

Anti-cancer Effect of the Ethyl Acetate Fraction from *Opuntia humifusa* on A-549 Human Lung Cancer Cells

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Abstract The anti-cancer effects of the ethyl acetate (EtOAc) fraction (E-OHS) from the ethanol extract of *Opuntia humifusa* against A-549 human lung cancer cells were investigated. Selective cytotoxicity was assessed in RAW 264.7 cells and A-549 cells using the MTS assay. After Annexin V-FITC/PI staining, apoptosis induction was confirmed by flow cytometry, and apoptotic nuclear fragmentation was examined using DAPI staining. Furthermore, induction of cell cycle arrest was confirmed by flow cytometry after PI staining, and anti-metastasis was evaluated using wound healing assay. The molecular mechanisms underlying apoptosis, cell cycle arrest, anti-metastasis, and upstream signaling were elucidated using western blot. E-OHS induced anti-cancer activity in a dose-dependent manner in A-549 cells, whereas it did not exhibit cytotoxicity in RAW 264.7 cells. Annexin V-FITC/PI and DAPI assays showed that number of apoptotic bodies increased in a dose-dependent manner. Western blot analysis showed that pro-caspase-9, pro-caspase-3, and PARP decreased, whereas cleaved-caspase-12, Bax, and cleaved PARP increased. This suggests that apoptosis was jointly induced through mitochondrial-mediated intrinsic pathway and endoplasmic reticulum (ER) stress-related pathway. Flow cytometry analysis showed that the distribution of G1/S phase cells increased in the E-OHS-treated group, whereas the distribution of G2/M phase cells decreased, confirming that E-OHS induced cell cycle arrest by inhibiting transition of G1/S phase. Western blot analysis reconfirmed at the molecular level that the cell cycle was arrested in the G1/S phase, as supported by the decrease in CDK2, CDK4, and cyclin A2. Wound-healing assay showed that the migration ability of A-549 cells was inhibited with E-OHS, and western blot analysis confirmed the decrease in integrin β 1, MMP-9, and HIF-1 α , demonstrating that E-OHS inhibits A-549 cell metastasis. Finally, western blot analysis showed a dose-dependent decrease in ERK1/2, confirming that induction of apoptosis and cell cycle arrest, as well as anti-metastasis, are influenced by upstream signaling pathway.

Keywords: anti-cancer, ethyl acetate fraction, apoptosis, cell cycle arrest, anti-metastasis, *Opuntia humifusa*

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1. Introduction

Lung cancer was the most commonly diagnosed cancer in 2022, with an estimated 2.5 million new cases worldwide, accounting for 1 in 8 cancer cases (12.4% of all cancer cases worldwide). It was followed by female breast cancer (11.6%), colorectal cancer (9.6%), prostate cancer (7.3%), and stomach cancer (4.9%). Lung cancer was also the leading cause of cancer deaths, accounting for an estimated 1.8 million deaths (18.7%). It was followed by colorectal cancer (9.3%), liver cancer (7.8%), female breast cancer (6.9%), and stomach cancer (6.8%) [1]. The age-standardized 5-year survival rates for lung cancer were generally low, in the range of 10–20% in both developed and developing countries. In Korean men, lung cancer had the second-lowest 5-year survival rate after

pancreatic cancer, and in Korean women, it had the third-lowest survival rate after pancreatic cancer and liver cancer [2]. Lung cancer is the leading cause of cancer death in both men and women in the United States, accounting for approximately 20% of all cancer deaths. The overall survival rate is only about 25%, which is significantly lower than other major cancers such as colon cancer (approximately 65%) or breast cancer (approximately 91%). This low survival rate suggests that effective treatment of lung cancer remains a major challenge due to the difficulty of early diagnosis, limited efficacy of existing treatments, and side effects [3]. According to the International Agency for Research on Cancer (IARC), among the four major subtypes of lung cancer (adenocarcinoma, squamous cell carcinoma, small cell carcinoma, and large cell carcinoma), adenocarcinoma has become the most common subtype in both men and women. As of 2022, 45.6% of male lung cancer patients

were adenocarcinoma, and 59.7% of female lung cancer patients were adenocarcinoma. In 2020, 39.0% of male lung cancer patients and 57.1% of female lung cancer patients were adenocarcinoma. According to IARC, 70% of lung cancer patients in non-smokers were adenocarcinoma [4,5]. A-549 cells are a human adenocarcinoma cell line widely used as a representative model of non-small cell lung cancer (NSCLC), which accounts for approximately 85% of all lung cancers. These cells are characterized by an epithelial-like morphology and harbor a well-defined genetic background, including wild type *TP53* and oncogenic *KRAS* mutations [6]. Due to their consistent responsiveness to various anti-cancer agents, A-549 cells serve as reliable *In Vitro* model for evaluating cellular responses such as apoptosis and cell cycle arrest. Therefore, in the present study, A-549 cells were selected as an appropriate model to investigate the therapeutic potential of ethyl acetate (EtOAc) fraction (E-OHS) of ethanol extracts from *Opuntia humifusa* stem in human lung cancer. *O. humifusa* is a plant belonging to the Cactaceae family. In Korea, this plant and its fruit are called cheonnyeoncho, and in English, it is called creeping prickly-pear. It is native to the eastern United States, northeastern Mexico, and southeastern Canada, and is currently reported to inhabit southern Europe, South Africa, Australia, and Japan. *O. humifusa* has anti-oxidant, anti-bacterial, and anti-cancer activities, and research on its various physiological activities is actively being conducted [7,8,9,10,11]. However, there is no study on the effect of *O. humifusa* on lung cancer. Therefore, this study aimed to investigate the anti-cancer effect of the E-OHS obtained from *O. humifusa* on A-549 human lung cancer cells. The E-OHS contains flavonoids and their derivatives, so it is expected to have anti-oxidant, anti-bacterial, anti-inflammatory, and anti-cancer activities and can be a good candidate for developing an effective anti-cancer agent [12,13]. In this study, the E-OHS will be treated to A-549 cells, a human lung cancer cell line, to systematically investigate induction of apoptosis and cell cycle arrest and inhibition of metastasis using MTS assay, flow cytometry, DAPI staining, wound-healing assay, and western blot analysis. The MTS assay measures cell viability by assessing the metabolic activity of living cells. This is achieved by quantifying the absorbance of formazan, which is produced when cellular enzymes reduce the MTS reagent [14,15]. Apoptosis is quantitatively evaluated using Annexin V-FITC and Propidium Iodide (PI) staining followed by flow cytometry, which detects changes in phosphatidylserine exposure and membrane integrity [16,17]. Cell cycle distribution is also analyzed via flow cytometry after staining nuclear DNA with PI, allowing assessment of cell cycle arrest at specific phase [18]. DAPI staining, a method that utilizes a fluorescent dye binding to nuclear DNA, is employed to visualize nuclear condensation and fragmentation associated with apoptosis under a fluorescence microscope [19]. The wound-healing assay is performed to evaluate cell migration by monitoring the closure of an artificially introduced gap in a confluent cell monolayer over time [20]. Finally, western blot analysis is used to measure the levels of proteins involved in apoptosis, cell cycle arrest, metastasis inhibition, and upstream signaling pathways. Proteins are separated by SDS-PAGE, transferred to a membrane, and probed with

