H9 Induces Apoptosis via the Intrinsic Pathway in Non-Small-Cell Lung Cancer A549 Cells

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Introduction

The main characteristics of cancer are abnormal growth, invasion, and metastasis. Cancer cells move around the body through blood vessels and lymphatic ducts. Globally, lung cancer is a leading cause of cancer-related deaths [14], and it can be categorized into two histological groups: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). Roughly 85% of all lung cancers are categorized as NSCLC, which has a 5-year survival rate [15]. The high mortality of this cancer type is due to difficulties in early diagnosis and its great potential to invade locally and metastasize to distant organs [21]. Many treatments are available to extend patient life and remove the cancer, including surgery, radiation, and drugs [30]. However, these therapies often lead to diminished quality of life owing to side effects. Herbal extracts have been used as alternative remedies for various diseases because they have fewer side effects than commercial anticancer agents [24]. The traditional/medicinal herbal extracts consist of many metabolites, such as flavonoids, glycosides, polyphenols, and other secondary metabolites that offer a variety of benefits, including antibacterial, antiallergic, antioxidative, and anticancer effects [3, 17]. As an alternative therapy, herbal extracts have been an important source of cancer treatments, some of which are currently used in clinical...
practice [8]. In recent years, natural herbal extracts have been extensively screened to develop potential anticancer agents [6, 25, 34].

Among the molecular mechanisms, apoptosis is a highly controlled process of cell death. Apoptotic cells have distinguishable changes, such as chromatin condensation, nuclear fragmentation, and blebbing [23]. Apoptosis involves both intrinsic and extrinsic pathways that serve to eliminate heavily damaged cells. Over the years, apoptosis has been shown to be the major mechanism to eliminate cancer cells [9]. Traditional herbs have been confirmed to have anticancer benefits through apoptosis [1].

In these studies, we investigated the anticancer effect of H9 on human lung cancer cells. To this end, this study aimed to assess H9-mediated toxicity and apoptosis in A549 cells.

Materials and Methods

Reagents and Antibodies
CellTiter 96 AQ One Solution Cell Proliferation Assay Reagent (MTS; 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) was purchased from Promega (Madison, WI, USA). Propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) stain and were purchased from Sigma (St. Louis, MO, USA). NE-PER Nuclear and Cytoplasmic Extraction Reagents were purchased from Pierce (Rockford, IL, USA). Antibodies specific to PARP, caspase-3, caspase-9, p53, Bcl-2, Bcl-xL, Bax, proliferating cell nuclear antigen, and cytochrome C were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody and anti-mouse IgG HRP-conjugated secondary antibody were purchased from Millipore (Billerica, MA, USA).

Reagents and Antibodies

Plant Materials
Oriental Herbal Materials, Dongguk University (Ilsan, Korea) were authenticated by Dr. Seong-Hyun Jung (Department of Oriental Medical Hospital, Dongguk University). The Oriental name, Grams, and % of the main ingredients of H9 and their proportions (w/w) were determined as follows: 12% Psoraleae Semen, 8% Evodia Fruit, 12% Fennel, 12% Nutmeg, 20% Ginseng, 8% Alpiniae Officinarum Rhizome, 4% Sparganium Rhizome, 12% Curcuma Root, and 12% Cinnamon Bark. The herbal ingredients were obtained from the Oriental Medical Hospital, Dongguk University (Ilsan, Korea) and were authenticated by Dr. Seong-Hyun Jung (Department of Oriental Herbal Materials, Dongguk University).

Methods of Extraction

The ethanol extracts from the plants listed above were prepared as follows. The dried and pulverized medicinal herbs were mixed together. Materials of 8 kg were soaked with 40% ethanol, and then extracted for 3 h at 90–100°C. This extract was filtered through a 11 µm microfilter, extracted for 3 h at 90–100°C, vacuum evaporated at 60°C using a rotary evaporator, lyophilized, and then reconstituted in distilled water for in vitro studies.

Cell Culture

We purchased human NSCLC cell line A549 cells from the American Type Culture Collection (ATCC; Rockville, MD, USA). These cells were cultured in RPMI medium (Welgene Incorporation, Daegu, Korea) containing 10% (v/v) heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT, USA). The cells were incubated at 37°C in saturating humidity of 5% CO₂/95% air.

