**Lethariella zahlbruckneri** Acetone Extract-Induced Apoptosis of MCF-7 Human Breast Cancer Cells Involves Caspase Cascade and Mitochondria-Mediated Death Signaling

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**Abstract**

*Lethariella zahlbruckneri* has been traditionally used in tea and medicines in China. This study aimed to evaluate the anti-cancer properties of *L. zahlbruckneri* acetone extract (AEL) and to explore its potential mechanisms on MCF-7 human breast cancer cells. The polyphenol and flavonoid concentrations of were 14.4 mg gallic acid equivalent/g and 6.5 mg quercetin equivalent/g, respectively. AEL inhibited the growth of MCF-7 cells in a dose- and time-dependent manner. AEL significantly induced apoptotic cell death, resulting in an increase in the sub-G1 apoptotic cell population, apoptotic DNA fragmentation, and a morphological change. Pretreatment with a caspase inhibitor modestly attenuated the AEL-induced increase in the sub-G1 cell population, implying that caspases play a partial role in AEL-induced apoptosis. Moreover, AEL-induced apoptosis was associated with changes of caspase activities, up-regulation of the apoptotic protein (Bax), and down-regulation of the anti-apoptotic protein (Bcl-2). AEL also induced apoptosis-inducing factor-release from mitochondria, indicating apoptosis stimulation through a caspase-independent pathway. These results suggest that AEL exerts its anti-cancer effects on MCF-7 human breast cancer cells through mitochondrial caspase-dependent and caspase-independent apoptotic pathways.

**Keywords:** *Lethariella zahlbruckneri*, MCF-7 cells; Cytotoxicity; Apoptosis

**Introduction**

Breast cancer is the most common form of cancer affecting women worldwide [1]. It is extremely difficult to treat because it involves several distinct classes of tumors that exhibit different treatment responses [2]. The anti-cancer drug tamoxifen has been reported to be effective in only one-third of breast cancer cases [3]. However, it causes some serious side effects, including stroke, blood clots, and endometrial cancer. Thus, new alternative agents for the prevention and treatment of breast cancer are in great demand. A variety of dietary substances, including plant extracts, fruits, and vegetables, are associated with a lower risk of breast cancer development [4]. Plant extracts have been historically considered important alternative remedies for various types of cancers, while medicinal plants constitute the main source of new pharmaceuticals and healthcare products [5,6].

Lichens, which represent the symbiotic association of a fungus with an algal partner, are important constituents of many ecosystems [7]. Lichens are the earliest colonizers of terrestrial habitats on earth and are distributed worldwide from arctic to tropical regions and from the plains to the highest mountains [8]. They produce secondary metabolites of various chemical classes, including aliphatic, cycloaliphatic, aromatic, and terpenic compounds [9]. These compounds are distinct from those of higher plants and show interesting biological and pharmaceutical activities, including anti-viral [10], antioxidant [11], and anti-herbivore [12] properties. Despite the large number of lichen species (20,000) identified thus far, only a few systematic chemical studies have been undertaken on these species.

One lichen, *Lethariella zahlbruckneri* (*L. zahlbruckneri*), can be found only at high altitudes. Although it has traditionally been used in tea and medicines in China, little is known about its biological activities. We recently published what seems to be the first report that *L. zahlbruckneri* acetone extract (ALE) inhibits the growth of human colon cancer cells via apoptosis [13]. However, no study has examined the anticancer activity of *L. zahlbruckneri* or its precise mechanisms of action in breast cancer cells. In this study, we demonstrate that AEL inhibited the growth of MCF-7 human breast cancer cells by inducing apoptosis and we provide insights into its mechanism of action.

**Materials and Methods**

**Plant material**

*L. zahlbruckneri* was obtained from Yunnan province in China in 2005 and identified by Lisong Wang. Voucher specimens are deposited in lichen herbarium of Kunming Institute of Botany, CAS, duplicated to Korean Lichen Research Institute (KoLRI) at Sunchon National University, Korea.

**Preparation of AEL**

The AEL was prepared using a previous method [13]. Briefly, the dried *L. zahlbruckneri* (5 g) was extracted with 100 mL acetone at 40°C for 3 h. The extracts was concentrated under reduce pressure and stored at 4°C until use (yield 0.276 g).

**Determination of total polyphenol content**

The total phenolic content of AEL was determined by the Folin-Ciocalteu method.