specific primary and secondary antibodies [21].

2. Materials and Methods

2.1. Preparation of the E-OHS of *O. humifusa*

The preparation of the E-OHS from *O. humifusa* followed the method used in the previous study [22].

2.2. Cell Lines and Reagents

A-549 human lung cancer cells Paraformaldehyde Solution in PBS (GeneAll, Korea) and AntiFade Mounting Medium (with DAPI) (MedChem Express, USA) were used.

2.3. Cell Culture

For culture of A-549 RPMI 1640, FBS, and penicillin were used. For culture of RAW 264.7, DMEM, FBS, and penicillin were used. The A-549 cells were cultured in an incubator at 37°C and 5% CO₂, and the medium composition was RPMI 1640 90%, FBS 10% and penicillin 1%. The A-549 cells were subcultured every week at 1:3 split ratios using 0.05% trypsin, and every 3 days, medium was changed. The cells were treated with 0.1% DMSO or various concentrations of E-OHS derived from *O. humifusa*. Normal RAW 264.7 cells were cultured in an incubator at 37°C, 5% CO₂, and the medium contained 90% DMEM, 10% FBS, and 1% penicillin. The RAW 264.7 cells were subcultured every week at 1:4 split ratios using 0.05% trypsin, and every 2 days, medium was changed. The cells were treated with 0.1% DMSO or various concentrations of E-OHS derived from *O. humifusa*.

2.4. The Cytotoxic Effect of E-OHS on RAW 264.7 Normal Cells

To evaluate the cytotoxicity of E-OHS against normal cells viability was assessed in RAW 264.7 macrophage cells. A total of 1×10^5 cells/well were seeded into 96-well plates and incubated for 24 h to allow cell stabilization. The cells were then treated with E-OHS at concentrations 0, 0.1% DMSO, 25, 50, 75, and 100 µg/mL (0.1% DMSO as the vehicle control) and further incubated for 12 h. After treatment, 20 µL of MTS solution (Cell Titer 96® Aqueous One Solution, [3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) to each well. The plate was then incubated for an additional 2 h at 37°C in a CO₂ incubator. Finally, absorbance was measured at 490 nm using the cell imaging multi-mode reader (BioTek, USA).

2.5. The Cytotoxic Effect of E-OHS on A-549 Cancer Cells

A total of 1×10^5 cells/well were seeded into 96-well plates and allowed to adhere for 24 h. The E-OHS was then applied at concentrations of 0, 0.1% DMSO, 25, 50, 75, and 100 µg/mL (0.1% DMSO as the vehicle control), and the cells were incubated for 12 h. Subsequently, 20 µL of MTS solution (Cell Titer 96® Aqueous One Solution)

was added to each well, followed by 2 h incubation at 37°C in a CO₂ incubator. Absorbance was measured at 490 nm using a cell imaging multi-mode plate reader (BioTek, USA).

2.6. Apoptosis Analysis

A-549 cells treated with various concentrations of E-OHS (0, 0.1% DMSO, 50, 75, 100 µg/mL) for 12 h were resuspended using 0.05% trypsin, centrifuged at 5,000 rpm for 5 min using a centrifuge (Hanil, Korea), washed with PBS, and centrifuged again. The supernatant was discarded, and 200 µL of Annexin V binding buffer was added to resuspend the cells. Annexin V-FITC and PI (5 µL each) were added, and the samples were incubated in the dark at room temperature for 15 min. Apoptosis was analyzed using a flow cytometer (NovocyteAdvanteon, Agilent).

2.7. Analysis of Nuclear Changes Using 4', 6-Diamidino-2-phenylindole (DAPI) Staining

To observe the morphological changes of the nucleus, A-549 cells were seeded on sterile coverslips in 6-well plates and treated with various concentrations of E-OHS (0, 0.1% DMSO, 25, 50, 75, 100 µg/mL) for 12 h, and then the supernatant was removed with a suction device (LabTek, Korea). The cells were then washed with PBS and fixed with PBS containing 4% paraformaldehyde for 20 min at room temperature. Finally, the cells were permeabilized with -20°C cold 100% methanol for 20 min and stained with DAPI mounting medium (abcam, United Kingdom). The morphological changes of the nucleus were observed using a cell imaging multi-mode reader (BioTek, USA).