Cell Viability Assays

Cell viability was observed by MTS dye-reduction assay, which measures the mitochondrial respiratory function. The A549 (1.0 × 10⁴) cells were seeded in medium in 96-well plates, incubated overnight, and then treated with various concentrations of H9, as mentioned in the figure legends, for 24 h. Cell viability was calculated by MTS metabolism, as previously reported [1]. In brief, the effect of H9 on cell viability was assessed by an electron-coupling reagent containing MTS and PMS (99 to 1 ratio). These reagents mixed with medium were treated (100 µl to each well), and the cells were incubated for another 1 h. Optical density was measured at 492 nm using an ELISA reader (Apollo LB 9110; BerthoI, Technologies GmbH, Germany). The percentage of viable cells was estimated by comparison with the untreated controls. The viability assay was repeated three times.

DAPI Staining

Apoptotic nuclear morphology was observed using DAPI staining. The A549 cells were seeded on coverslips in 6-well plates and treated with specified concentrations of H9 for 24 h. The coverslips in the 6-well plates were washed with phosphate-buffered saline (PBS). Then, the A549 cells were fixed with 4% JC-1 (5,5',6,6'-tetrachloro-1',3',3'-tetraethyl benzimidazolycarbocyanine chloride) was purchased from Enzo (Farmingdale, NY, USA)

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paraformaldehyde and stained with DAPI staining solution. The coverslips were then washed with PBS, dried completely, and mounted on microscope slides with a mounting solution. The stained cells were observed using fluorescence microscopy (Olympus, Japan).

**Cell Cycle and Apoptosis Analyses by Flow Cytometry**

The cell cycle and apoptosis were analyzed by PI staining and flow cytometry. The A549 cells (1.0 × 10^6 cells/well) were seeded in 6-well plates and exposed to various concentrations of H9 for 24 h. The cells were harvested with trypsin-ethylenediamine tetra acet acid (EDTA) and fixed with 80% ethanol. After fixation, the cells were washed twice with cold PBS, centrifuged, and the supernatants were eliminated. The pellet was resuspended and stained with PBS containing 50 µg/ml PI and 100 µg/ml RNase A, for 20 min in the dark. The DNA content was analyzed by flow cytometry using a FACScalibur instrument and CellQuest software (BD Bioscience, San Jose, CA, USA).

**Reverse-Transcription Polymerase Chain Reaction (RT-PCR)**

The cells treated with H9 were harvested. RNA was extracted using an easy-BLUE Total RNA Extraction Kit (iNtRon Biotechnology, SungNam, Korea) according to the manufacturer’s instructions, as previously reported [1]. The cDNA products were obtained using M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA, USA). Using a PCR Thermal Cycler Dice instrument (TaKaRa, Otsu, Shiga, Japan), we performed a RT-PCR analysis with the following primer sets: Fas 5'-AGG GAT TGG AAT TGA GGA AG-3' (forward), 5'-AGT GGC TTT GTC GGT GTA CT-3' (reverse); FasL 5'-AGT CCA CCC CCT GAA AAA AA-3' (forward), 5'-ATT CCA TAG GTG TCT TCC CA-3' (reverse); TRAIL 5'-GTC TAG TGG ACT CCT AT-3' (forward), 5'-GCT GCA ACT GTG AAC CT-3' (reverse); and GAPDH 5'-GGC TGC TTT TAA CTC CAC TCC CA-3' (forward), 5'-GTC TGG TCT GGT GGA TT-3' (reverse). GAPDH was used as an internal control.

**Detection of Caspase-3/-9 Activity**

A549 cells were plated at 1 × 10^5 cells/well in 60 µ plates. After H9 treatment for 24 h, control and treated cells were harvested with trypsin-EDTA. The activity of caspase-3 was measured using a Caspase-3 Colorimetric Assay kit (BioVision, Mountain View, CA, USA) in accordance with the manufacturer’s protocol. The absorbance was measured at 405 nm, using a UV spectrophotometer (Biochrom WPA Bio-wave II, Cambridge, UK).

