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A polysaccharide fraction of adlay seed (*Coix lachryma-jobi* L.) induces apoptosis in human non-small cell lung cancer A549 cells

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ABSTRACT

Different seed extracts from *Coix lachryma-jobi* (adlay seed) have been used for the treatment of various cancers in China, and clinical data support the use of these extracts for cancer therapy; however, their underlying molecular mechanisms have not been well defined. A polysaccharide fraction, designated as CP-1, was extracted from the *C. lachryma-jobi* L. var. using the ethanol subsiding method. CP-1 induced apoptosis in A549 cells in a dose-dependent manner, as determined by MTT assay. Apoptotic bodies were observed in the cells by scanning electronic microscopy. Apoptosis and DNA accumulation during S-phase of the cell cycle were determined by annexin V-FITC and PI staining, respectively, and measured by flow cytometry. CP-1 also extended the comet tail length on single cell gel electrophoresis, and disrupted the mitochondrial membrane potential. Further analysis by western blotting showed that the expression of caspase-3 and caspase-9 proteins was increased. Taken together, our results demonstrate that CP-1 is capable of inhibiting A549 cell proliferation and inducing apoptosis via a mechanism primarily involving the activation of the intrinsic mitochondrial pathway. The assay data suggest that in addition to its nutritional properties, CP-1 is a very promising candidate polysaccharide for the development of anti-cancer medicines.

1. Introduction

The ancient crop, coix (*Coix lachryma-jobi* L.; also known as adlay seed), is widely cultivated in the warm regions of Asia, Africa and the Mediterranean Rim. The grain is prepared by roasting and may be eaten dry, used as porridge, or processed into flour [1]. In addition to polysaccharides, coix is rich in protein, fat, carbohydrates, amino acids, vitamins and inorganic salts. In traditional Chinese medicine, coix is used as a diuretic, an anti-inflammatory drug, an anticancer drug, an analgesic, and a nutrient [2]. Studies have shown that coix contains a large number of lipopolysaccahrides, including palmitic acid, stearic acid, octadecadienoic acid, oleic acid, and linoleic acid [3], and it also contains oligosaccharides with free radical scavenging and other antioxidant properties [4].

A number of studies on the bioactivities of compounds isolated from coix have been reported. The methanol extract of adlay seed suppressed cyclooxygenase-2 (COX-2) expression in human lung cancer cells and displayed significant anti-proliferative effects [5]. The oil extract of adlay seed has been shown to inhibit fatty acid synthase (FAS), and it is used for anti-neoplastic therapy [6]. Other laboratories have revealed that dehulled adlay suppressed early events in colon carcinogenesis and also reduced COX-2 protein expression [7]. The polysaccharide of coix has been confirmed to possess hypoglycemic function [8] and to improve the immune system [9]. Therefore, we used coix polysaccharides obtained by specific extraction methods to elucidate its cellular anti-cancer mechanism in A549 cells by alkaline gel electrophoresis of single cells (comet assay), flow cytometric analysis and other methods.

2. Materials and methods

RPMI 1640 medium was purchased from Thermo (Beijing, China). Revert Aid First Strand cDNA Synthesis Kit was from Thermo (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Gibco GRL (Grand Island, NY, USA). Penicillin–streptomycin solution, trypsin, phosphate buffered saline (PBS), dimethyl sulfoside (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT), low-melting point agarose, normal-melting point agarose, and cell lysis solution were purchased from Solarbio (Beijing, China). Propidium iodide was purchased from Sigma–Aldrich (St. Louis, MO, USA). FITC Annexin V Apoptosis Detection Kit I was purchased from BD (Franklin Lakes, NJ, USA). RNeasy Mini Kit was purchased from Qiagen (Hilden, Germany). Antibodies to β-actin,

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caspase-3 and caspase-9 were purchased from Bioworld (St. Louis Park, MN, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

2.1. Isolation and purification of the CP-1 polysaccharide

The CP-1 polysaccharide was extracted from adlay seed (*C. lachryma-jobi* L.) by decoction and alcohol sedimentation techniques. Briefly, the seed was ground and mixed with distilled water at a grain:water ratio of 1:20 w/v. The mixture was incubated in a water bath at 90 °C with stirring for 3 h. After 3 h, the mixture was cooled to room temperature (25 ± 2 °C), filtered through a gauze and centrifuged at 4000 r/min for 15 min. The supernatant was collected, concentrated with a rotary evaporator, precipitated with four volumes of 75% ice-cold ethanol, and freeze-dried (Thermo Scientific, Rockford, IL, USA).

