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# Antiproliferative potential of *Artemisia capillaris* polysaccharide against human nasopharyngeal carcinoma cells

Guofang Feng[a], Xiaoqing Wang[b], Changxuan You[c], Xiaohua Cheng[d], Zeli Han[a], Liang Zong[a], Chengyong Zhou[a], Meikui Zhang[e,*]

[a] Department of Otolaryngology Head and Neck Surgery, First Affiliated Hospital of Chinese PLA General Hospital, Beijing 100048, China
[b] Department of Pharmacology, First Affiliated Hospital of Chinese PLA General Hospital, Beijing 100048, China
[c] Department of Oncology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China
[d] Beijing Institute of Otolaryngology, Beijing Tongren Hospital, Capital Medical University, Beijing 100005, China
[e] Tel-consultation Medical Center, Chinese PLA General Hospital, Beijing 100853, China

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ABSTRACT

One water-soluble polysaccharide fraction (WACP) was purified from the *Artemisia capillaris* by DEAE-cellulose anion-exchange and Sephacryl S-400 gel-permeation chromatography. Based on gas chromatography (GC) analysis, WACP was an arabinogalactan (AG), consisting of arabinose and galactose in the ratio of 4:2. Its molecular weight was about $5.8 \times 10^4$ Da. The present study demonstrated the anticancer potential of WACP on human nasopharyngeal carcinoma CNE-2 cells and elucidated its possible mechanisms. MTT and flow cytometric analysis identified that WACP had a potent anti-proliferation activity on CNE-2 cells by inducing apoptosis, which was parallel with the morphological changes of CNE-2 cells under inverted microscope. In addition, WACP triggered the apoptosis via the mitochondrial apoptotic pathway, which included the loss of mitochondrial membrane potential, release of cytochrome c and activation of caspase-3/9. Taken together, these results suggest that WACP has anticancer potential in the treatment of human nasopharyngeal carcinoma.



## 1. Introduction

Nasopharyngeal carcinoma (NPC) is the most common squamous cell carcinoma that occurs in the epithelial lining of the nasopharynx and exhibits varying degrees of differentiation (Wei & Sham, 2005). NPC differs significantly from other cancers of the head and neck in its occurrence, causes, clinical behavior, and treatment. It is vastly more common in certain regions of East Asia and Africa than elsewhere, with viral, dietary and genetic factors implicated in its causation. The World Health Organization classifies NPC in three types: typical squamous cell carcinomas (type I), keratinizing undifferentiated carcinoma (type II) and nonkeratinizing undifferentiated carcinoma (type III) (Fang et al., 2012). Usually, NPC is treated by surgical resection, radiotherapy and chemotherapy. However, the current therapeutic options for nasopharyngeal carcinoma are often disappointing. Cisplatin is the most effective cytotoxic agent used in NPC treatment. However, inherent and acquired resistance to the drug limits its applications in NPC chemotherapy (Xie et al., 2008). The co-administration of radiotherapy and adjuvant chemotherapy with cisplatin is the standard treatment for NPC, but the 5-year survival rate is only about 50–60%. One obstacle ahead is the high risk of locoregional relapse and distant metastasis (Hu et al., 2008; Wei & Sham, 2005). Therefore, it is important to search for better drugs for the NPC chemotherapy.

One of the promising therapeutic approaches on NPC is drug screening from natural products, such as components from traditional Chinese medicine. Nowadays many biological polysaccharides have been isolated from mushrooms, fungi, yeast, algae, lichens, and plants, due to their healthy benefits for human (Markova et al., 2003; Murata, Shimamura, Tagami, Takatsuki, & Hamuro, 2002). Most polysaccharides derived from plants are relatively nontoxic and do not cause significant side effects. These could allow development of an effective natural anticancer with few side effects.

