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Exopolysaccharides from *Rhizopus Nigricans* Inhibit Proliferation and Induce Apoptosis in Human Lung Adenocarcinoma A549 Cells

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Abstract
Objective: To analyse the effects of an exopolysaccharide (EPS2-1) obtained from the fermentation broth of *Rhizopus nigricans* on the viability and apoptosis of human lung adenocarcinoma (A549) cells.
Methods: Lung cancer cells were cultured *in vitro* and divided into the control and exopolysaccharide-treated groups. CCK-8 and colony formation assays were performed to measure the proliferation of A549 cells. Caspase activity was measured using a colorimetric assay. The apoptosis rate, reactive oxygen species (ROS) production, and mitochondrial membrane potential (MMP) were evaluated using flow cytometry.
Results: EPS2-1 effectively suppressed A549 cell proliferation ($p < 0.05$). At the same time, EPS2-1 significantly increased the enzyme activity of caspase-8 and caspase-3 compared with that in the control group ($p < 0.05$) and facilitated the apoptosis of A549 cells. Furthermore, EPS2-1 increased the accumulation of intracellular ROS and induced a reduction in MMP in A549 cells. These findings suggest that EPS2-1 initiated apoptosis in A549 cells via the mitochondrial pathway.
Conclusion: EPS2-1 significantly inhibited A549 cell proliferation and induced apoptosis via ROS accumulation and mitochondrial dysfunction, suggesting its potential as an anti-cancer agent. This study provides the first evidence that *Rhizopus nigricans*-derived polysaccharides exhibit induce-apoptosis activity on A549 cells through ROS-mediated mitochondrial dysfunction, establishing a novel pharmacological foundation for fungal polysaccharide research and development of natural antitumor agents.
Keywords: *Rhizopus stolonifer*, exopolysaccharide, lung neoplasms, apoptosis

Introduction

Lung cancer is the leading cause of cancer-related deaths, with non-small cell lung cancer (NSCLC) accounting for 80% of lung cancer cases.[1,2] Although many treatment strategies are used to treat lung cancer, including chemotherapy, surgery, and radiotherapy, their efficacy remains poor. Therefore, the development of effective anti-lung cancer drugs with high biological activity and low toxicity is essential.[3]

Apoptosis is a vital physiological process in the development and growth of the body.[4] The evasion of apoptosis perturbs the balance between cell growth and death, resulting in the occurrence of cancer. Therefore, triggering apoptosis is an effective drug target for therapeutic intervention of cancers.[5,6]

Polysaccharides are macromolecular polymers derived from microorganisms, plants, and animals, which have excellent bioactivity and biocompatibility.[7–9] The immunomodulatory,[10] anti-inflammatory,[11] and antioxidant[12] activities of natural polysaccharides have been widely applied in the medical and health product industries. Researchers have successfully isolated numerous anti-cancer polysaccharides from species such as *Dendrobium officinale*,[13] *Astragalus*,[14] and *Ganoderma lucidum*.[15] Polysaccharides exert anti-cancer effects by suppressing cell proliferation, inducing apoptotic cell death, triggering autophagy in tumour cells, reducing the metastasis of cancer cells, balancing the tumour microenvironment, protecting the intestinal barrier, and enhancing immunity. Moreover, the combination of glycans and chemotherapy drugs can improve their anti-cancer activities and reduce the incidence of side effects involving the immune system.[16] Fungal polysaccharides are classified into yeast glycans, mold glycans, and mushroom glycans. In addition, fungal glycans exist in the fruiting bodies, fermentation broth, and mycelia. Fungal polysaccharides can effectively suppress growth and trigger apoptosis in tumour cells through intrinsic mitochondrial signalling pathways and external apoptosis provoked by tumour necrosis factor.[17] In summary, due to their excellent biological activities and low toxicity, these agents may have potential as antitumour drugs.

In 2015, Yu et al. purified a homogenous exopolysaccharide (EPS1-1) from *R. nigricans* with a Mw of 9.7×10^3 g/mol. At the same time, they found EPS1-1 inhibited the proliferation of human colorectal cancer HCT-116 cells, and induced S phase cell cycle arrest and apoptotic cell death through mitochondrial signalling pathway.[18] Furthermore, EPS1-1 can suppress the viability and invasion of MCF-7 cells through the Akt signalling pathway,[19] inhibit inflammatory injury of gastric mucosa cells, and restrain the development of chronic atrophic gastritis.[20] Recently, our group purified the novel exopolysaccharide EPS2-1 from *R. nigricans*, which has a weight average molecular weight of 32.083 kDa.[21] EPS2-1 inhibited the growth of mouse forestomach carcinoma cells and increased the growth of macrophages. In addition, EPS2-1 exhibited antioxidant activity. However, the effects of EPS2-1 on apoptosis have not yet been elucidated. In the present study, we investigated the apoptotic activity of EPS2-1 in A549 cells and elucidated the potential molecular mechanism involved.