The molecular mass of CP-1 was determined to be 12 kDa by gel filtration. The percentage of total sugar was determined to be 88.5% by the phenol–sulfuric acid method. The optical rotation was [α] 20 D + 190. The component sugars of CP-1 were determined by gas chromatography (GC). The coixans were hydrolyzed with acid, reduced and acetylated, indicating that the neutral sugar components were rhamnose, arabinose, xylose, glucose and galactose. Using Fourier transform infrared spectroscopy (FTIR) (Bruker, Ettlingen, Germany), the structure of CP-1 was determined to possess a backbone composed of β-D-glucopyranosyl (Glcp) residues. Ultraviolet spectrophotometry (Agilent Santa Clara, CA, USA) confirmed the absence of protein and nucleic acid.

2.2. Cell culture

The human non-small cell lung cancer A549 cell line was routinely cultured in our laboratory. The cell line was grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1x penicillin/streptomycin (100 U/mL P + 0.1 mg/mL S) at 37 °C in a 5% CO_2 atmosphere.

2.3. Cell viability and proliferation

The effect of CP-1 on the viability of A549 cells was assessed by MTT assay [10]. Briefly, exponentially growing cells in 96-well plates were treated with different concentrations (10–300 μg/mL) of CP-1 in complete medium. Control cells were grown in medium not containing CP-1. MTT (20 μL, 5 mg/mL) was added after incubating the cells for 24, 48 or 72 h, following which the plates were incubated for 4 h, the medium was aspirated, and 150 μL of dimethyl sulfoxide (DMSO) were added into each well. The absorbance was measured at 570 nm on a 96-well microplate reader. All experiments were performed three times. The cell viability was calculated as follows:

$$\text{the ratio of cell viability } (\%) = \frac{A-B}{C-B} \times 100\%$$

where *A* is the average optical density of CP-1-treated cells, *B* is the average optical density of the control wells (culture medium without cells), and *C* is the average optical density of the negative control (culture medium containing DMSO).

2.4. Morphologic observations

A549 cells (5×10^6 cells/mL) were grown on cover slips in 6-well plates and treated with CP-1 at concentrations of 0, 100, 200 and 300 μg/mL. The morphological changes were observed under a scanning electron microscopic (SEM) (HITACHI, Tokyo, Japan).

2.5. Cell cycle analysis

A549 cells (1×10^6 cells/mL) were seeded in 100 mm dishes and exposed to CP-1 (0, 100, 200 and 300 μg/mL). The cells were washed in phosphate buffered saline (PBS) and collected by trypsinization, fixed in 70% glacial ethanol, washed in PBS, resuspended in 1 mL of PBS containing 50 U/mL RNase and 50 μg/mL propidium iodide (PI), and then incubated for 40 min in the dark at 4 °C. Cell cycle analysis was performed by flow cytometry (BD, Franklin Lakes, NJ, USA), and the population of cells in each phase was calculated using the the Modifit LT software program. Each experiment was conducted three times.

2.6. Flow cytometric analysis of apoptosis

A549 cells (1×10^6 cells/mL) were washed twice with cold PBS, resuspended in 1× Binding Buffer, and then stained with 5 μL of FITC Annexin V and 5 μL PI, which were included in the FITC Annexin V Apoptosis Detection Kit I, at RT (25 °C) for 15 min in the dark. The stained cells were analyzed by flow cytometry (BD, Franklin Lakes, NJ, USA) within 1 h. The experiments were repeated three times.