*Artemisia capillaris* Thunb. is an annual glabrous herb used in the herbal medicine field, which can dispel wind-heat, subdue hyperactivity of the liver and improve eyesight (Cha et al., 2005; Wang et al., 2000). Phytochemical studies on *A. capillaris* revealed the occurrence of volatile essential oil, coumarins, flavonol glycosides and a group of unidentified aglycones (Fakeya, Yoshitomo, & Haruji, 1976; Yamahara, Kobayashi, Matsuda, Katayama, & Fujimura, 1989). However, to the best of our knowledge, no information has been reported on the purification and antitumor activity

* Corresponding author. Tel.: +86 010 66937639; fax: +86 010 68174828.
*E-mail address:* meikuizhang@gmail.com (M. Zhang).

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of the polysaccharide from *A. capillaris*, let alone its anti-cancer mechanism. Herein we purified and characterized the polysaccharide from *A. capillaris* and investigated the anti-tumor effect of *A. capillaris* polysaccharide against human nasopharyngeal carcinoma CNE-2 cells. Our results provided the first evidence that *A. capillaris* polysaccharide induced apoptosis in CNE-2 cells.

## 2. Materials and methods

### 2.1. Materials and chemicals

The plant *A. capillaris* was purchased from Shanxi Hunyuan Astragalus Co. (Shanxi, China). The aerial part of dried herb was ground in a mechanical grinder and stored in an air tight container for further use. DEAE-cellulose and Sephacryl-S400, T-2000, T-500, T-110, T-70 and T-40 were obtained from Pharmacia Co. Ltd. (Uppsala, Sweden). Monosaccharide standards, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), streptomycin and penicillin were from Gibco Co. (Grand Island, NY, USA). Aqueous solutions were prepared with ultra-pure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All the other reagents were of analytical grade.

### 2.2. Preparation of the polysaccharide

The dried aerial parts of *A. capillaris* (1.4 kg) were first pre-extracted twice with 95% ethanol for 2 h to remove lipids, and then refluxed twice with 80% ethanol for 2 h to remove monosaccharide, disaccharide, oligosaccharide and polyphenol in the samples. The remaining powders were dried, and refluxed with hot distilled water (6000 ml) twice at 90 °C for 4 h. The combined water extract was concentrated in a rotary evaporator under reduced pressure at 50 °C and filtered through Whatman filter paper. The proteins in the filtrate were removed three times by Sevag reagent (Staub, 1965). After removal of the Sevag reagent, the extract was mixed with 3-fold volumes of 95% EtOH at 4 °C overnight and followed by centrifugation at 4000 × *g* for 10 min to afford the crude polysaccharides (CACP, 35.56 g).

The crude polysaccharide fraction CACP (522.4 mg) dissolved in distilled water were applied to a DEAE-cellulose ($OH^-$ form) column (2.5 cm × 40 cm) and eluted with distilled water at a flow rate of 0.8 ml/min. The obtained fractions were combined based on the total sugar content quantified by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The relevant fractions were concentrated and further loaded onto a Sephacryl-S400 column (2.5 cm × 100 cm) with water as the mobile phase, and collected, concentrated using a rotary evaporator at 40 °C, dialyzed against distilled water and lyophilized to obtain the purified polysaccharide (WACP) as a brown powder.

### 2.3. Physicochemical analysis of WACP

#### 2.3.1. Main component analysis

Total sugar was determined by the phenol–sulfuric acid method, using Glc as the standard (Dubois et al., 1956). The protein contents were determined according to the method of Bradford with bovine serum albumin (BSA) as the standard (Bradford, 1976). Uronic acid content was determined according to a meta-hydroxydiphenyl colorimetric method, using glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991). On the basis of the sulfate content, the sulfate content was determined by barium chloride–gelatin assay (Sun et al., 2009).