2.8. Cell Cycle Analysis

A-549 cells were treated with various concentrations of E-OHS (0, 0.1% DMSO, 50, 75, 100 µg/mL) for 12 h, then harvested using trypsin 0.05% and washed with cell-based assay buffer. Cells were resuspended at a concentration of 4×10^5 cells/mL, fixed in cold fixative, and incubated at -20°C for at least 2 h. After centrifugation at 5,000 rpm for 5 min using a centrifuge (Hanil, Korea), the fixative was removed and resuspended in staining buffer containing RNase A and PI. The mixture was incubated for 30 min at room temperature in the dark. Cell cycle distribution was analyzed using a flow cytometer.

2.9. Wound-healing Analysis

A-549 cells were seeded into 6-well plates at a density of 5×10^5 cells/mL and cultured until 90% confluency. After stabilization for 24 h, the medium was replaced with a serum-free medium containing various concentrations of E-OHS (0, 0.1% DMSO, 25, 50, 75, 100 µg/mL). A linear scratch was introduced in the center each well using a sterile 200 µL pipette tip (0 h). The wound closure was monitored using a cell reader at 0 and 12 h post-treatment. The migration rate was calculated by measuring the

change in wound width over time.

2.10. Western Blot Analysis

A-549 cells were treated with E-OHS (0, 0.1% DMSO, 50, 100 µg/mL) for 12 h and washed with PBS. Total proteins were extracted on ice using lysis buffer containing RIPA buffer, phosphatase inhibitor, and protease inhibitor. Protein concentrations were determined by the Brad-ford assay (Bio-Rad). Proteins were separated via 12% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in TBST and incubated overnight at 4°C with primary antibodies diluted in either 3% BSA in TBST or 5% non-fat milk in TBST, according to the datasheet. After washing, membranes were incubated with secondary antibody at room temperature for 1 h. Immunoreactive bands were visualized using an WesternBright ECL kit (Advansta, USA).

2.11. Statistical Analysis

The differences between the control group and the experimental group were evaluated using Student's t-test, and statistical significance was considered as $P < 0.05$. The results were expressed as the mean $\pm$ standard deviation, and statistical significance was considered as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3. Results

3.1. Cytotoxicity Analysis of E-OHS on RAW 264.7 Macrophages Using MTS Analysis

To evaluate the cytotoxicity of E-OHS, RAW 264.7 macrophages were treated with various concentrations of E-OHS (0, 0.1% DMSO, 25, 50, 75, and 100 µg/mL) for 12 h. As shown in Figure 1, no cytotoxic effects were observed in any E-OHS treatment group compared to the control group. Based on these results, 100 µg/mL was selected as the highest treatment concentration in subsequent experiments.

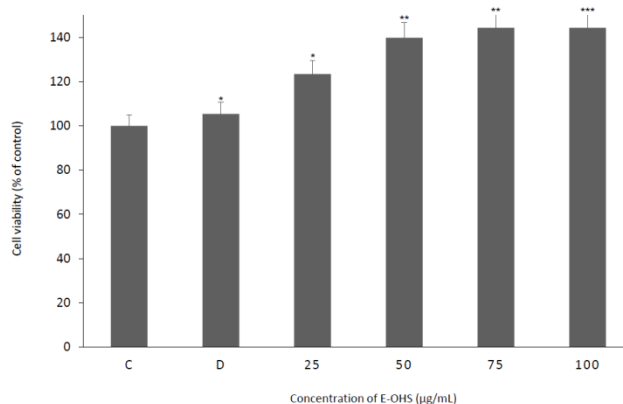


Figure 1. Effects of E-OHS on viability of RAW 264.7 macrophage cells measured by cytotoxicity assay for 12 h. The results are presented as the mean $\pm$ standard deviation (SD) of three independent experiments. Statistical significance was determined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

3.2. Analysis of the Effect of E-OHS on A-549 Cell Viability Using MTS Assay

A-549 cells were treated with various concentrations of E-OHS (0, 0.1% DMSO, 25, 50, 75, and 100 $\mu\text{g/mL}$) for 12 h, and cytotoxic effects were assessed using MTS assay. As shown in Figure 2, E-OHS decreased A-549 cell viability in a concentration-dependent manner. In particular, E-OHS at concentrations of 75 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ significantly reduced cell viability.

3.3. Flow Cytometric Analysis of E-OHS-induced Apoptosis in A-549 Cells

To further analyze the apoptotic effects of E-OHS, A-549 cells were treated with various concentrations of E-OHS (0, 0.1% DMSO, 50, 75, and 100 $\mu\text{g/mL}$) for 12 h. The cells were then stained with Annexin V-FITC and PI, followed by flow cytometry. Annexin V binds to phosphatidylserine (PS) exposed in early apoptotic cells, whereas PI identifies late apoptotic or necrotic cells. Based on quadrant analysis (LL: live cells, LU: necrotic cells, RL: early apoptotic cells, and RU: late apoptotic cells) (Figure 3), E-OHS treatment significantly increased early and late apoptosis in a dose-dependent manner. These results indicate that E-OHS effectively induces apoptosis in A-549 cells.

3.4. DAPI staining Analysis of Apoptotic Features in A-549 Cells Treated with E-OHS

To examine nuclear morphological changes in A-549 cells exposed to different concentrations of E-OHS (0, 0.1% DMSO, 50, 75, and 100 $\mu\text{g/mL}$), DAPI staining was performed. As shown in Figure 4, E-OHS-treated cells

exhibited characteristic apoptotic features, including chromatin condensation and apoptotic body formation. The concentration-dependent increase in the intensity and frequency of these features supports the apoptosis-inducing potential of E-OHS.

3.5. Western Blot Analysis of Apoptosis-related Protein Levels in A-549 Cells

To elucidate the apoptosis-related signaling pathway, A-549 cells were treated with different concentrations of E-OHS (0, 0.1% DMSO, 50, and 100 $\mu\text{g/mL}$) for 12 h, followed by western blot analysis. In E-OHS-treated cells, the levels of pro-caspase-9 and pro-caspase-3 were significantly reduced or eliminated (Figures 5(A) and 5(B)). The level of cleaved caspase-12 increased in a dose-dependent manner (Figure 5(C)). As shown in Figure 5(D), Bax expression increased in a dose-dependent manner. Finally, as shown in Figure 5(E), higher concentrations of E-OHS decreased PARP levels, while C-PARP levels increased. Taken together, these results suggest that E-OHS induces apoptosis in A-549 cells via the mitochondrial intrinsic pathway and ER stress-related pathway.