2.7. Measurement of DNA damage by the comet assay

DNA damage (caused by apoptosis) was evaluated by alkaline single cell gel electrophoresis (comet assay). The CP-1 treated and untreated cells were plated at a density of 1×10^6 cells per dish and left to adhere overnight. The cells were then trypsinized, resuspended in PBS and counted. 0.5% normal agarose in PBS (100 μL) prewarmed at 45 °C was dropped onto the slides and covered with a glass coverslip. The coverslips were removed after allowing the agarose to set at 4 °C for 10 min. Then, 10 μL of the cells were mixed with 75 μL of 0.7% low melting point agarose in PBS at 37 °C, spread onto the slides, and left for 10 min at 4 °C. A final layer of 75 μL of 0.7% low melting point agarose was applied in the same way. The slides from which the coverslips had been removed were immersed in chilled lysis buffer containing 10% DMSO at 4 °C for 2 h, then placed in electrophoresis buffer (1 mmol/L of EDTA, 300 mmol/L of NaOH, pH > 13) in a tank at 4 °C for 40 min to allow alkaline unwinding of the DNA. Electrophoresis was performed for 20 min at 25 V and 300 mA. The slides were then transferred to 0.4 mmol Tris-buffer (pH 7.5), washed three times and gently dried. Comets were stained with propidium iodide (2 μg/mL) and analyzed by confocal laser scanning microscopy (Nikon, Tokyo, Japan). Image analysis and tail length analysis were

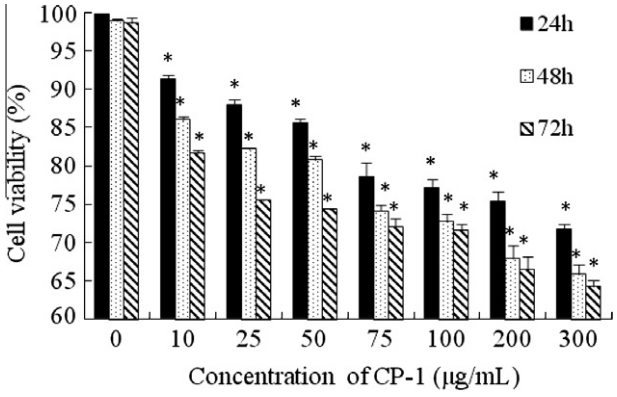

Fig. 1. Effect of CP-1 on the viability of A549 cells at different concentrations and time points ($n = 3$). *$P < 0.05$ vs. control.