#### 2.3.2. Monosaccharide composition analysis

The monosaccharide composition of WACP was determined according the method described by Zhang, Xiao, He, and Sun (2011), with some modification. Briefly, sample (10 mg) was hydrolyzed with l ml of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h in a sealed glass tube. After the removal of the excess amount of TFA by co-evaporation at reduced pressure with ethyl alcohol, the subsequent treatment of the resultant dry hydrolysate with acetic anhydride and pyridine afforded the corresponding alditol acetate which was analyzed by an Agilent 6890 system gas chromatography (Agilent Technologies, Palo Alto, CA, USA) fitted with an HP-1MS capillary column (30 m × 0.25 mm ID, film thickness 0.20 μm) and a flame-ionization detector (FID). The column temperature was fixed at 150 °C for 1 min, increased to 240 °C at 15 °C/min, and fixed at 240 °C for 3 min. The injector and detector temperatures were fixed at 250 °C. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. The standard monosaccharides were then measured following the same procedure and inositol was used as the internal standard.

#### 2.3.3. Molecular weight determination

The homogeneity and molecular weight of WACP was determined by gel permeation chromatography on a Waters 515 instrument equipped with the GPC software (Millennium$^{32}$), a Waters 2410 RI detector and a TSK G-3000 SWXL column (7.8 mm × 300 mm). The columns were calibrated with T-series Dextran (T-2000, T-500, T-110, T-70 and T-40). 0.2 M phosphate buffer (pH 7.0) was used as the eluant and the flow rate was 0.7 ml/min. A 20-μl aliquot was injected for each run.

### 2.4. Cell line and cell culture

The human nasopharyngeal carcinoma cell line CNE-2 cell was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and grown in DMEM, supplemented with 10% FBS and antibiotics. Cells were maintained in a humidified incubator at 37 °C under a 5% $CO_2$ atmosphere.

### 2.5. MTT assay for cell viability

The viability of CNE-2 cells was assessed by MTT assay (Zhang et al., 2012). Briefly, CNE-2 cells were incubated in 96-well plates at an initial density of $2 \times 10^5$ cells/ml for 24 h, and then the cells were washed twice with phosphate-buffered saline (PBS) before the medium was removed. Cells were then incubated with fresh medium containing 0–200 μg/ml of WACP for 24 h, 48 h, and 72 h, respectively. Subsequently 20 μl MTT (5 mg/ml) was added to each well and incubated for 4 h at 37 °C with 5% $CO_2$. The culture media was then discarded and the insoluble formazan was collected and dissolved in dimethylsulfoxide (DMSO, 0.5%). Once the blue crystals had dissolved, the optical density was determined on a microreader (Bio-Rad Co.) at 570 nm. Cell growth inhibition rate (%) was calculated using the following equation: Inhibitory rate (%) = $(1 - A_{\text{treatment}}/A_{\text{control}}) \times 100\%$. The experiments were repeated three times for each cell line.

### 2.6. Cytomorphology observation

To detect morphological evidence of apoptosis, CNE-2 cells were seeded in 50-ml culture bottle ($5 \times 10^5$ cells/bottle) and then incubated with fresh medium containing WACP (50, 100 and 200 μg/ml) for 48 h. Cells were directly observed under inverted microscope.

### 2.7. Flow cytometric analysis of apoptosis

Detection of apoptosis was performed using a commercially available Annexin V-FITC apoptosis detection kit (Sigma, Aldrich). Briefly, tumor cells ($4 \times 10^5$) were incubated with test sample (50, 100 and 200 μg/ml) or PBS (control) for 48 h. Then cells were harvested, washed with ice-cold PBS and resuspended in 200 μl binding buffer at $2 \times 10^5$ cells/ml. The samples were incubated with 5 μl of Annexin V-FITC and 5 μl propidium iodide (PI) in the dark for 15 min at room temperature. Finally the stained cells were analyzed directly on a FACSCAN flow cytometer (Becton Dickinson, San Jose, CA, USA) by using cellquest software (Zhang et al., 2012). The results are shown as a dotplot graph. In each graph, the percentages of apoptotic cells are indicated in lower right quadrant, the *Y*-axis corresponds to relative PI staining, and the *X*-axis corresponds to the log of the FITC signal.