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Ciocalteau method with some modifications [14]. In a 10 mL test tube, 0.1 mL of AEL, 8.4 mL of distilled water, 0.5 mL of 2 N Folin-Ciocalteu phenol reagent (Sigma-Aldrich, Co. St. Louis, Mo, USA) and 0.5 mL of 20% Na2CO3 were added and mixed. After exactly 2 Hour, the absorbance was read at 725 nm. Total phenolic concentration was calculated from a calibration curve that was obtained using gallic acid (GAE) as a standard.

**Determination of total flavonoid content**

Aluminum chloride colorimetric method was used for flavonoids determination [15]. 0.1 mL of AEL was diluted with 80% aqueous ethanol 0.9 mL. An aliquot of 0.5 mL was added to test tubes containing 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M aqueous potassium acetate and 4.3 mL of 80% ethanol. After 40 min at room temperature, the absorbance was determined spec-trophotometrically at 415 nm. Total flavonoid concentration was calculated using quercetin (QE) as standard.

**Cell culture and cell growth**

The MCF-7 human breast cancer cell lines were purchased from the Korea Cell Line Bank, Seoul National University. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 μg/mL) in a humidified 5% CO2 at 37°C.

Cell growth was determined by a trypan blue assay. The cancer cells were seeded at a concentration of 1 × 106 cells in 24-well tissue culture plates and incubated with various concentrations of AEL for different time periods. The cells were collected and dyed in trypan blue solution.

**Cell cycle analysis for sub-G1 population**

Cells were seeded at a density of 1 × 106 cells per well 6-well plates, and cultured for 24 h in RPMI-1640. After culturing, the cells were treated with the indicated concentrations of AEL for 48 h. The cells were then collected and fixed in ice-cold 70% ethanol in media and stored at 4°C overnight. After resuspension, the cells were washed and incubated with 1 μL of RNase (1 mg/mL) (Sigma, St. Louis, USA), 20 μL of propidium iodide (1 mg/mL) (Sigma, St. Louis, USA), and 500 μL of PBS at 37°C for 30 min. The DNA contents of stained cells were analyzed using a flow cytometer (Backman Coulter EPICS XL, USA).

**Detection of morphological apoptosis**

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using bis-benzimide (Hoechst 33258) staining. Briefly, the cells were seeded in 6-well plates at a density of 1 × 106 cells per well 6-well plates, and cultured for 24 h in RPMI-1640. After culturing, the cells were treated with the indicated concentrations of AEL for 48 h. The cells were then collected and fixed in ice-cold 70% ethanol in media and stored at 4°C overnight. After resuspension, the cells were washed and incubated with 1 μL of RNase (1 mg/mL) (Sigma, St. Louis, USA), 20 μL of propidium iodide (1 mg/mL) (Sigma, St. Louis, USA), and 500 μL of PBS at 37°C for 30 min. The DNA contents of stained cells were analyzed using a flow cytometer (Backman Coulter EPICS XL, USA).

**Detection of DNA fragmentation**

DNA fragmentation was evaluated by enzyme-linked immunosorbent assay (ELISA) using the Cell Death Detection ELISA Plus Kit (Roche Applied Science, IN, USA) according to the instruction manual. The relative amounts of mono- and oligonucleosomes generated from the apoptotic cells were quantified using monoclonal antibodies directed against DNA and histones by ELISA. Briefly, the cytoplasmic fractions of the untreated control cells and cells that were treated with AEL for 48 h were transferred onto a streptavidin coated 96-well plate and incubated for 2 h at room temperature with a mixture of peroxidase conjugated anti-DNA and biotin-labelled anti-histidine.

The plate was washed thoroughly, incubated with 2.2-azino-di-(3-ethylbenzthiazoline sulphonate) diammonium salt and absorbance was measured at 405 nm with a reference wavelength of 490 nm using a microplate reader (Molecular Devices, Inc. USA). All conditions were repeated in triplicate in at least two independent experiments and the mean values were calculated.

**Assay for caspase activity**

This assay was based on the ability of enzyme to cleave chromophore from the enzyme substrate, Ac-DEVD-pNA (for caspase-7), Ac-IETD-pNA (for caspase-8), and Ac-LEHD-pNA (for caspase-9). The cells were seeded at a density of 2 × 104 cells in a 100 mm dish and then cultured for 24 h in RPMI-1640. After culturing, the cells were treated with the indicated concentrations of AEL for 48 h and then collected by centrifugation. The cells were incubated with the peptide substrate in lysis buffer for 30 min on ice, followed by centrifugation at 10,000g for 5 min at 4°C. The protein content of the supernatant was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). The supernatant containing 50 μg of protein was mixed with DTT in 2 × reaction buffer as well as 10 μM concentrations of the different substrates. After incubation, the release of p-nitroaniline was monitored at 405 nm using a microplate reader (Molecular Devices, Inc., US).