3.6. Flow Cytometric Analysis of Cell Cycle Arrest in E-OHS-Treated A-549 Cells

DNA contents and cell counts were assessed in A-549 cells treated with various concentrations of E-OHS (0, 0.1% DMSO, 50, 75, and 100 $\mu\text{g/mL}$) using PI staining and flow cytometry. As shown in Figure 6, most cells in the control group (untreated and 0.1% DMSO) were distributed in the G2/M phase, whereas in the E-OHS-treated group, most cells were distributed primarily in the G1/S phase in a dose-dependent manner.

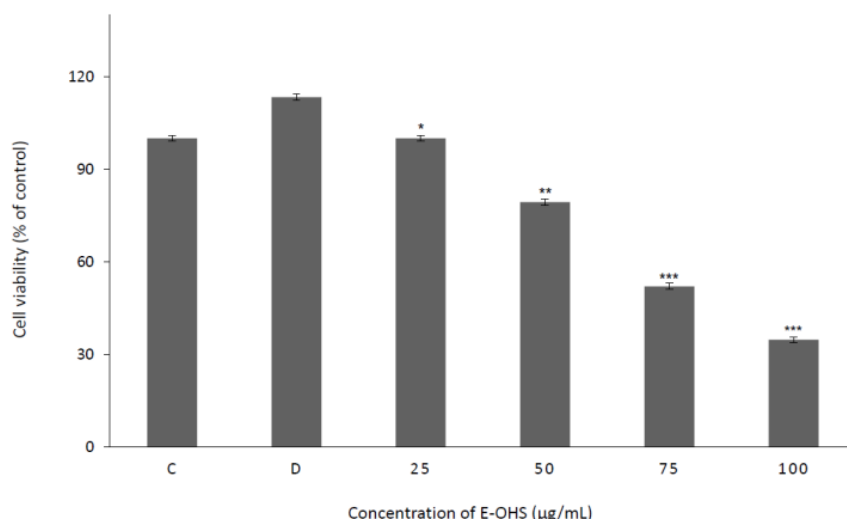


Figure 2. Effect of E-OHS on viability of A-549 cells for 12 h. The cells were treated with varying concentrations (0, 0.1% DMSO, 25, 50, 75, or 100 $\mu\text{g/mL}$) of E-OHS for 12 h. Cell viability was assessed using MTS assay. The values are presented as the mean $\pm$ S.D. of three independent experiments. Statistical significance was determined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

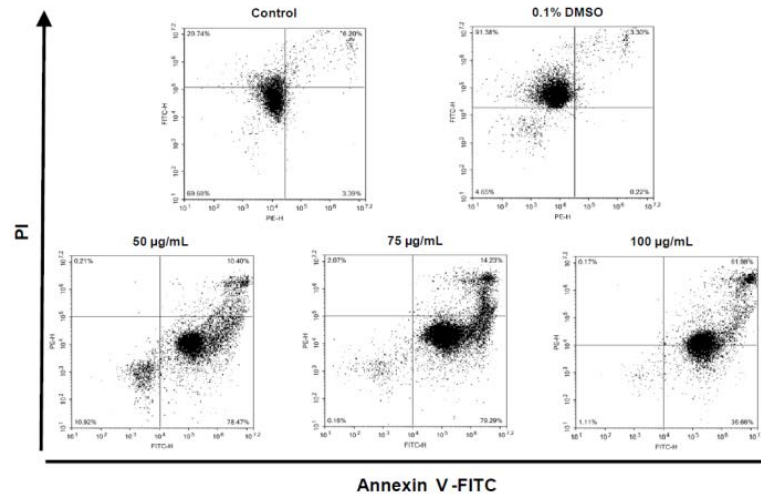


Figure 3. Flow cytometric analysis of apoptosis progression in A-549 cells treated with E-OHS. The cells were exposed to various concentrations (0, 0.1% DMSO, 50, 75, or 100 µg/mL) for 12 h. The cells were labeled with monoclonal antibodies specific for Annexin V-FITC and PI. Based on the staining pattern, cells were classified as follows: Annexin-/PI- (LL), indicating viable cells; Annexin+/PI- (LR), indicating cells undergoing early apoptosis; Annexin-/PI+ (UR), indicating late apoptotic cells; Annexin+/PI+ (UL), indicating necrotic cells

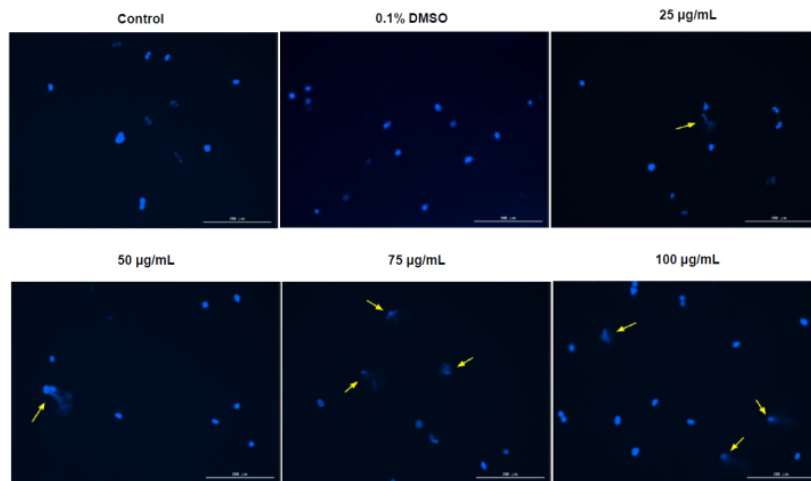


Figure 4. DAPI staining analysis of apoptotic death of A-549 cells. Induction of apoptosis in A-549 cells. The cells were treated with E-OHS (0, 0.1% DMSO, 25, 50, 75, or 100 µg/mL) for 12 h and then stained with the DNA-specific fluorochrome DAPI. Apoptotic bodies are indicated by yellow arrows