### 2.8. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured by the incorporation of cationic fluorescent dye, rhodamine 123 (Wu et al., 2012). After 48 h incubation in normal medium with WACP at the concentrations of 50, 100 and 200 μg/ml, CNE-2 cells were harvested and incubated for 15 min at 37 °C with 5 μM rhodamine 123 in the dark. Cells were then washed twice with PBS and analyzed immediately by flow cytometry (BD FACScan™, USA).

### 2.9. Analysis of intracellular cytochrome c release

After the cells were treated with WACP at designed concentration of 50, 100 and 200 μg/ml for 48 h, the cells were harvested by centrifugation at $1000 \times g$. And the resulting released cytochrome c in supernatant was determined by a commercial available cytochrome c assay kits according to the manufacturer's directions.

### 2.10. Measurement of caspase-3 and -9 activities

The activities of caspase-3 and 9 were determined by colorimetric assays using commercial available assay kits according the manufacturer's protocol. After the treatment with WACP (50, 100 and 200 μg/ml) for 48 h, cells were harvested and lysed by the addition of lysis buffer. 20 μg of cell lysates was added to the reaction buffer in 96-well microtiter plates, which contained caspase-3 or -9 substrates, and then incubated for 2 h at 37 °C. Thereafter, the absorbance at 405 nm was measured with a microplate reader. The results are represented as the percentage of change of activity compared to the untreated control.

### 2.11. Statistical analysis

The results are represented as the mean ± SD. All statistical methods were performed by the statistical software Statistical Package for Social Sciences 13.0 (SPSS, Chicago, IL). The difference between two mean values was analyzed by Student's *t*-test and was considered to be statistically significant when $p < 0.05$.

## 3. Results

### 3.1. Purification and characterization of WACP

In the present study, the crude water-soluble polysaccharides (CACP) were isolated from the aerial parts of *A. capillaris* by defatting with ethanol, hot water extraction, deprotein by Sevag method, ethanol precipitation and drying in vacuum, and the yield was about 2.54% of the plant raw material. Furthermore, CACP were fractionated by DEAE-cellulose anion-exchange chromatography, and eluted with distilled water to yield one main fraction, which were collected based on total carbohydrate elution profile detected by the phenol–sulfuric acid method. Then the polysaccharide fractions were pooled, dialyzed, lyophilized and further purified by Sephacryl S-400 gel-permeation chromatography into one major fraction, name as WACP. WACP had no absorption at 280 or 260 nm in the UV spectrum, which indicated absence of protein and nucleic acid. The percentages of polysaccharide existed in WACP was determined by the phenol–sulfuric acid method to be 92.4%. Moreover there no uronic acid and sulfate radical present in WACP, evaluated by the meta-hydroxydiphenyl colorimetric method and barium chloride–gelatin assay, respectively. The HPLC profile showed a single and symmetrically sharp peak, indicating that WACP was a homogeneous polysaccharide. Correlation with the calibration curve of Dextran standards, its molecular weight was about $5.8 \times 10^4$ Da. Based on the monosaccharide composition, WACP was an arabinogalactan (AG) with arabinose and galactose in the ratio of 4:2. In addition, WACP showed negative Fehling's reagent and iodine–potassium iodide reactions, indicating that they did not contain reducing sugar and starch-type polysaccharide.

### 3.2. Effect of WACP on the cell viability of CNE-2 Cells

After CNE-2 cells were exposed to WACP (50, 100 and 200 μg/ml) for 24, 48 and 72 h, the cell viability was determined by the MTT assay. The anti-proliferative effect of WACP on CNE-2 cells was shown in Fig. 1. WACP was found to have significant inhibitory effect on the proliferation of CNE-2 cells in a concentration dependent manner. A maximum inhibitory rate of 65.4% was observed in CNE-2 cells at 200 μg/ml after 48 h incubation with WACP. The data revealed a significant concentration-effect and time-effect correlation of antiproliferation in WACP-treated CNE-2 cells.