**Assay for caspase inhibitor activity**

The cells were seeded at a density of 5 × 105 cells per well in a 24-well plate, then cultured for 24 h in RPMI-1640. The cells were pre-incubated with z-Val-DMK for 2 h and then treated with the indicated concentrations of AEL for 48 h. The cells were collected and fixed in ice-cold 70% ethanol in media, and then stored at 4°C overnight. After resuspension, the cells were washed and incubated with 1 μL of RNase (1 mg/mL) (Sigma, St. Louis, USA), 20 μL of propidium iodide (1 mg/mL) (Sigma, St. Louis, USA), and 500 μL of PBS at 37°C for 30 min. After staining, flow cytometry was used to analyze the sub-G1 DNA content.

**Western blotting analysis**

The cells were seeded at a density of 2 × 105 cells in a 100 mm dish, and then cultured for 24 h in RPMI-1640. After culturing, the cells were treated with the indicated concentrations of AEL for 48 h, followed by centrifugation. The resulting pellets were lysed by lysis buffer (50 mM Tris- HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF; 30 mM Na3P2O7, 1 mM PMSF, and 2 μg/mL of aprotinin) for 30 min on ice. To examine the release of apoptosis-inducing factor (AIF) from the mitochondria into the cytosol, preparation of cytosolic extracts was carried out according to the manual provided in the mitochondria isolation kit (Pierce, Rockford, IL, USA). The protein content of the supernatant was measured using a BCA protein assay kit. The protein samples were then loaded at 10 μg of protein/lane and then separated by 12% SDS-PAGE at 100 V of constant voltage/slab for 1.5 h. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes. After blocking with 2.5% and 5% bovine serum albumin (BSA) for 1 h at 37°C, the membranes were incubated with primary antibody (anti-PARP, anti-Bax, anti-Bcl2, and anti-AIF) at 4°C overnight. Finally, the membranes were then treated with horseradish peroxidase-coupled
secondary antibodies for 1 h at 4°C. The membranes were washed with T-TBS after each antibody binding reaction. Detection of each protein was performed using an ECL kit (Santa Cruz, CA, USA).

**AIF translocation**

MCF-7 cells were seeded in 6-well plates at seeding densities of 5 × 10⁴ cells per well, and followed by treatment with AEL for 48 h. After harvesting, the cells were washed twice with PBS and then blocked with blocking buffer (2% BSA in T-TBS) for 1 h. The cells were incubated with AIF primary antibody overnight at 4°C, followed by anti-rabbit secondary antibody for 1 h. AIF translocate ion was analyzed under a fluorescence microscope (Olympus Optical Co. Ltd. Japan).

**Statistical analysis**

The data were analyzed by Student’s t-test to evaluate significant differences. A level of ‘p < 0.05 ’p < 0.01, and ‘p < 0.001 was regarded as statistically significant.

**Results**

**Total phenolic and flavonoid content of AEL**

The total phenolic and flavonoid content of the AEL were determined to evaluate the phytochemical constituents. Various phenolic compounds have been extensively studied to determine their range of biochemical and pharmacological properties such as antibacterial, antifungal, anti -inflammatory, antiviral, antioxidant, and anticancer activities [16,17]. Total polyphenol and flavonoid concentrations of AEL were found to be 14.4 ± 1.05 mg GAE/g and 6.5 ± 1.94 mg QE/g, respectively (Table 1).

**AEL inhibits MCF-7 cell growth**

To test whether AEL inhibits MCF-7 cell growth, the cells were exposed to 10-100 μg/mL of AEL for 24, 48 and 72 h. As shown in Figure 1,

<table>
<thead>
<tr>
<th>AEL (μg/mL)</th>
<th>Total polyphenols (mg GAE/g)</th>
<th>Total flavonoids (mg QE/g)</th>
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<tbody>
<tr>
<td>10</td>
<td>14.4 ± 1.05</td>
<td>6.5 ± 1.94</td>
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<td>30</td>
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(GAE, gallic acid equivalents. 
QE, quercetin equivalents. 
Values are mean ± S.D. (n=3) 

**Table 1:** Content of total polyphenols and flavonoids in AEL.

**Figure 1:** Effect of AEL on MCF-7 cell growth. MCF-7 cells were treated with various concentrations for 24, 48, and 72 h. Cell growth and viability was determined by trypan blue exclusion. Data values are expressed as mean ± SD of triplicate determinations. Significant differences were compared with the control at ‘p < 0.05, **p < 0.01, and ***p < 0.001 by Student’s t-Tests.