Materials and Methods

Materials and Reagents

All the experiments were conducted using A549 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China).

Cell line authentication was confirmed by STR profiling and mycoplasma contamination testing was performed prior to use. Foetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and phosphate-buffered saline (PBS) were obtained from Thermo Fisher Scientific (Waltham, New York, USA). Dimethyl sulfoxide (DMSO) was purchased from Solarbio (Beijing, China). The crystal violet staining solution, trypsin, CCK-8, cell apoptosis, caspase activity, reactive oxygen species (ROS), and mitochondrial membrane potential (MMP) detection test kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Extraction and Purification of EPS2-1

EPS2-1 was obtained according to our previously reported method.[21] The *R. nigricans* fermentation broth was filtered and precipitated with four volumes of 95% ethanol overnight. The resulting precipitate was re-dissolved in distilled water, deproteinized using the Sevag method, and decolorized via D301R resin chromatography. Crude polysaccharides were fractionated on a DEAE-52 Sepharose column (eluted with Tris-NaCl buffer) to yield EPS-1 and EPS-2. EPS2-1 was separated from EPS-2 using Sephadex G-100 and G-75 columns.

Cell Culture and Viability Assay

A549 cells were maintained in DMEM.[22] The cytotoxic effects of the polysaccharides were measured using a CCK-8 assay according to a previously reported method.[23] After being treated with EPS2-1 for 1 and 2 days, cells were incubated with CCK-8 for 120 min. Then, their absorbance was measured at 450 nm by Microplate Reader (Thermo Fisher Scientific, Waltham, New York, USA).

Colony Formation Assay

We measured cell proliferation using a colony formation test. The cancer cells were plated in six-well plates and incubated with EPS2-1 for 7 days. After washing with PBS, methanol and crystal violet were added to stain the colonies.[24]

Measurement of Apoptosis

A549 cells were incubated with EPS2-1 (0.1–0.3 mg/mL) in six-well plates for 24 h and washed with PBS. Cells (10×10^4) were double-stained with Annexin V-fluorescein isothiocyanate (FITC) (200 µL) and PI (propidium iodide) (10 µL) for 20 min at room temperature. Finally, A549 cells were analysed using flow cytometry (BD Biosciences, San Jose, CA, USA).

Caspase Activity Assay

The caspase activity was detected using a previously reported method.[25] After being treated with EPS2-1 (0.1–0.3 mg/mL) for one day in a six-well plate, cancer cells were washed with PBS and lysed. Then, lysates were centrifuged at 10000 × *g* for 15 min, and 10 µL of lysates were incubated with 2 mM substrates of caspases for 2 h.[26] Then the release of *p*-nitroanilide (*p*NA) was quantified using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 405 nm. In addition, caspase activity was expressed as the fold-change compared with the values obtained for the control group.[27]

ROS Detection

ROS was measured using DCFH-DA as previously described.[28] Briefly, cancer cells were incubated with EPS2-1 (0.2–0.6 mg/mL) for 1 d. Next, 1 mL of DCFH-DA was incubated with A549 cells for 0.5 h. After staining, the harvested cells were washed with DMEM to remove the residual probes. Finally, flow cytometry was used to detect the ROS content.

Mitochondrial Membrane Potential (MMP) Detection

After being treated with EPS2-1 (0.2–0.6 mg/mL) for 1 d, A549 cells were treated with 1000 µL JC-1 probe for 20 min. Staining buffer solution was used to wash the cancer cells three times, and lung cancer cells were analysed using flow cytometry.[29]

Statistical Analysis

GraphPad Prism 8.0 (San Diego, CA, USA) was used to analyse the experimental data. One-way ANOVA and the *t*-test were used to determine statistically significant differences. Experimental data were expressed as the mean ± standard deviation (SD), and statistical significance was defined as P values < 0.05.