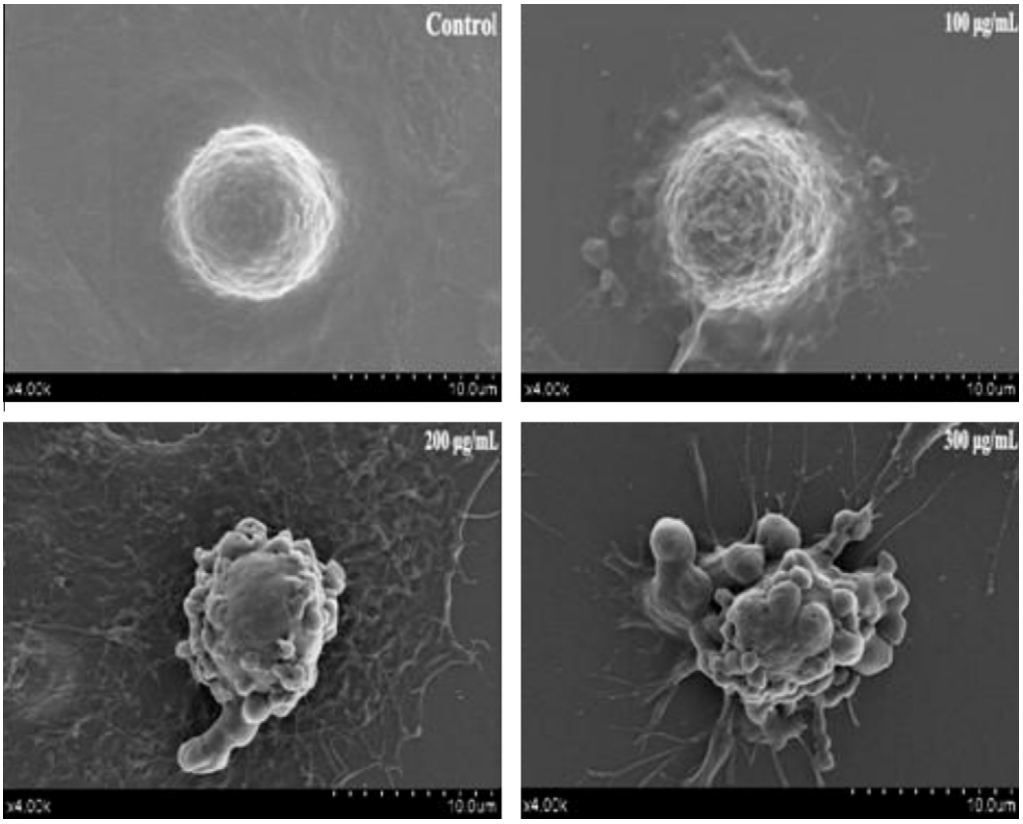

Fig. 2. SEM analysis of the morphology of A549 cells in response to treatment with CP-1. Control: dense microvilli with minor protrusions on the surface of the cells were seen. In the presence of 100 μg/mL CP-1, the microvilli became thinner, and the number of protrusions and their size increased markedly. In the presence of 200 μg/mL CP-1, wrinkles were formed on the cell surface. Following treatment with 300 μg/mL CP-1, apoptotic bodies were formed on and around the spherical cells.

performed with CASP 1.2.2 software (CaspLab, Germany), and 25 cells were randomly selected per sample.

2.8. Analysis of mitochondrial potential ($\Delta\Psi m$)

Changes in mitochondrial membrane potential ($\Delta\Psi m$) due to mitochondrial dysfunction were detected using rhodamine-123 [11]. Briefly, A549 cells (1×10^5 cells/mL) were incubated in the absence or presence of CP-1 (100, 200 and 300 μg/mL) in 100 mm dishes. The cells were trypsinized and washed with PBS, and then incubated at 37 °C for 10 min with rhodamine-123 (5 μg/mL). Finally, the cells were washed twice in PBS and observed by laser confocal scanning microscopy (Nikon, Tokyo, Japan) at an excitation wavelength of 488 nm.

2.9. Western blot analysis

A549 cells were treated with or without CP-1 as in other assays. The cells were washed with ice-cold PBS and lysed for 10 min in lysis buffer. Equal amounts (60 μg) of protein were separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose filters (GE, USA). After blocking in 5% non-fat milk in TBST buffer, the membranes were incubated overnight at 4 °C with primary antibodies to caspase-3 and caspase-9, followed by horseradish peroxidase-conjugated secondary antibodies. Bound antibodies were detected by analysis on an ImageQuant LAS4000 (GE, USA).

2.10. Statistical analysis

Each experiment was repeated at least three times. Numerical data are presented as the mean ± SEM. The difference between means was analyzed using one-way ANOVA. All statistical analyses were performed using SPSS 17.0 software (Chicago, IL, USA). $^*P < 0.05$ was considered significant.

3. Results

3.1. CP-1 inhibited the growth of A549 cells in vitro

MTT assays showed that A549 cell proliferation was significantly inhibited by CP-1 in a time- and concentration-dependent manner. Fig. 1 shows the viability of cells treated with CP-1 at various concentrations for 24, 48 and 72 h. The cell viability was 64.23% following CP-1 treatment at a concentration of 300 mg/mL for 72 h.

3.2. Morphological observations showed that CP-1 induced apoptosis in A549 cells

The morphology of A549 cells treated with or without CP-1 was studied by SEM (Fig. 2). Typical apoptotic morphological changes in response to CP-1 treatment were observed in A549 cells in a concentration-dependent manner, which suggested that CP-1 is an inducer of apoptosis in A549 cells.

3.3. Effects of CP-1 on the cell cycle distribution of A549 cells

The cell cycle distribution of A549 cells was analyzed by flow cytometry. The effect of CP-1 treatment for 48 h on cell cycle phase distribution is shown Fig. 3A. The sub-G_1 peak (apoptotic peak) was observed, which is normally regarded as one of the characteristics of apoptosis [12]. Compared with control cells, CP-1 caused