**Western Blot Analysis**

Cells were treated with specified concentrations of H9 for 24 h and then harvested. The cells were washed with ice-cold PBS and centrifuged (1,890 g, 5 min, 4°C). The cell pellets that were obtained were resuspended in a lysis buffer containing 50 mM Tris (pH 7.4), 1.5 M sodium chloride, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail. The cell lysates were incubated on ice for 1 h, and then clarified by centrifugation at 17,010 × g for 30 min at 4°C. The protein content was quantified using a Bradford assay (Bio-Rad, Hercules, CA, USA) and a UV spectrophotometer. The cell lysates were separated by 10-12% SDS polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to polyvinylidene difluoride membranes (PVDF; Millipore). The membranes were blocked in 5% non-fat dried milk dissolved in Tris-buffered saline, containing Tween-20 (2.7 M NaCl, 53.65 mM KCl, 1 M Tris-HCl, pH 7.4, 0.1% Tween-20), for 1 h at room temperature. The membranes were incubated overnight at 4°C with the specific primary antibodies. After washing three times, the secondary antibodies (HRP-conjugated anti-rabbit or antimouse IgG) were incubated with the membranes for 1 h at room temperature. After washing three times, the blots were analyzed using West-Zol plus a western blot detection system (iNtRON Biotechnology).

**Nuclear and Cytoplasmic Fractionation**

The H9-treated cells were collected and fractionated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific Inc, IL, USA), according to the manufacturer’s protocol. After fractionation, we calculated each extract concentration, performed a 15% Tris-glycine gel electrophoresis, and transferred them to a PVDF membrane. The membrane was blocked with 5% TBST skim milk and incubated with primary antibodies overnight. The membrane was washed with TBST and incubated with secondary antibodies for 1 h. After washing with TBST, we added a chemiluminescent substrate (Westzol; iNtRON Biotechnology).

**Analysis of Mitochondrial Transmembrane Potential (MTP)**

The MTP (ΔΨm) was evaluated by JC-1 staining and flow cytometry. JC-1 (5,5',6,6'-tetrachloro-1',1'-3',3'-tetrathyl-benzimidazolyl-carbocyanine chloride) was purchased from Enzo. The A549 cells were seeded in 6-well plates (1.0 × 10^5 cells/well) and treated with various concentrations of H9. The cells were harvested with trypsin-EDTA and transferred to 1.5 ml tubes. JC-1 (5 µg/ml) was added to the cells, mixed until completely dissolved, and incubated in the dark for 10 min in a 37°C incubator. The cells were centrifuged (300 × g, 5 min, 4°C), washed twice with PBS, and re suspending in 200 µl of PBS. The solutions were divided using a FACScalibur instrument and analyzed by CellQuest software (BD Bioscience). The entire protocol was performed in minimal light.

**Statistical Analysis**

Data are presented as the mean ± SEM from at least three independent experiments. Statistical significance was assessed with the Student’s t-test. *p < 0.05 or **p < 0.005 was considered statistically significant.
Results

Chemical Content of H9

H9 contains ethanol extracts from nine oriental medicinal herbs, as described in the Materials and Methods section (Table 1). We identified the potential medicinal components of the H9 extract using gas chromatography mass spectrometry (Fig. 1). Compounds were identified by comparing with the compounds in the Wiley 6th edition of the MS spectra library (search program hits that were >90% probable were viewed as likely hits). Table 2 lists the major components in the H9 extract, such as coumarin, isoeugenol, isoelemicin, and angelicin. It has been reported that coumarin and angelicin have anticancer properties [11, 35]. However, there are no reports regarding the effects of the other compounds, such as isoeugenol and isoelemicin, on cancer cells (Table 2).

Effect of H9 on Cell Viability

The cell viability of A549 cells with H9 treatment was analyzed by MTS assay. We evaluated the cell viability of H9-treated cells compared with untreated control cells. A549 cells were treated with H9 in various concentrations for 24 h. Compared with the other concentrations, a distinct decrease was not shown at concentrations lower than 50 μg/ml. Compared with the other concentrations, the cell viability was increased in concentrations of 50–200 μg/ml (Fig. 2A). A549 cells were treated for 24 h with up to 200 μg/ml of H9 for optical observation by phase-contrast microscopy. As the concentration of H9 increased, the cell density gradually decreased. In addition, cell shapes were changed into shrunken forms (Fig. 2B). DAPI staining revealed that nuclear condensation was significantly increased in H9-treated A549 cells, suggesting an inhibition of proliferation or progression of cell death (Fig. 2C).