### 3.3. WACP induces apoptosis in CNE-2 cells

Effective tumor growth inhibitory rate was observed in CNE-2 cells after treatments with WACP. To determine if WACP-induced cell death occurred through cytotoxic necrosis and/or apoptosis, the cells stained simultaneously with AnnexinV and PI were

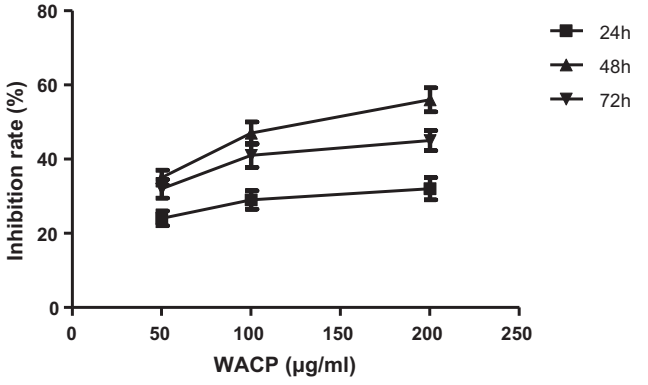


**Fig. 1.** Effects of WACP on the growth of CNE-2 cells. CNE-2 cells were treated with WACP (50, 100 and 200 μg/ml) for 24, 48 and 72 h, and then cell proliferation was determined by MTT assay. Each data are expressed as the mean ± SD obtained from triplicate experiments.

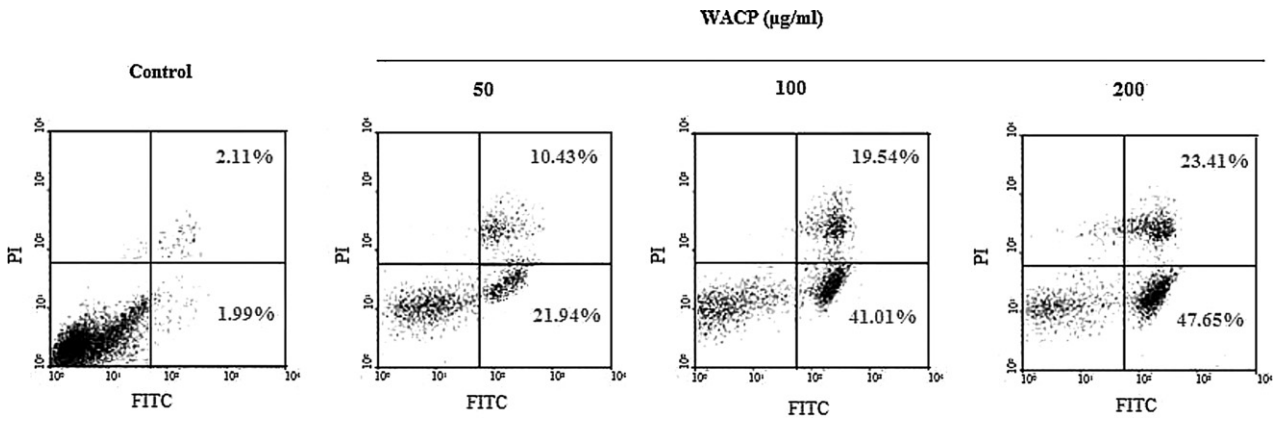


**Fig. 2.** Apoptosis in CNE-2 cells after 48 h treatment with WACP (50, 100 and 200 μg/ml) or PBS (control) by flow cytometry analysis.

harvested and assayed for apoptosis induction by flow cytometry. The results showed a significant amount of apoptotic cell death in the WACP-treated CNE-2 cells when compared with the normal cells (Fig. 2). Simultaneously, the decrease in the percentage of live cells was accompanied during incubation with all concentrations of WACP. All concentrations of WACP gave a higher percentage of apoptotic cells than necrotic cells. Moreover, typical morphological changes in CNE-2 cells associated with apoptosis such as cell shrinkage, membrane blebbing, cytoplasm aggregation and apoptotic body formation were more prominently observed 48 h after treatment with WACP at the concentration of 50, 100 and 200 μg/ml (Fig. 3).