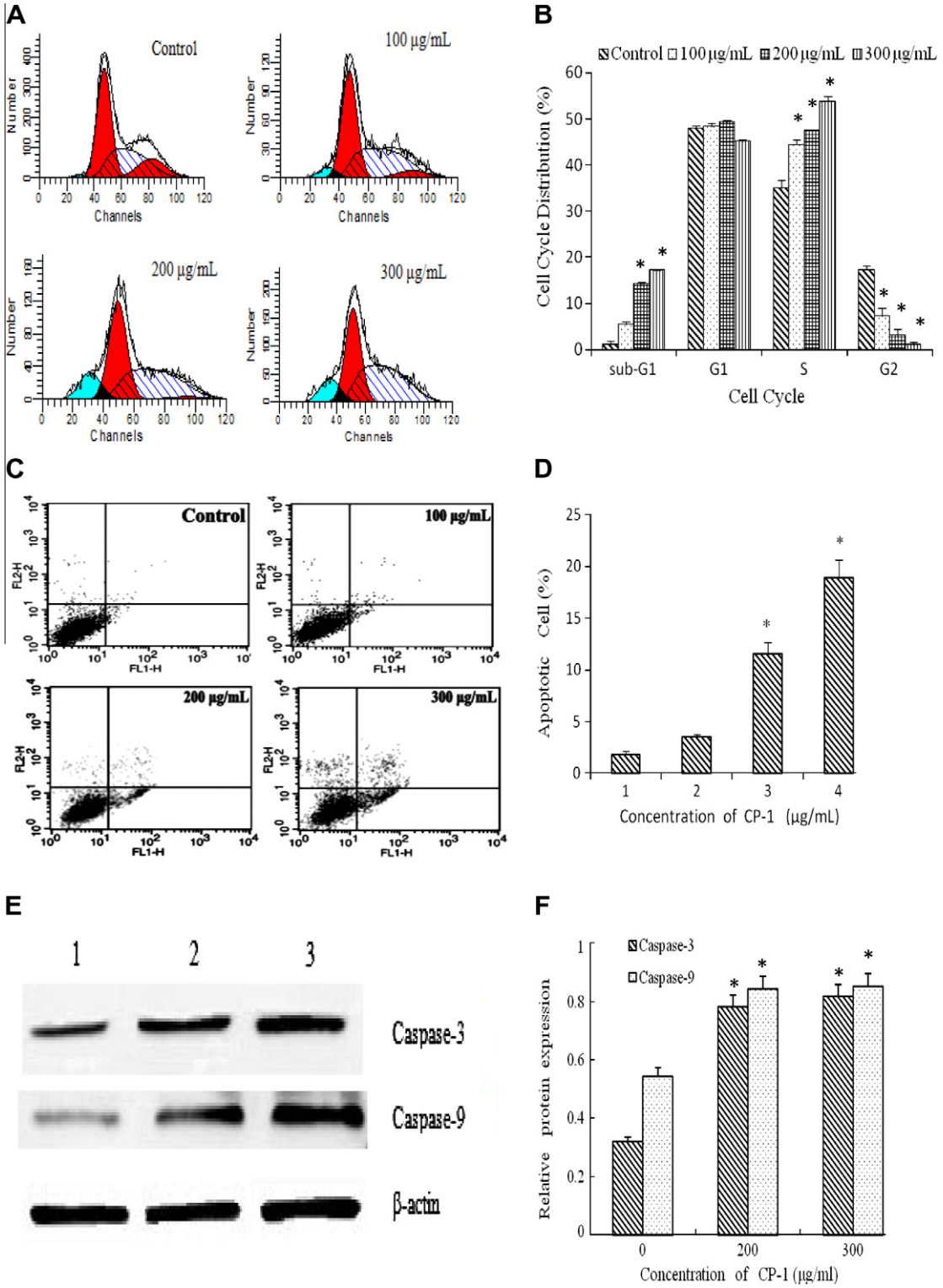

Fig. 3. Cell cycle distributions of A549 cells in the absence and presence of CP-1. (A) Flow cytometry histogram. (B) A DNA fluorescence intensity plot was used to determine the cell cycle distributions of A549 cells treated with CP-1 at concentrations of 0, 100, 200, 300 μg/mL. The sub-G_1 peak (apoptotic peak) was observed at CP-1 concentrations of 100, 200 and 300 μg/mL. (C) Apoptosis was assessed by flow cytometry after staining the cells with annexin V-FITC and PI. (D) Bar graph summarizing the percentage of apoptotic cells. (E) Lane M, marker; lane 1, control; lane 2, 200 μg/mL CP-1; lane 3, 300 μg/mL CP-1. A549 cells were treated with the indicated concentrations of CP-1. After 12 h, the cells were harvested and analyzed for the expression of caspase-3 and caspase-9 by western blotting. (F) Western blot showing the expression of caspase-3 and caspase-9. $^*P < 0.05$ vs. control.

the accumulation of cells in the S phase (Fig. 3A). In addition, the apoptotic fraction was markedly increased when CP-1 was applied. These results indicated that the reduced proliferation of A549 cells mediated by CP-1 was associated with cell cycle arrest in S phase.