To investigate whether or not AEL-induced cell growth inhibition is caused by apoptosis, we performed flow cytometry assay, DNA fragmentation assay, and hoechst 33258 staining. As shown in Figure 2A, MCF-7 cells exposed to 30, 50, and 100 μg/mL of AEL for 48 h resulted in accumulation of cells in sub-G1 in a dose-dependent manner. There was a significant increase in DNA fragmentation at concentrations of 50 and 100 μg/mL compared to untreated cells. MCF-7 cells exposed to 50 μg/mL and 100 μg/mL AEL showed a 1.8 and 1.9-fold increase in DNA fragmentation, respectively (Figure 2B). In addition, MCF-7 cells with condensed and fragmented nuclei and apoptotic bodies (arrows) were seen upon AEL treatment but not control treatment (Figure 2C).

**AEL-induced apoptosis involves caspase activities**

To address whether or not the apoptotic effects induced by AEL are associated with caspase activation, the activities of initiator caspase (caspases-8,-9) and effector caspase (caspase-3) were investigated by colorimetric protease assay. AEL markedly increased the caspase activities in a dose-dependent manner. AEL-treated cells experienced a 3.5-fold increase in caspase-3 activity at 100 μg/mL compared to untreated cells. Similarly, significant increases in the activities of caspase-8 (1.3 fold) and caspase-9 (2.5 fold) were observed at the same concentration (Figure 3).

To confirm the significance of caspase activities in AEL-induced apoptosis, we used a universal caspase inhibitor, z-VAD-fmk (10 μM). In the absence of z-VAD-fmk, approximately 12 and 15% of the cells treated with AEL at concentrations of 50 and 100 μg/mL were in sub-G1 phase, respectively, whereas only 2 and 5% of the cells were in sub-G1 phase in the presence of z-VAD-fmk (Figure 4).
AEL induces PARP cleavage

We examined the cleavage of a well-characterized caspase-3/7 substrate, poly (ADP-ribose) polymerase (PARP), from its 116-kDa intact form into an 89-kDa fragment by Western blotting. PARP was processed to its predicted cleavage product of 89 kDa after AEL treatment, but processing was not observed in the untreated cells (Figure 5).

AEL-induced apoptosis involves mitochondrial-mediated death signaling

To determine the mitochondrial apoptotic events in AEL-induced apoptosis, we examined changes in the levels of Bcl-2 family proteins such as Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) by Western blotting. As shown in Figure 6, AEL dose-dependently increased Bax expression but decreased Bcl-2 expression in MCF-7 cells. We next determined whether or not apoptosis-inducing factor (AIF), a caspase-independent apoptosis factor, plays a role in AEL-induced apoptotic cell death by measuring changes in the level of AIF in the cytosol by Western blotting, as well as translocation of AIF into the nucleus by immunostaining. AEL increased the levels of AIF protein in the cytosol (Figure 7A) and nucleus at concentrations of 50 and 100 μg/mL (Figure 7B).

Discussion

Natural products are a potential source of novel anticancer drugs and have contributed much to cancer chemotherapies such as etoposide, camptothecin, and paclitaxel [18]. According to a previous study, over 50% of anticancer drugs undergoing clinical trials are isolated from natural sources [19].

Most development of anticancer agents from plants has focused on the molecular mechanism by which an agent induces cytotoxicity and apoptosis in cancer cells [20]. Here, we report for the first time that AEL exhibited cytotoxicity and induced apoptosis in MCF-7 human breast cancer cells, as evidenced by an increase in the sub-G1 apoptotic cell population, apoptotic DNA fragmentation, and the appearance of

Figure 3: Effect of AEL on caspase activation in MCF-7 cells. Caspases-3, -8, and -9 activities were determined by enzyme activity assays using Ac-DEVD-pNA, Ac-LEHD-pNA, and Ac-IETD-pNA enzyme substrate, respectively (A, B, and C). Data values are expressed as mean ± SD of triplicate determinations. Significant differences were compared with the control at *p<0.05, **p<0.01, and ***p<0.001 by Student’s t-tests.

Figure 4: Effect of caspase inhibitor (z-VAD-fmk) on cell death induced by AEL. MCF-7 cells were pre-incubated with 10 μM z-VAD-fmk for 2 h and then treated with various concentrations of AEL. Data values are expressed as mean ± SD of triplicate determinations. Significant differences were compared with the control at *p<0.05, **p<0.01, and ***p<0.001 and by Student’s t-tests.

Figure 5: Western blot analysis of PARP cleavage in AEL-treated cells. MCF-7 cells treated with AEL for 48 h, after which cells were harvested and lysed. Protein lysate was subjected to 12% SDS-PAGE gel and immunoblotted with the corresponding antibodies.

