

Fig. 6. Effects of ADAPW on the protein expression levels of PI3K and pAkt in HOS cells.

400 μg/ml) led to a dose-dependent decrease of PI3K and pAkt at protein level. However, ADAPW had no effects on Akt protein expression at all indicated doses (Fig. 6). These experiments support the conclusion that ADAPW-induced apoptosis may be mediated by PI3K/Akt downregulation.

4. Conclusions

We successfully isolated and purified a homogeneous polysaccharide from the alkaline extraction of the roots of *D. asperoides* using DEAE-Sepharose CL-6B anion-exchange and Sephacryl S-300 HR gel filtration chromatography. HPGPC and GC analysis identified that ADAPW was a homogeneous polysaccharide with a molecular mass of 16 kDa and was composed mainly of glucose, rhamnose, arabinose and mannose in the molar ratio of 8.54:1.83:1.04:0.42. In this study, we aimed at examining the anti-proliferative activity of ADAPW against HOS cells and the underlying mechanisms. ADAPW exhibited evident growth inhibition on HOS cells in a concentration-dependent manner and dose-dependently induced apoptosis in HOS cells, as determined by FITC-Annexin V/PI double staining. Besides, ADAPW caused a considerable intracellular ROS production and induced a remarkable change in mitochondrial membrane potential. Furthermore, Western blot analysis demonstrated that ADAPW suppressed the protein expression of PI3K and pAkt. Therefore, we propose that ADAPW-induced apoptosis in HOS cells partly depends on the activation of the PI-3K/Akt signaling pathway. These findings suggest that ADAPW may be a promising chemotherapeutic agent in the fight against human osteosarcoma.

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