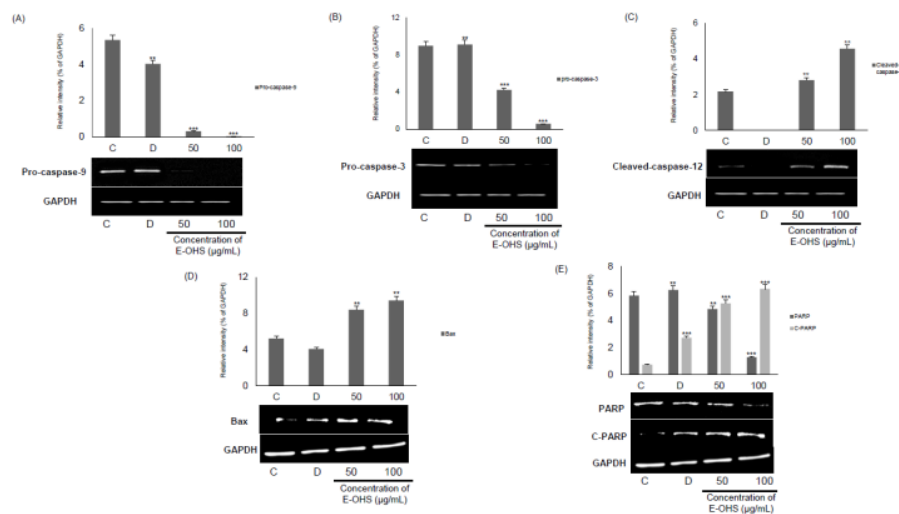


Figure 5. The modulation of apoptosis-related key biomarkers in A-549 cells treated with E-OHS (0, 0.1% DMSO, 50, or 100 µg/mL) for 12 h. The levels of pro-caspase-3 (A), pro-caspase-9 (B), caspase-12 (C), Bax (D), PARP, and cleaved-PARP (E) were analyzed using western blot analysis. Band densities were quantitated and displayed as bar graphs with GAPDH as an internal control. The results were obtained from three separate experiments and were expressed as the mean $\pm$ S.D. Statistical significance was determined as * p < 0.05; ** p < 0.01; *** p < 0.001

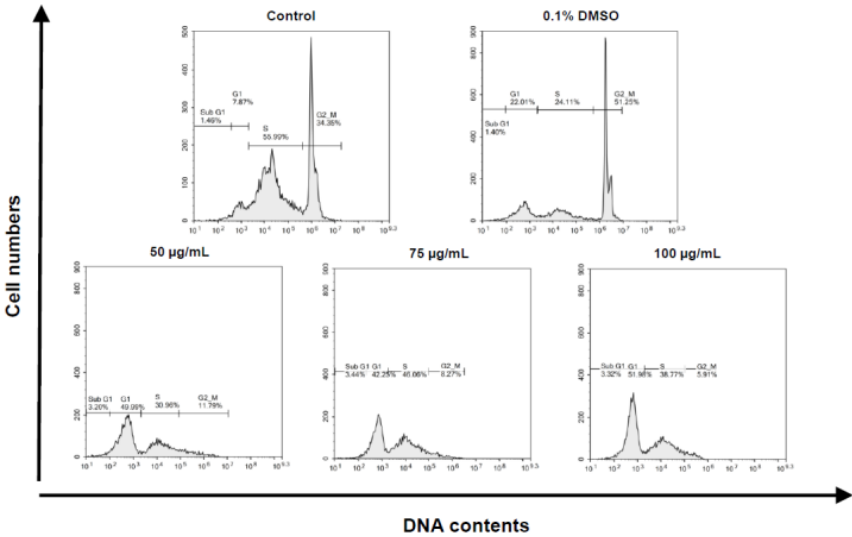


Figure 6. Flow cytometric analysis of cell cycle arrest in A-549 cells treated with E-OHS. The cells were exposed to various concentrations (0, 0.1% DMSO, 50, 75, or 100 µg/mL) for 12 h. Histograms represent Sub G1, G1, S, and G2/M phases of A-549 cells. Results are expressed as percentage of total treated cells. Three experiments showed similar results

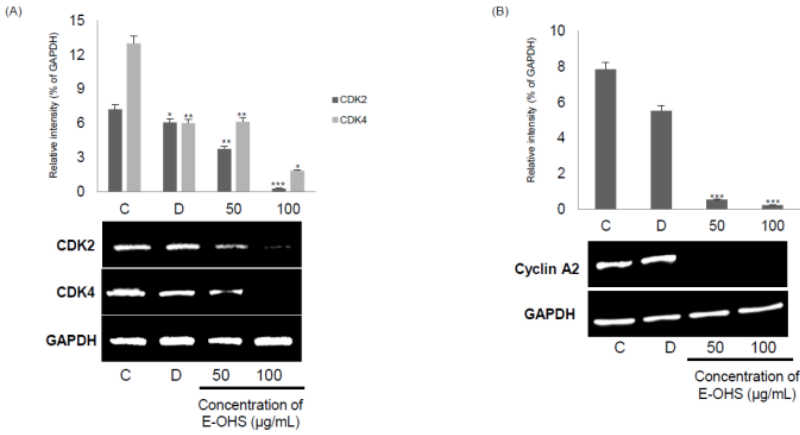


Figure 7. The modulation of cell cycle arrest-related key biomarkers in A-549 cells treated with E-OHS (0, 0.1% DMSO, 50, or 100 µg/mL) for 12 h. The levels of CDK2, CDK4 (A), and cyclin A2 (B) were analyzed using western blot analysis. Band densities were quantitated and displayed as bar graphs with GAPDH as an internal control. The results were obtained from three separate experiments and were expressed as the mean $\pm$ S.D. Statistical significance was determined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