Figure 6: Expression level of Bcl-2 family proteins in AEL-treated MCF-7 cells. MCF-7 cells treated with AEL for 48 hrs. Protein lysate was subjected to 12% SDS-PAGE gel and immunoblotted with the corresponding antibodies.
apoptotic bodies. We showed that AEL inhibited the growth of MCF-7 cells in a dose- and time-dependent manner. Suppression of the cell cycle in cancer cells is considered to be one of the most effective strategies for controlling cancer cell growth [21]. AEL treatment showed a concentration-dependent increase in the apoptotic sub-G1 DNA fraction and DNA fragmentation. Treatment of MCF-7 cells with AEL induced typical morphological changes, including cell shrinkage, chromatin condensation, and formation of apoptotic bodies. These data demonstrate that AEL induced MCF-7 cells apoptosis in a dose-dependent manner.

Caspases, which are cytoplasmic aspartate-specific cysteine proteases, play an important role in apoptosis [22]. Initiator caspases such as caspases-8 and -9 activate executioner caspases such as caspases-3 and -7 by proteolytic cleavage, after which the executioner caspases cleave PARP from its 116-kDa intact form into an 89-kDa fragment [23]. Caspase-3 seems to play a central role in chemotherapy-induced apoptosis. It is known that activation of caspase-7, a member of the caspase 3 subfamily, cleaves PARP in caspase-3-deficient MCF-7 cells [24]. In the present study, AEL was found to elicit not only the activation of effector caspase-7 but also the initiators caspases-8 and -9 in MCF-7 breast cancer cells, and this effect was dose-dependent. AEL (100 μg/mL) significantly increased the activities of caspases-8, 9, and 7 in MCF-7 cells by 3.5-, 1.3-, and 2.5-fold, respectively, compared to control cells. These results suggest that apoptosis of MCF-7 cells induced by AEL is mediated through activation of caspase. Furthermore, PARP was processed to its predicted cleavage product of 89-kDa after AEL treatment, but the processing was absent in the untreated cells. To address the significance of caspase activation in AEL-induced apoptosis, we used a general and potent inhibitor of caspase, z-VAD-fmk. AEL significantly increased the sub-G1 population of MCF-7 cells; however, incubation of cells in the presence of a caspase family inhibitor, z-VAD-fmk, decreased the sub-G1 population. These results clearly indicate that AEL-induced apoptosis was dependent on caspase activation.

In mammalian cells, mitochondria are involved in the apoptosis signal transduction pathway [18]. Mitochondria-dependent apoptosis is mediated by at least two different pathways. The major one, the classical apoptotic pathway, involves cleavage and activation of caspase-3/7 as well as regulation of the Bcl-2 family. The second pathway involves nuclear translocation of mitochondrial proteins such as AIF, which can induce large-scale DNA fragmentation independently of caspase [25]. AEL dose-dependently increased the expression of the pro-apoptotic protein Bax, whereas it decreased expression of the anti-apoptotic protein Bcl-2 in MCF-7 cells. Additionally, we showed that AEL increased the level of AIF protein and translocation into the nucleus, implying that AEL may also have induced apoptosis through a caspase-independent pathway.

Phenolic compounds are the most numerous and ubiquitous secondary metabolites in plants. Recently, there has been increasing interest in the identification of plant polyphenols, owing to their wide range of biochemical and pharmacological effects such as antibacterial, antiviral, immune-stimulating, estrogenic, antiproliferative, and cytotoxic activities in several tumor cells [17,26-28]. In this study, AEL was found to contain phenolics and flavonoids. Phenolics arrest the cell cycle and activate apoptotic signal transduction pathways in cancerous cells [29]. In particular, flavonoids are known to exert antioxidant activity through multilateral mechanisms such as inhibition of tumor cell proliferation or induction of apoptosis via suppression of DNA, RNA and protein synthesis or an increase in the concentration of cAMP [30,31]. Further, numerous lichens contain various phenolic compounds with anticancer activity including usnic acid, lecanoric acid, glycyrrhizinic acid, salazinic acid, lobarian acid, eremic acid, and vulpinic acid [32,33]. Therefore, future studies are necessary to determine which compound(s) of AEL is involved in its antiproliferative effect of breast cancer cells.

In conclusion, these results indicate that the anti-cancer effects of AEL were attributable to the induction of apoptosis via caspase-dependent and caspase-independent mitochondrial pathways in MCF-7 human breast cancer cells. Although further studies on the bioactive components of AEL are needed to support these findings, our study provides useful insights with possible therapeutic implications against breast cancer.

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References
Dietary factors for cancer prevention