### *3.4. WACP induces mitochondrial dysfunction and cytochrome c release in CNE-2 cells*

To evaluate the influence of WACP on the function of mitochondria, change in mitochondrial potential was analyzed by employing a fluorescent dye rhodamine 123. As shown in Fig. 4, WACP dose-dependently induced mitochondrial transmembrane depolarization, characterized as the decrease of mitochondrial membrane potential. In addition, WACP induced a significant increase in release of cytochrome c in the cytosolic fraction of CNE-2 cells in a dose-dependent manner, which was consistent with the changes of the mitochondrial membrane potential. These data indicated that WACP-induced apoptosis was accompanied by the

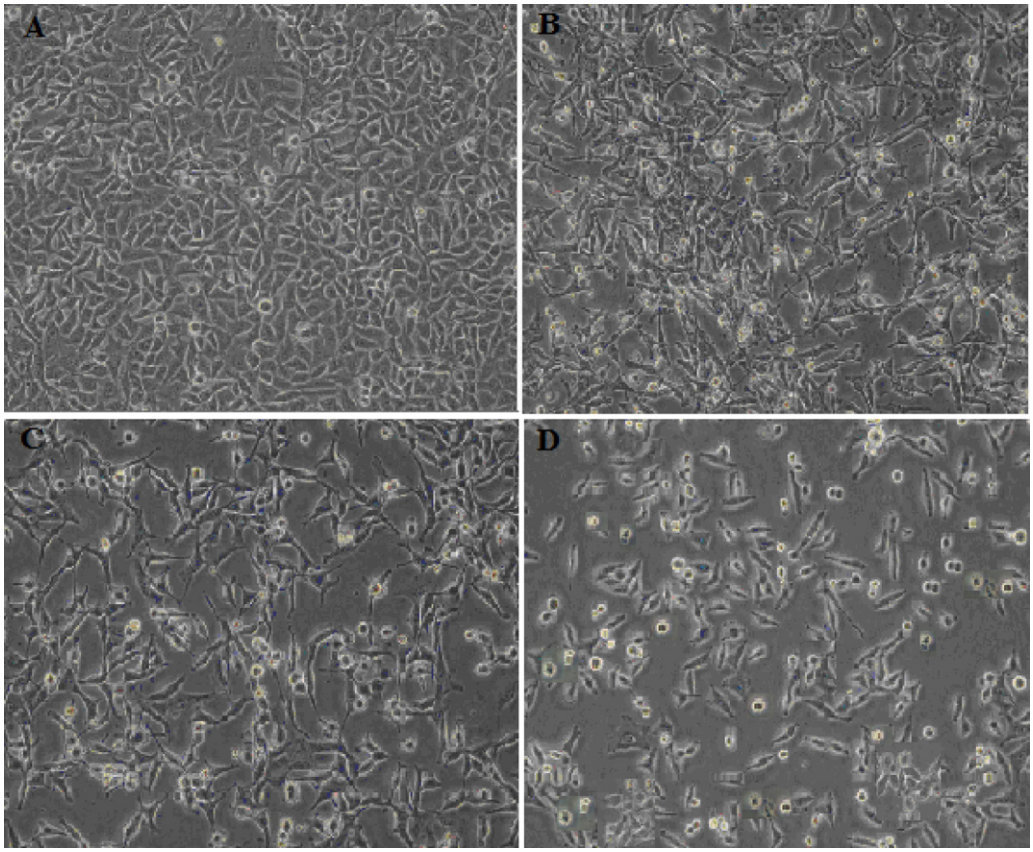


**Fig. 3.** Effect of WACP on cell morphological changes. CNE cells were treated with WACP at the indicated concentrations for 48 h, and the morphological changes were observed by inverted microscope. A: control; B: 50 μg/ml; C: 100 μg/ml; D: 200 μg/ml.