Table 2. Volatile compounds identified in H9.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Relative (%)</th>
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</thead>
<tbody>
<tr>
<td>Unknown</td>
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<td>1.454</td>
</tr>
<tr>
<td>Unknown</td>
<td>28.28</td>
<td>4.431</td>
</tr>
<tr>
<td>Coumarin</td>
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</tr>
<tr>
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<td>Methylisoeugenol</td>
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<tr>
<td>Unknown</td>
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<td>1.22</td>
</tr>
<tr>
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<td>39.69</td>
<td>23.12</td>
</tr>
<tr>
<td>Isoelemicin</td>
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<td>1.717</td>
</tr>
<tr>
<td>Angelicin or psolarene</td>
<td>45.23</td>
<td>12.35</td>
</tr>
<tr>
<td>Angelicin or psolarene</td>
<td>47.15</td>
<td>14.15</td>
</tr>
</tbody>
</table>

H9-Induced Apoptosis in A549 Cells

PI staining was performed to determine whether H9-mediated cell death was due to cell cycle arrest or apoptosis (Fig. 3A). PI staining revealed that H9 enhanced cell numbers at the sub-G$_1$ phase, where hypodiploid (≤2N) fragmented DNA is a marker of apoptosis [18]. However, H9 inhibited the G$_0$/G$_1$ cell population (Fig. 3B). In order to check the modulators involved in the early cell cycle, such as the sub-G$_1$ and G$_0$/G$_1$ phases, western blot analysis was performed to detect the cell-cycle-dependent kinases (CDKs) and the cell-cycle-dependent kinase inhibitors (CDKIs) (Fig. 3C). H9 enhanced the expression levels of p53 and its target CDKIs (p21/p27) and suppressed cyclin D; these findings suggested that H9 inhibited the progress of the G$_0$/G$_1$ phase and resulted in enhanced cell numbers at the sub-G$_1$ phase (Figs. 3A and 3B).

H9-Treated A549 Cells Evaded Extrinsic Pathways

In order to investigate whether H9 would induce apoptosis mediated via the extrinsic signaling pathways, we revealed cell surface death receptors and their interacting factors. PCR analysis revealed that H9 treatment downregulated the expression levels of Fas/Fasl, FADD, DR5, and TRAIL/TRAIL receptors (Fig. 4A). These results suggested that A549 lung cancer cells might suppress or evade extrinsic pathways by downregulating cell surface death receptors.
Since extrinsic pathways were not involved in the H9-induced apoptotic process, we focused on the intrinsic signaling pathways. H9 treatment led to the processing of caspase-3, caspase-9, and PARP in a dose-dependent manner (Fig. 4B). H9 treatment also increased the activation of caspase-3 and caspase-9 (Fig. 4C). Caspase-3 cleaved PARP, which resulted in cell death [20]. In order to elucidate whether H9 activates the mitochondrial pathway, we examined the expression levels of factors related to the intrinsic pathways. We examined the valence between expressions of pro-apoptosis and pro-survival proteins by western blot analysis. The pro-apoptotic factor Bax expression level was increased by H9, while H9 decreased the anti-apoptotic factor Bcl-xL expression level (Fig. 5A). To elucidate the pathway underlying apoptosis, we observed

![Graph](image)

**Fig. 2.** Antiproliferative and pro-apoptosis effects of H9 on A549 cells.

(A) The cell viability was calculated by MTS assay. A549 cells were treated for 24 h with various concentrations of H9. Data are expressed as the mean ± SEM; †*p < 0.05, ‡*p < 0.005, compared with the untreated control. (B) Morphological changes were imaged by phase-contrast microscopy. A549 cells were treated with H9 for 24 h. (C) Apoptotic nuclei after treatment with H9, as detected by fluorescence micrographs. A549 cells were treated with H9 for 24 h and stained with 4,6-diamidino-2-phenylindole.
mitochondrial cytochrome C release into the cytosol following H9 treatment on A549 cells for 24 h. As shown in Fig. 5A, cytochrome C was released into the cytosol in a dose-dependent manner. The mitochondrial outer membrane permeabilization is essential to cytochrome C release [31]. Released cytochrome C triggers executor caspase activation [31]. For that reason, we examined the mitochondrial membrane potential collapse and cytochrome C release. We performed JC-1 staining. In apoptotic cells, JC-1 dye exists as a monomer, but JC-1 aggregation occurs in viable cells.