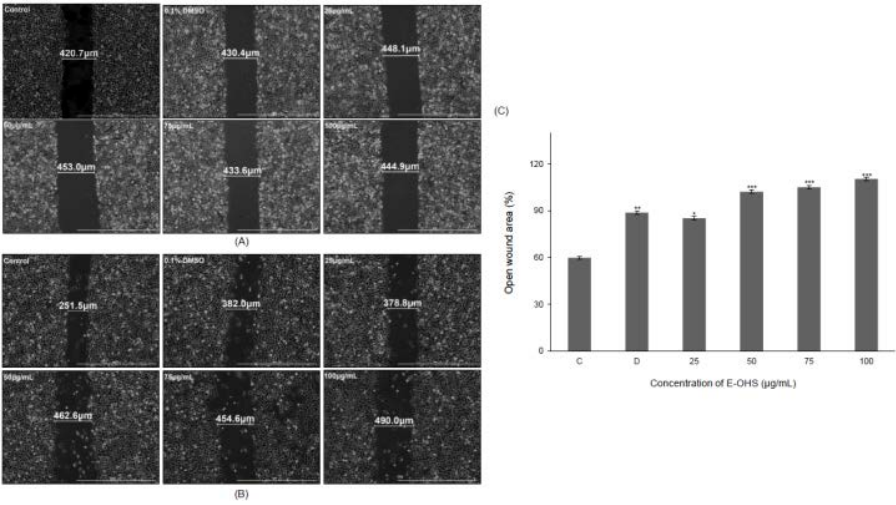


Figure 8. Wound healing assay to determine the inhibitory effect of E-OHS on A-549 cell migration. A-549 cells were treated with various concentrations of E-OHS (0, 0.1% DMSO, 25, 50, 75, and 100 µg/mL) for 12 h, and then a wound healing assay was performed to evaluate cell migration. The open wound area was quantified and expressed as a percentage of the initial wound area. Similar results were observed in three experiments. The results were obtained from three separate experiments and are expressed as the mean $\pm$ SD. Statistical significance was determined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

3.7. Western Blot Analysis of Cell Cycle Arrest-related Protein Levels in A-549 Cells

Western blot analysis was performed to elucidate changes in the levels of cell cycle arrest-related mediators in A-549 cells treated with different concentrations of E-OHS (0, 0.1% DMSO, 50, and 100 $\mu\text{g/mL}$) for 12 h. As shown in Figure 7(A) and Figure 7(B), the levels of CDK2, CDK4, and cyclin A2 decreased in a dose-dependent manner, with a significant decrease observed at 100 $\mu\text{g/mL}$. CDK2 and CDK4 are protein kinases that play a crucial role in the cell cycle, particularly during the G1/S transition, and cyclin A2, in complex with CDK2, is essential for initiating and regulating DNA synthesis during the S phase. Therefore, these results support the induction of cell cycle arrest at the G1/S phase.

3.8. Anti-metastatic Effect of E-OHS on A-549 Cells Assessed by Wound-healing Assay

To investigate the anti-metastatic potential of E-OHS, a wound healing assay was performed on A-549 cells treated with various concentrations of E-OHS (0, 0.1% DMSO, 25, 50, 75, and 100 $\mu\text{g/mL}$). As shown in Figure 8(A), migration was evaluated based on the wound healing area at 0 h. After 12 h of treatment, E-OHS significantly inhibited cell migration in a concentration-dependent manner (Figure 8(B)). Compared with the control group, the E-OHS-treated group showed a decreased motility as indicated by decreased wound closure. As shown in Figure 8(C), the open wound area (%) in cells treated with E-OHS was significantly increased in a dose-dependent manner compared with the control group (C). Specifically, wound closure was significantly inhibited at concentrations of 50, 75, and 100 $\mu\text{g/mL}$ of E-OHS, with more than 100% inhibition during the initial culture period. The DMSO control (D) showed a slight increase in open wound area compared to the untreated control, but this effect was significantly

increased by E-OHS treatment. These results suggest that E-OHS has a potential anti-metastatic effect by inhibiting the migration ability of A-549 cells.

3.9. Western Blot Analysis of Anti-metastasis-related Protein Levels in A-549 Cells

To further confirm the anti-metastatic activity observed in the wound healing assay, the levels of HIF-1 α , integrin β 1, and MMP-9 proteins, which are mediators of anti-metastasis, were measured by western blot analysis in A-549 cells treated with different concentrations of E-OHS (0, 0.1% DMSO, 50, and 100 $\mu\text{g/mL}$) for 12 h. HIF-1 α plays a significant role in cancer metastasis by promoting cancer cell migration, survival, and proliferation. Integrin β 1 plays a significant role in cancer metastasis by facilitating cancer cell adhesion, invasion, and migration. MMP-9 plays a significant role in cancer metastasis by breaking down the extracellular matrix (ECM) and facilitating cancer cell invasion and migration. As shown in Figures 9(A), 9(B), and 9(C), the concentrations of HIF-1 α , integrin β 1, and MMP-9 decreased in a dose-dependent manner, particularly at a concentration of 100 $\mu\text{g/mL}$. Therefore, these results support that E-OHS effectively induces anti-metastasis in A-549 cells.

3.10. Western Blot Analysis of Upstream Signaling-related Protein Levels in A-549 Cells

To investigate the levels of mediators involved in the upstream signaling pathway in A-549 cells treated with different concentrations of E-OHS (0, 0.1% DMSO, 50, and 100 $\mu\text{g/mL}$) for 12 h, western blot analysis was performed. As shown in Figure 10, ERK1/2 was decreased in a dose-dependent manner in A-549 cells treated with E-OHS. This suggests that the upstream signaling pathway involving ERK1/2 may affect E-OHS-induced apoptosis, cell cycle arrest, and anti-metastatic activity in A-549 cells.