3.4. CP-1 induced apoptosis in A549 cells

Investigation of the effect of CP-1 on apoptosis in A549 cells by annexin V-FITC/PI staining (Fig. 3C and D) showed that the

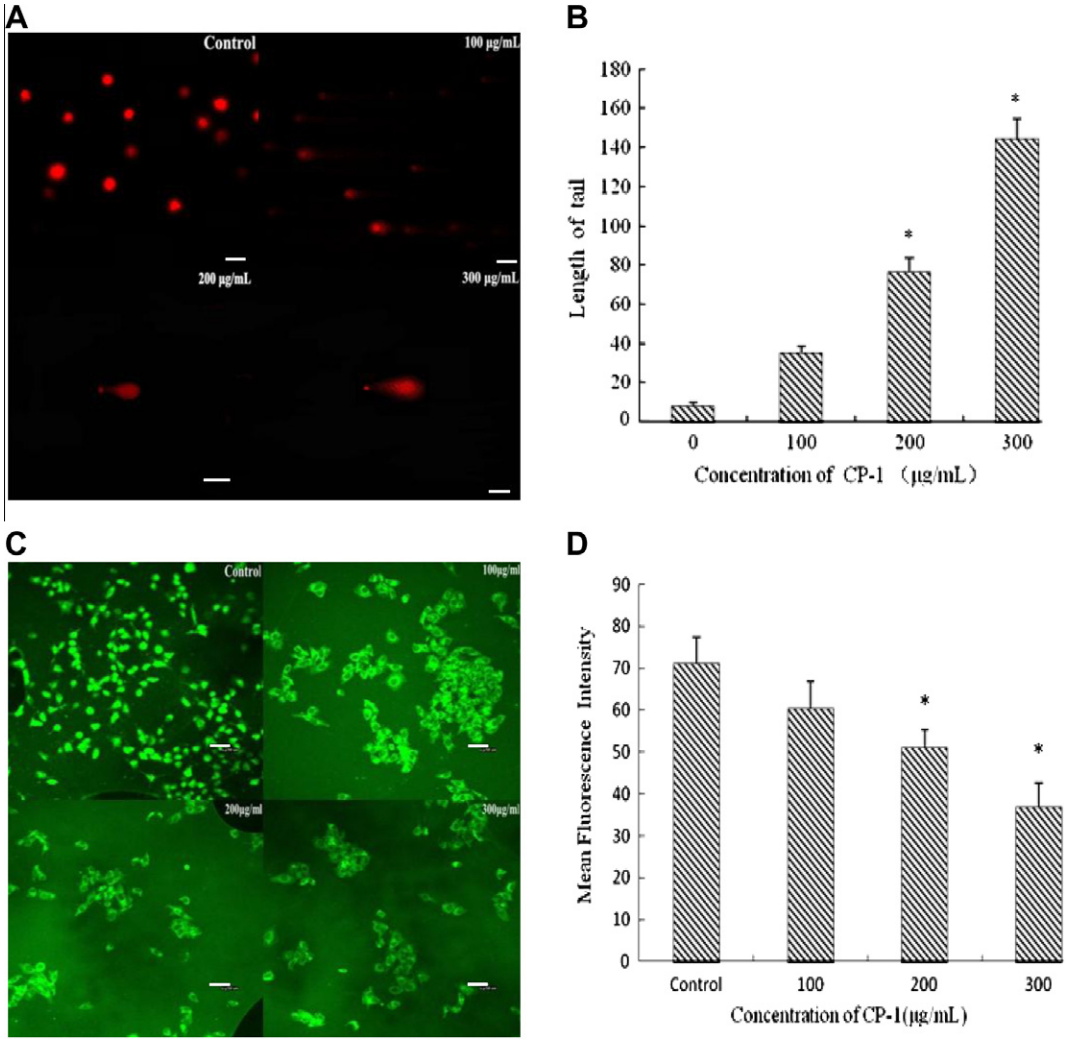

Fig. 4. (A) Comets were analyzed by confocal laser scanning microscopy at 543 nm. (B) The length of comet tails increased in the presence of 200 and 300 μg/mL CP-1. (C) Loss of the mitochondrial membrane potential ($\Delta\Psi m$) was observed at 488 nm (scale bar: 50 μM). (D) The mean fluorescence intensity of 20 cells selected at random was determined. $^{*}P < 0.05$ vs. control.

apoptotic ratio increased in CP-1-treated A549 cells compared with untreated cells (17.05 at 300 μg/mL). These results demonstrated that CP-1 suppressed A549 cell proliferation by inducing apoptosis.

3.5. Measurement of DNA damage by the comet assay

The extent of DNA damage was evaluated by the comet assay after incubating A549 cells in the presence or absence of CP-1 (Fig. 4A). CP-1 was shown to extend the length of comet tail length (Fig. 4B).

3.6. Analysis of mitochondrial membrane potential ($\Delta\Psi m$) in A549 cells

The mitochondrial membrane potential ($\Delta\Psi m$) of cells was measured using the dye, rhodamine-123 (Fig. 4C), and a decrease in mean fluorescence intensity was observed following treatment of the cells with CP-1. The bar graph demonstrates the loss of mitochondrial membrane potential ($\Delta\Psi$) (Fig. 4D) due to mitochondrial membrane depolarization, which is considered to be an initial and irreversible step of apoptosis [13]. The data indicate that apoptosis induced by CP-1 lead was accompanied by alterations in the mitochondrial membrane potential ($\Delta\Psi m$).

3.7. Analysis of caspase-3 and caspase-9 protein expression by western blotting

In order to understand the mechanism of CP-1-induced apoptosis, we examined the expression of caspase-3 and caspase-9 proteins. Western blotting revealed that caspase-3 and caspase-9 proteins increased dramatically in the 200 and 300 mg/mL CP-1-treated groups, compared with control cells (Fig. 3E and F). These results suggest that the increased expression of caspase family proteins may contribute to A549 cell apoptosis induced by CP-1.