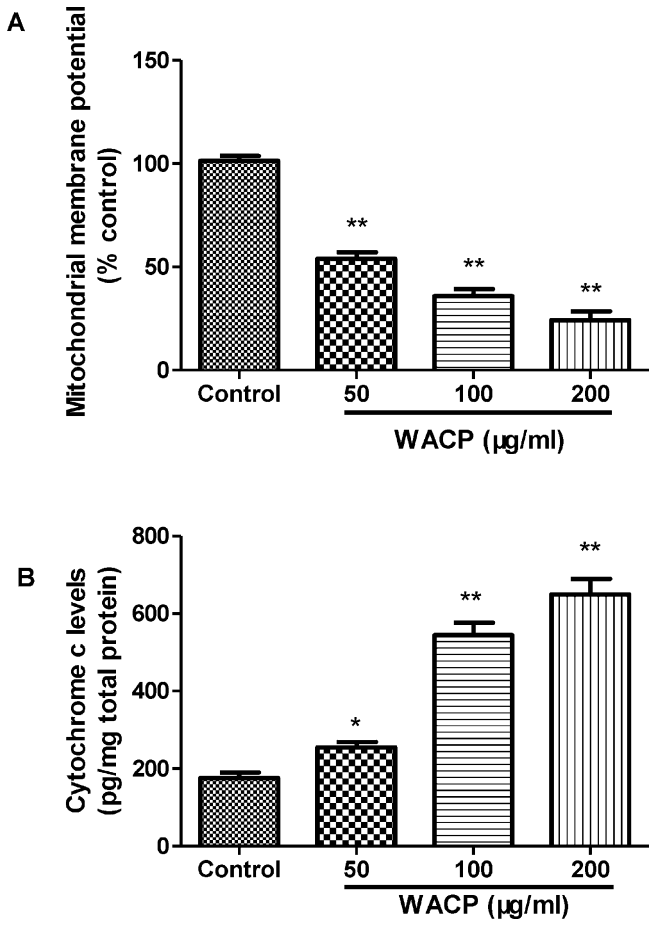


**Fig. 4.** Effect of WACP on mitochondrial membrane potential and cytochrome c release of CNE-2 cells. Each data are expressed as the mean ± SD obtained from triplicate experiments. (A) CNE-2 cells exposed to WACP (50, 100 and 200 μg/ml) or not for 48 h were incubated with rhodamine 123. Then, the fluorescence intensity was measured. (B) The released cytochrome c into cytosol in CNE-2 cells treated with WACP (50, 100 and 200 μg/ml) or not was determined by cytochrome c ELISA kit. Each data are expressed as the mean ± SD obtained from triplicate experiments. $^{*}p < 0.05$ and $^{*}p < 0.01$ versus the control.