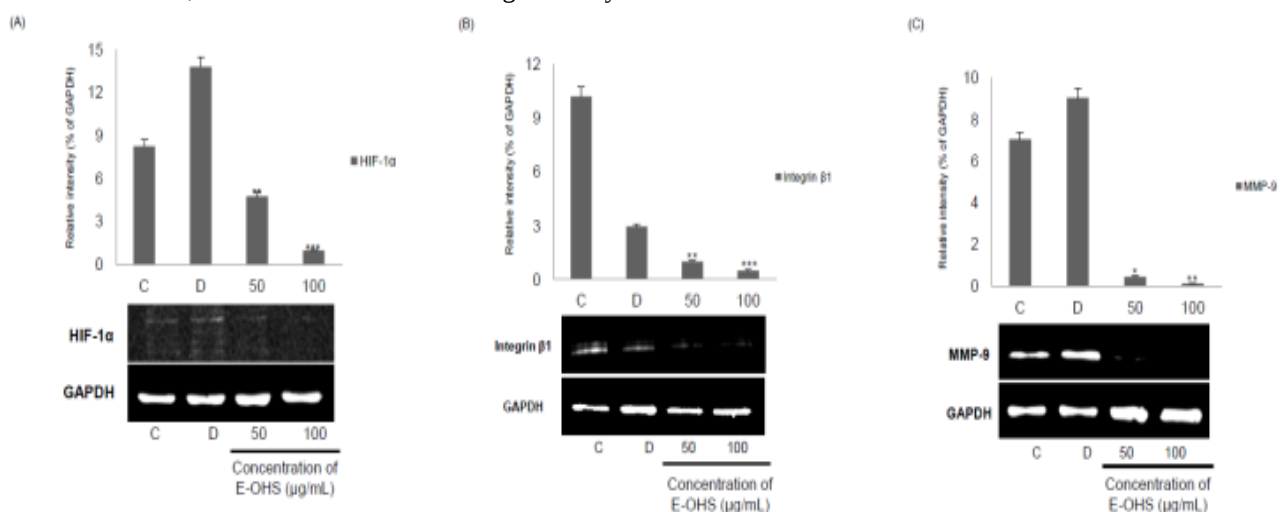


Figure 9. The modulation of anti-metastasis related key biomarkers in A-549 cells treated with E-OHS (0, 0.1% DMSO, 50, or 100 $\mu\text{g/mL}$) for 12 h. The levels of HIF-1 α (A), Integrin β 1 (B), ERK1/2 (C) and MMP-9 (D) were analyzed using western blot analysis. Band densities were quantitated and displayed as bar graphs with GAPDH as an internal control. The results were obtained from three separate experiments and were expressed as the mean $\pm$ S.D. Statistical significance was determined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

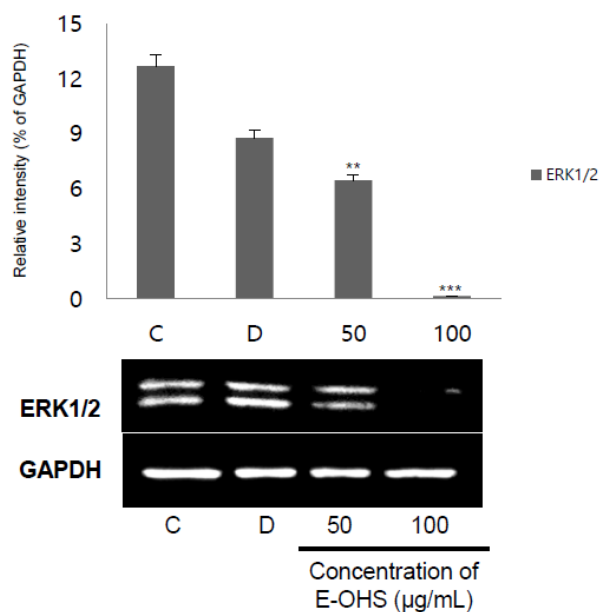


Figure 10. Modulation of key biomarker related to upstream signaling pathways in A-549 cells treated with E-OHS (0, 0.1% DMSO, 50, or 100 µg/mL) for 12 h. ERK1/2 levels were analyzed using western blot analysis. Band densities were quantified and expressed as bar graphs with GAPDH as an internal control. Results were obtained from three individual experiments and are presented as mean $\pm$ SD. Statistical significance was defined as * p < 0.05; ** p < 0.01; *** p < 0.001

4. Discussion

The results of the current study suggest that E-OHS possesses a multifaceted anticancer activity capable of contributing to the development of a safe and a natural therapeutic agent. While previous research has investigated the biological activities of *O. humifusa*, particularly its anti-inflammatory and antibacterial properties, its therapeutic potential in oncology remains insufficiently elucidated [9,10,23,24,25,26,27,28]. Recently, E-OHS has been reported to exhibit a biological activity against PANC-1 pancreatic cancer cells [22]. The present study expands the field of natural product-based anticancer research by demonstrating that E-OHS exerts selective cytotoxicity toward A-549 lung cancer cells while sparing normal macrophage cells [29]. This study, also, supports the multifaceted anticancer effects of E-OHS by providing a comprehensive analysis that goes beyond simple antiproliferative activity assessment to include molecular mechanism elucidation and metastasis inhibition. Crucially, this study goes beyond simply highlighting the biological activities of *O. humifusa* and provides a foundation for its development as a candidate anticancer agent by elucidating the molecular pathways through which E-OHS exerts its therapeutic effects.