4. Discussion

MTT assays showed that CP-1 inhibited the proliferation of A549 cells in a time- and concentration-dependent manner, with the highest inhibition observed at a CP-1 concentration of 300 μg/mL for 72 h. Cell cycle arrest in S phase and the induction of apoptosis in A549 cells by CP-1 was demonstrated by cell cycle analysis and annexin V-FITC/PI staining assays, respectively, using flow cytometry.

The single cell gel electrophoresis assay (also known as the comet assay) is a technique that detects DNA damage, including DNA strand breaks and alkali labile lesions, with high visual resolution

[14]. The movement of the DNA from the head to the tail has been described as the most obvious characteristic of apoptotic cells in the comet assay [15]. Our results showed that the different lengths of the heads and tails of the comets were proportional to the degree of DNA fragmentation. Furthermore, apoptotic cells were clearly distinguishable between the control and test groups in images from the comet assay.

The caspase gene family plays a central role in mitochondria-mediated apoptosis [16–18]. Caspase-3 contributes to the execution of apoptosis via the activation, hydrolysis and proteolysis of specific substrates such as DNA-dependent protein kinases [19]. Caspase-9 is the protease that activates caspase-3. Our western blotting analysis showed that CP-1 increased the expression of caspase-3 and caspase-9, suggesting that coix polysaccharides mediate apoptosis via a caspase-dependent pathway. Furthermore, the disruption of mitochondrial membrane potential, which typically leads to the activation of caspase-3 and caspase-9 [20], by CP-1, further suggested that a mitochondria-dependent pathway was involved in the induction of apoptosis by CP-1.

The CP-1 polysaccharide fraction interferes with the growth, metabolism and proliferation of non-small cell lung cancer A549 cells, thus leading to the induction of apoptosis in these tumor cells. The mechanism by which CP-1 induces apoptosis in A549 cells is worthy of further investigation because of its potential anti-tumor effect.

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