Results and Discussion

EPS2-1 Reduced the Viability of A549 Cells

Several polysaccharides reduce cancer cell viability. For instance, polysaccharides from red ginseng,[30] *Bletilla striata*,[31] and *Echinodontium tinctorium*[32] have been shown to inhibit tumour cell growth. To evaluate the growth inhibition ability of EPS2-1, tumor cells were incubated with various concentrations of EPS2-1 for 1 and 2 days and then analysed. The results showed that the viability of A549 cells was 77.29%, 45.49%, 63.25%, 54.56%, and 59.47% after incubation with exopolysaccharides at concentrations ranging from 0.2–1.0 mg/mL for 1 day (Figure 1A). Furthermore, the survival rate of A549 cells was 113.87%, 111.32%, 69.43%, 49.31%, and 39.71% after incubation with polysaccharides (0.1–0.8 mg/mL) for 2 days (Figure 1B). These data demonstrated that EPS2-1 effectively suppressed the proliferation of A549 cells, and the inhibitory activity of EPS2-1 on cell growth was better than that of EPS1-1. In addition, after treatment with EPS2-1 for 7 days, the colony formation assay showed that EPS2-1 significantly suppressed ($P < 0.05$) the formation of cancer cell colonies (Figure 1C and 1D). The observed discrepancy in the inhibitory activities of EPS2-1 and EPS1-1 may be attributed to their structural differences. EPS2-1 has a heteropolysaccharide structure with a molecular weight of 32.8 kDa, consisting of mannose, galactose, glucose, arabinose, and fucose. Structural analysis revealed the backbone of EPS2-1 was →2)-α-D-Man*p*-(1→2,6)-α-D-Man*p*-(1→3,6)-β-D-Gal*p*-(1→3,6)-β-D-Gal*p*-(1→. EPS1-1 demonstrated different characteristics, with a lower molecular weight (9.7 kDa), and it was primarily composed of glucose, mannose, galactose, and fructose. The structural complexity of these polysaccharides potentially confers their increased propensity to interact with apoptosis-related receptor systems.

Induce-apoptosis Activity of EPS2-1 on A549 cells

The promotion of apoptosis is a key mechanism of action for antitumour drugs. Annexin V/PI can detect apoptosis in A549 cells.[33] As displayed in Figure 2A, the total populations of apoptotic cells were 5.12%, 15.04%, and 24.5% after treatment

with 0.1, 0.2, and 0.3 mg/mL EPS2-1, respectively. Our experimental data indicated that EPS2-1 triggered apoptosis in A549 cells, suggesting that its inhibitory effect on proliferation is relevant to the induction of apoptosis. Our results align with previous studies on fungal exopolysaccharides, such as exopolysaccharides from *Trichoderma pseudokoningii*, which similarly increased the proportion of total apoptotic cells from 2.45% to 32.1%, 35.1%, and 46.4% at concentrations of 0.25, 0.5, and 1.0 mg/mL.[29] Therefore, fungal glycans are expected to become promising anti-cancer agents.