It is noteworthy that no cytotoxicity was observed in RAW 264.7 macrophages [30,31,32]. Cytotoxicity of E-OHS on A-549 cells was analyzed using MTS assay, and cell viability was decreased in a dose-dependent manner. To determine whether the cell death occurring in these cells was apoptosis or necrosis, flow cytometry was used. Results showed that both early and late apoptosis increased in a dose-dependent manner in E-OHS-treated

cells, confirming that apoptosis was the cause of cell death. Subsequently, western blot analysis was performed to identify the involved apoptotic pathways. One of the most notable aspects of E-OHS is its ability to induce apoptosis via both the intrinsic mitochondrial pathway and the endoplasmic reticulum (ER) stress-mediated pathway. This pattern has also been confirmed in other studies [33,34,35]. The mitochondrial pathway is characterized by the activation of the pro-apoptotic protein Bax and the sequential activation of pro-caspase-9 and pro-caspase-3, which is well known as a classical mechanism of intrinsic apoptosis [36,37,38,39]. PARP plays a crucial role in DNA damage detection and repair, and an increase in cleaved PARP is known to inhibit cancer cell proliferation and DNA repair capacity. The decrease in PARP and the increase in cleaved PARP indirectly support the functional role of activated caspase-3 in inducing apoptosis. This pattern has also been reported in other studies [40,41,42,43]. Meanwhile, the induction of apoptosis through caspase-12 activation suggests that E-OHS may induce apoptosis under ER stress by exacerbating the unfolded protein response (UPR) or oxidative stress [44,45].

Another important implication of these findings is the effect of E-OHS on cell cycle regulation. G1/S transition inhibition is closely linked to tumor growth suppression. Impaired G1/S transition inhibition is a common feature in many cancers and contributes to uncontrolled cell proliferation [46,47,48]. CDK4 and CDK2 play complementary roles in cell cycle regulation. CDK4 controls the early phase (G1) of the cell cycle, while CDK2 controls the late phase. The Cyclin A2-CDK2 complex is involved in the initiation and progression of DNA synthesis [49]. The decrease in CDK4, CDK2, and cyclin A2 observed in western blot analysis, coupled with the inhibition of G1/S transition observed in flow cytometry, suggest that cell cycle arrest primarily occurs in the G1/S phase. Meanwhile, other finding obtained by treating A-549 cells with the *Orostachys japonicus* EtOAc fraction showed that transition inhibition occurs primarily in the G2/M phase, whereas the results of this study showed a distinct difference that transition inhibition occurs primarily in the G1/S phase [50].

Furthermore, wound healing assays confirmed that E-OHS dose-dependently inhibited A-549 cancer cell migration. Subsequently, western blot analysis confirmed that E-OHS dose-dependently reduced metastasis-related mediator proteins, such as HIF-1 α , integrin β 1, and MMP-9, in A-549 cancer cells. These results complementarily support the anti-metastatic effect of E-OHS in A-549 cancer cells. HIF-1 α is a transcription factor strongly induced in a hypoxic tumor microenvironment, and its expression is particularly significant in lung cancer, which has a poor prognosis. HIF-1 α is a transcription factor that is strongly induced in a hypoxic tumor microenvironment, and is particularly significant in lung cancer, which has a poor prognosis. HIF-1 α acts as a transcription factor that binds to DNA and is involved in various cellular processes such as angiogenesis, metabolism, and cell survival [51,52]. Integrin β 1, one of the downstream targets of HIF-1 α , mediates the interaction between cells and the extracellular matrix (ECM) to promote cancer cell adhesion and invasion. In particular, the β 1 subunit

combines with various α subunits to form various integrin complexes, which are involved in cell adhesion, migration, and differentiation [53,54]. MMP-9 is an ECM-degrading enzyme that specifically targets collagen IV, disrupting the basement membrane and ECM structure, thereby promoting invasion, metastasis, tumor growth, and angiogenesis. MMP-9 activity is closely related to the progression of various malignancies, and inhibition of this signaling axis can effectively limit tumor progression by attenuating cell motility, angiogenesis, and invasion [55,56].

Additionally, when western blot analysis was performed to identify the upstream signaling pathway, E-OHS dose-dependently decreased ERK1/2 protein in cancer cells. ERK1/2 is an important member of the MAPK family and is activated by the Ras-Raf-MEK-ERK signaling pathway, which is widely involved in cell proliferation, differentiation, survival, apoptosis, cell cycle, and metastasis [57,58,59]. Therefore, the results of the present study suggest that E-OHS can suppress the proliferation and survival of A-549 lung cancer cells while inducing apoptosis, cell cycle arrest, and metastasis inhibition by controlling the expression of ERK1/2, an upstream signaling mediator.

Collectively, these findings show that E-OHS exerts anti-cancer effects through three complementary mechanisms: induction of apoptosis, cell cycle arrest, and metastasis inhibition in A-549 lung cancer cells.

5. Conclusion

In conclusion, this study demonstrated that E-OHS derived from *O. humifusa* exhibited significant anti-cancer effects on A-549 human lung cancer cells, while not exhibiting cytotoxicity in normal RAW 264.7 macrophages. Specifically, E-OHS induced apoptosis via the mitochondrial intrinsic pathway and ER stress-mediated pathways, as evidenced by upregulation of Bax and cleaved caspase-12, downregulation of pro-caspase-9 and pro-caspase-3, and increased cleaved PARP. Furthermore, E-OHS was demonstrated to induce cell cycle arrest by downregulating the expression of CDK4, CDK2, and cyclin A2, thereby inhibiting the G1/S phase transition and subsequent S phase progression. Furthermore, E-OHS was demonstrated to induce anti-metastasis by downregulating the expression of pro-metastatic signaling mediators including HIF-1 α , integrin β 1, and MMP-9. Finally, it was, also, demonstrated that E-OHS could induce apoptosis, cell cycle arrest, and metastasis inhibition by downregulating the expression of ERK1/2, an upstream signaling mediator. Collectively, this study suggests that E-OHS has the potential as a natural agent with anti-lung cancer activity.

Conflict of Interest Statement

Authors declare no conflict of interest.

Authors' Contributions

EJY was involved in designing the work, data collection, data analysis, interpretation, and drafting the article. MCC was involved in data analysis and interpretation. DSL was involved in designing the work, data analysis, interpretation, and drafting the article.

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