JC-1 is a lipophilic cationic dye that can enter and accumulate in the mitochondrial matrix. Healthy mitochondria have a negative charge. In apoptotic cells, the mitochondrial membrane potential collapses. The orange fluorescence of JC-1 shows intact mitochondria, whereas green fluorescence shows monomeric JC-1 that remain unprocessed owing to the collapse of the mitochondrial membrane potential. The condensational form of JC-1 emits orange fluorescence at 590 nm (FL-2) and the monomer emits green fluorescence at 525 nm (FL-1) [29]. To measure the mitochondrial potential change by H9 treatment, we conducted JC-1 staining and FACS analysis using the green and orange channels. As shown in Fig. 5B, JC-1 staining revealed that JC-1 aggregation was reduced by H9 treatment and the peaks were shifted to the left. This result suggested that the mitochondrial potential loss was triggered at a higher concentration of H9 compared with the control.

Discussion

Traditional/herbal medicinal herbal extracts have been used in Asian countries. These extracts can overcome the adverse effects of chemotherapy. Medicinal herbal extracts may be
used to give additive or synergistic preventive effects for the inhibition of tumor growth [4]. H9 is composed of ethanol extracts from mixtures of Psoraleae Semen, Evodia Fruit, Fennel, Nutmeg, Ginseng, Alpiniae Officinarum Rhizome, Sparganium Rhizome, Curcuma Root, and Cinnamon Bark. There have been several reports on the subject of these materials [16, 19, 33], but this study is the first to test the effect of this mixed extract, brown-colored H9. Table 2 shows that the major components in H9 extract are coumarin, isoeugenol, isoelemicin, and angelicin. The cell viability of A549 cells treated with H9 was analyzed by MTS assay. However, it seems that results of the cell viability assay did not match with microscopic data, specifically at 200 µg/ml concentration (Figs. 2A and 2B). These results suggest that, as shown in Table 2, H9 has many phytochemicals or compounds such as coumarin, isoeugenol, isoelemicin, and angelicin that can affect the electron-coupling reaction between MTS and PMS reagent, which results in the mismatch between MTS and microscopic data.

Among the compounds of H9, coumarin and angelicin have antitumor activities. Coumarin is widely distributed as a plant. Coumarin has been examined for anticoagulation, antiviral, anti-inflammatory, antibacterial, and anticancer activities [5, 7, 12, 13]. Angelicin has been isolated from diverse plants. It is being used for its antimicrobial and central inhibitory activities, plus its inhibitory role in cell proliferation [11, 26, 28]. Angelicin increases cytotoxicity and induces apoptosis in human SH-SY5Y neuroblastoma cells; this effect is mediated by the activation of the intrinsic...
Apoptotic pathway and indicates that angelicin activates the caspase-3-dependent apoptosis via the mitochondria-mediated apoptotic pathway. However, angelicin treatment did not induce an increase in the levels of Fas/FasL activation and caspase-8 [27]. In other words, angelicin-induced apoptosis is caspase-dependent, rather than being dependent on the Fas-receptor-mediated signaling pathways. H9-induced apoptosis was also caspase-dependent rather than being dependent on death-receptor-mediated signaling pathways (Fig. 4).

Apoptosis is triggered by two major pathways that play essential roles in cell survival, growth, development, and tumorigenesis [32]. One is the extrinsic pathway and the other is the intrinsic pathway. The extrinsic pathway is begun by the death receptor located on the cell membrane [10]. The intrinsic pathway is generated by mitochondrial dysfunction and mitochondrial membrane potential collapse [2].

A schematic summary of H9-induced cell death is presented in Fig. 6. H9 treatment induces p53, a well-known tumor suppressor protein that controls the cell cycle and initiates apoptosis in cancer cells [22]. In our case, H9 induced the intrinsic apoptotic pathway, not the extrinsic pathway. Well-known death receptors/ligands and FADD were downregulated in a dose-dependent manner by H9 treatment, suggesting that H9-treated A549 cells evaded the extrinsic pathway. On the other hand, H9 activated the intrinsic apoptotic pathway. H9 treatment altered the expression levels of Bax and Bcl-xL proteins embedded in the mitochondrial membrane; it also induced mitochondrial potential loss, induced mitochondrial cytochrome C release into the cytosol, activated caspase-9 and caspase-3 and the processing of PARP in A549 cells, and led to the successful induction of apoptosis.

In summary, our study demonstrated that H9 has anti-proliferative and anticancer effects on A549 non-small-cell lung cancer cells. H9 can function as a potential anticancer agent.
**Acknowledgments**

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**References**