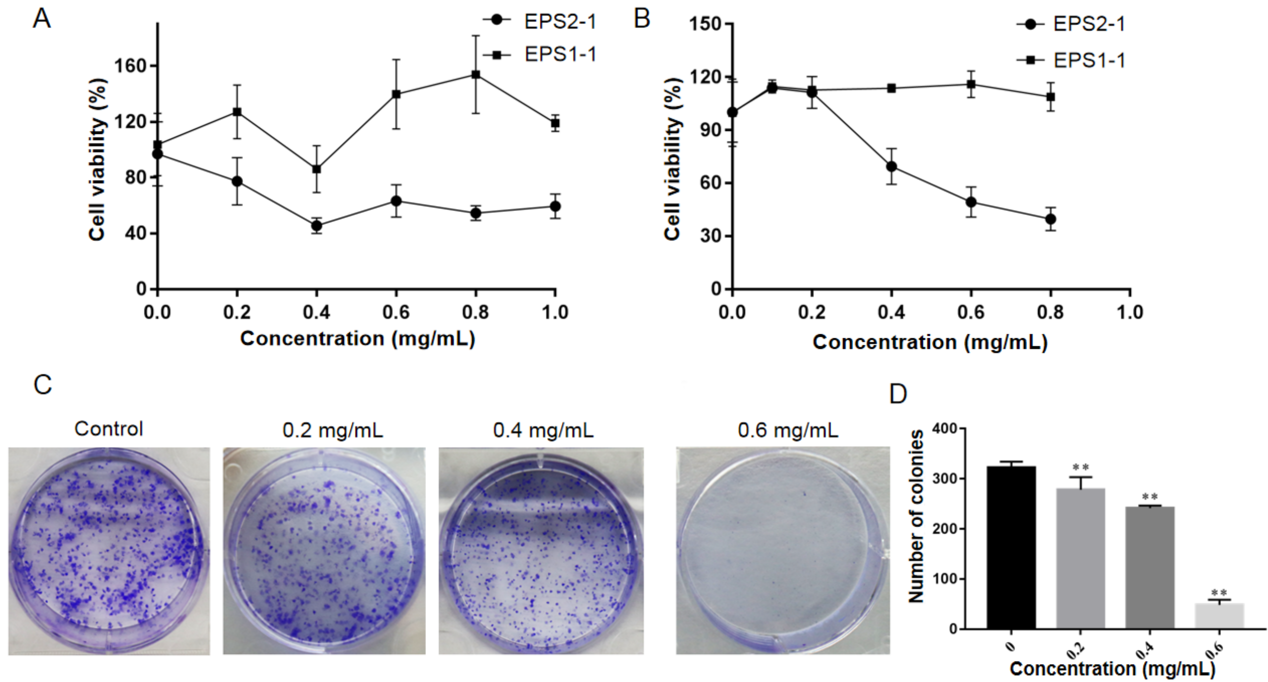

Fig. 1 **EPS2-1 reduced A549 cell proliferation, as determined using CCK-8 and colony formation assays (*P* < 0.05). (A, B) The cell proliferation was evaluated using a CCK-8 assay. (C, D) Tumour cells were incubated with polysaccharides for 7 days, and the numbers of colonies were recorded. **P* < 0.05 and ***P* < 0.01.**

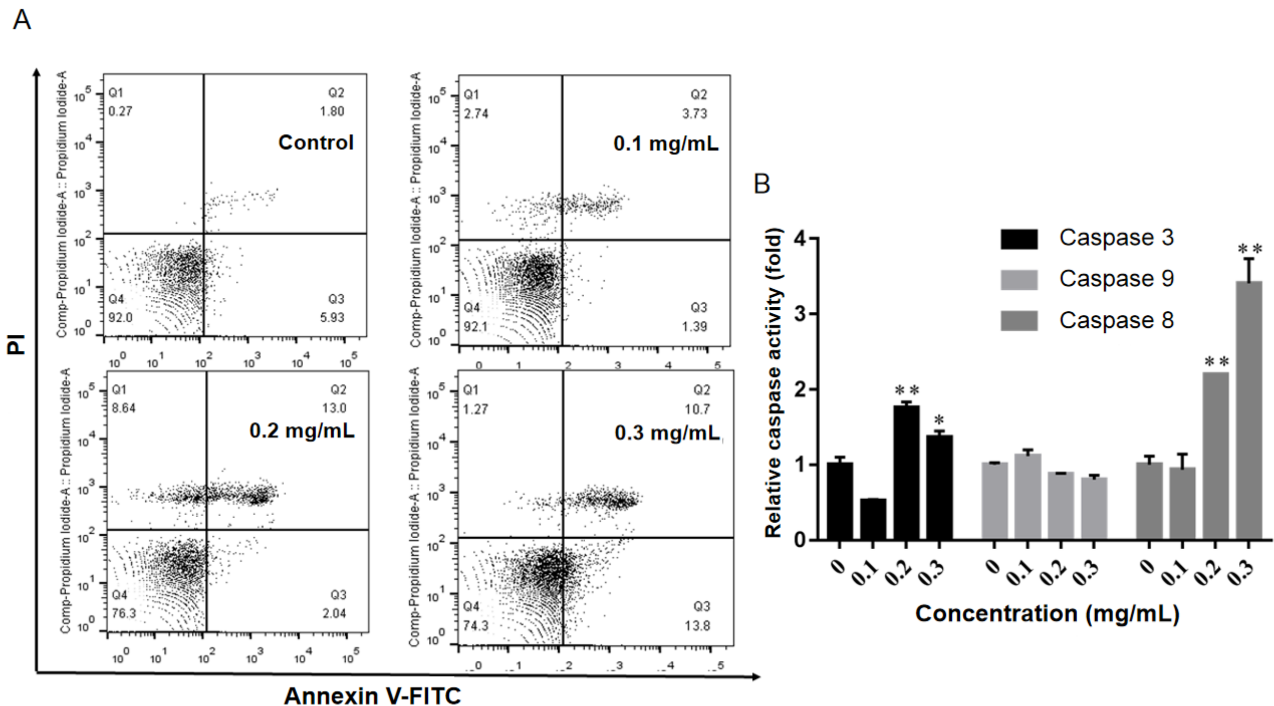

Fig. 2 **EPS2-1 triggered apoptosis in A549 cells, as determined using apoptosis rate and caspase activity assay (*P* < 0.05). (A) A549 cells were stained with Annexin V-FITC (fluorescein isothiocyanate) /PI and evaluated using flow cytometry. (B) The activities of the caspases were measured using colorimetric analysis. **P* < 0.05 and ***P* < 0.01.**