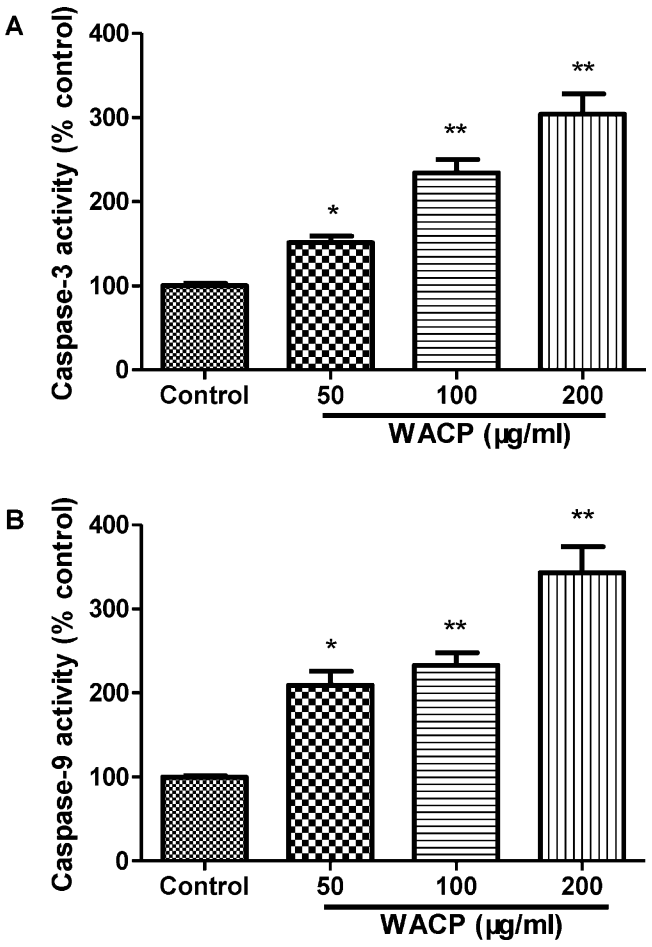


**Fig. 5.** Effects of WACP on (A) caspase-3 and (B) caspase-9 activation in CNE-2 cells. Each data are expressed as the mean ± SD obtained from triplicate experiments. $^{*}p < 0.05$ and $^{*}p < 0.01$ versus the control.

collapse of mitochondrial membrane potential, which would later lead to the release of cytochrome c and trigger the cleavage and activation of mitochondrial downstream caspases and the onset of apoptosis.

### *3.5. WACP induces the activation of caspase-3 and caspase-9 in CNE-2 cells*

To further elucidate whether caspase-3 and caspase-9 mediated the apoptosis induced by WACP, the activities of caspase-3 and 9 were determined using a fluorometric immunosorbent enzyme assay kit. Following 48 h treatment of CNE-2 cells with various concentrations of WACP, significant increases of both caspase-9 and -3 activities were detected and found to be in a dose-dependent manner compared with the control (Fig. 5), which was in parallel with the percentage of apoptotic cells in CNE-2 cells. These results indicated that the signaling cascade leading to apoptosis in WACP-treated cells involves the activation of common executors of apoptosis – caspase-3 and caspase-9.

## 4. Discussion

In this investigation, one neutral polysaccharide, WACP, was isolated from the *A. capillaris* through DEAE–cellulose column and Sephacryl S-400 column. WACP has a molecular weight of $5.8 \times 10^4$ Da and was mainly composed of arabinose and galactose in the ratio of 4:2. MTT assay proved for the first time that WACP had a potent anti-proliferation activity on human nasopharyngeal carcinoma CNE-2 cells. Similarly we found that WACP induced dose-dependent apoptosis in CNE-2 cells after 48 h incubation, indicating its potential application value in the therapy for cancer. Apoptosis involves a series of cellular biochemical events leading to a variety of characteristic morphological changes such as membrane blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation (Hickman, 1992; Kerr, Winterford, & Harmon, 1994). Microscopic observation showed the CNE-2 cells under WACP treatment displayed the characteristic apoptosis morphology. Furthermore mitochondria play an important role in apoptotic pathways by releasing cytochrome c, leading to the activation of the caspase cascade (Gil, Almeida, Oliveira, & Rego, 2003). The results demonstrated that WACP could disrupt the functions of mitochondria at the early stages of apoptosis and subsequently coordinate caspase-3 and caspase-9 activation through the release of cytochrome c.

Taken together, apoptosis, cytochrome c release, mitochondrial membrane depolarization, and caspase-3/9 activation were all observed in the cells treated with WACP. These results strongly indicated the involvement of mitochondria-mediated pathway in WACP-induced apoptosis in CNE-2 cells. The signaling pathway involves the loss of mitochondrial membrane potential, release of cytochrome c into cytosol, and activation of caspase-3/9 cascades

responsible for apoptosis. Further work should be performed using in vivo models to better understand the potential application of WACP in the management of NPC.

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