EPS2-1 Activated Caspase Activities in A549 Cells

Caspases can be classified as apoptosis activators and executioners; caspases 8, 9, and 10 are essential activators, and caspases 3, 6, and 7 are classic executioners. The activation of caspase-9 can activate caspase-3, resulting in the disintegration of nuclear proteins and the cytoskeleton.[34,35] Although many fungal polysaccharides show anti-cancer effects, their molecular mechanisms differ. To confirm whether EPS2-1-induced apoptosis was related to caspase cascade activation, we measured the enzyme activity of caspases using a colorimetric assay. As shown in Figure 2B, EPS2-1 significantly increased the enzyme activities of caspase-8 and caspase-3 compared with those in the control group ($P < 0.05$) at concentrations of 0.2 and 0.3 mg/mL. This result was different from the findings of a previous report; a polysaccharide from *T. pseudokoningii* increased the enzyme activity of caspase-9 and caspase-3 and induced apoptosis in breast cancer cells, whereas the activity of caspase-8 remained unchanged.[29]

Induction of ROS Generation by EPS2-1

The accumulation of ROS can induce apoptosis in cells, which occurs in the mitochondria.[36] Mitochondria remain a key source of ROS, which can induce damage to DNA, proteins, and cell membranes. ROS are cleared in the body by enzymes such as superoxide dismutase and glutathione. In addition, ROS generation can directly induce MMP loss.[37] Fungal polysaccharides mediate apoptosis through ROS accumulation. Pharmacological studies have demonstrated that *Hericium erinaceus* polysaccharides dose-dependently elevate ROS levels in colorectal cancer cells (DLD1 and HCT116), thereby activating mitochondrial apoptosis via caspase-9 and Bcl-2/Bax axis modulation.[28]

To study the role of ROS in the apoptosis of A549 cells, we determined the ROS content in A549 cells using flow cytometry. The experimental data (Figure 3A) indicated that the levels of intracellular ROS in the lung cancer cells increased with increasing concentrations of EPS2-1. Our

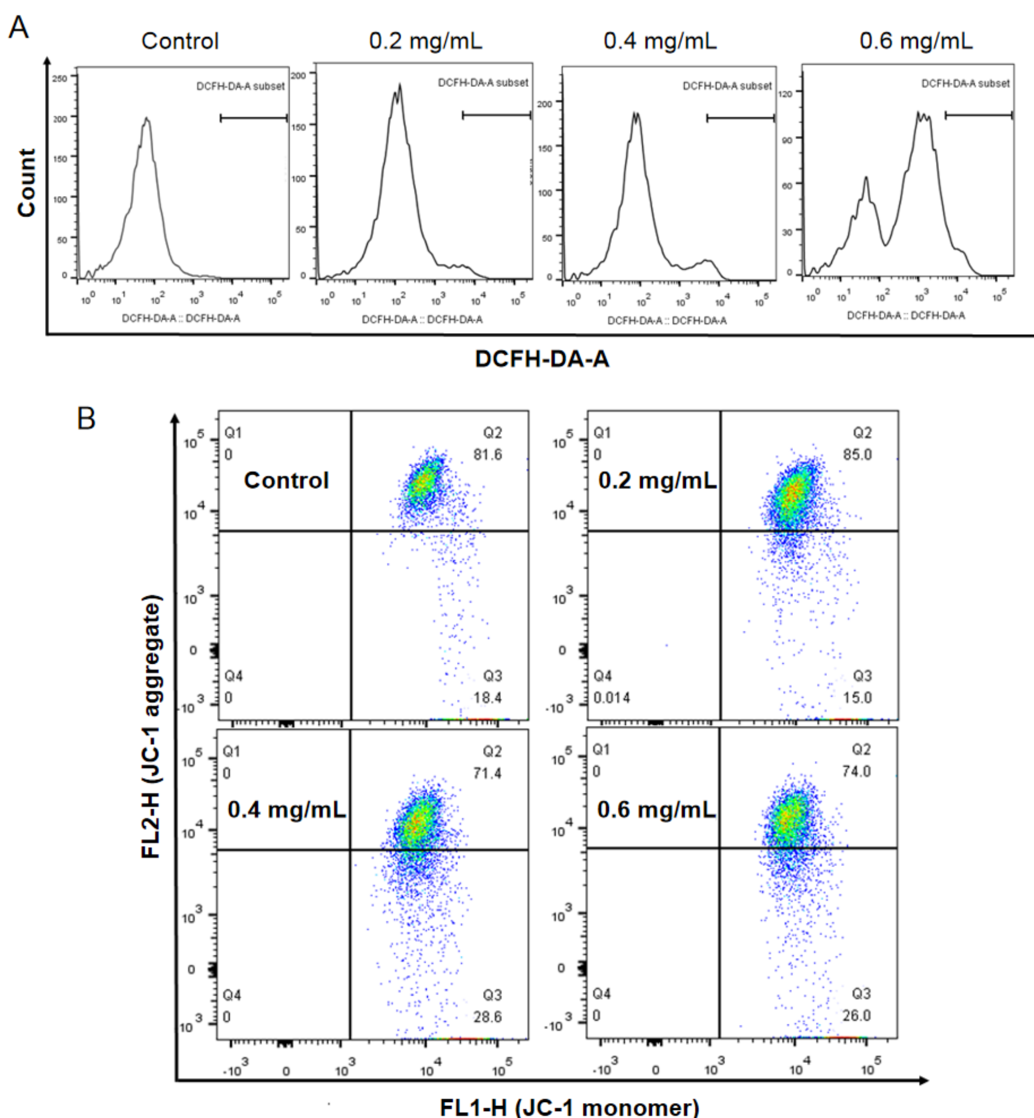

Fig. 3 **EPS2-1 induced the generation of ROS and disruption of the MMP in lung cancer cells. Cells were incubated with EPS2-1 for one day and then were harvested. (A) Cells were incubated with probes and then evaluated using flow cytometry. (B) A549 cells were incubated with JC-1 probes and then measured.**

data showed that EPS2-1 increased the accumulation of intracellular ROS in A549 cells. Therefore, the accumulation of intracellular ROS may constitute a shared mechanism by which fungal polysaccharides induce apoptosis in tumour cells.

EPS2-1 Induced the Loss of MMP

Apoptosis occurs through both extrinsic and intrinsic pathways. The intrinsic pathway, driven by ROS and MMP collapse, involves Bax oligomerization antagonized by Bcl-2, which triggers cytochrome c release. This activates caspase-9 via apoptosome formation and propagation of effector caspases (caspase-3 and caspase-7). The Bcl-2/Bax ratio determines mitochondrial permeability. The extrinsic pathway initiated by death receptors activates caspase-8 to cleave executioner caspases. Both pathways converge upon caspase activation, with intrinsic signalling central to ROS-mediated apoptosis.[38,39]

To determine whether apoptosis in A549 cells is related to the loss of MMP, flow cytometry was performed. Figure 3B showed that the amount of green fluorescence-positive cells was increased from 18.4% in untreated cells to 28.6% and 26% in the exopolysaccharides treated group (0.4 and 0.6 mg/mL). In summary, the experimental results demonstrated that EPS2-1 induced ROS accumulation, resulting in MMP collapse and subsequent activation of the caspase cascade. These findings suggest that EPS2-1 may initiate apoptosis via the mitochondrial pathway. It should be noted that the present study did not investigate the expression levels of apoptosis-related proteins (e.g., Bcl-2, Bax, and cleaved caspase-9). The precise molecular mechanism underlying EPS2-1-induced apoptosis, particularly regarding mitochondrial pathway regulation, remains to be elucidated through proteomic analysis and siRNA validation experiments.

Conclusion

In the present study, we found that EPS2-1 suppressed the viability and colony formation of A549 cells. EPS2-1 activated caspase-8 and caspase-3, and induced apoptosis in lung cancer cells. Furthermore, EPS2-1-induced apoptosis in lung cancer cells was related to MMP depletion and increased ROS production. This is the first study to demonstrate the anti-lung cancer activity of polysaccharides from *Rhizopus nigricans*. The molecular mechanism involved in the apoptosis induced by EPS2-1 requires further research. In summary, the findings of the present study suggests that EPS2-1 is a promising antitumour agent.

Acknowledgments

Not applicable.

Ethics Approval and Consent to Participate

As this study did not involve the use of human or animal subjects, no ethical approval was required or sought.

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Disclosure of Conflict of Interest

None.

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