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*Full Length Research Paper*

# Cytotoxic Effects of *Angelica decursiva* on Osteosarcoma Cells: Induction of Apoptosis and Inhibition of Proliferation

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**The *Angelica decursiva* has been used in Korean traditional medicine as an antitussive, an analgesic, an antipyretic and a cough remedy. However, its anticancer activity has been unknown. In this study, we have examined the cytotoxic activity of ethanol extract of *A. decursiva* root (EEAD), and the mechanism by which EEAD suppresses cell growth in Saos2 human osteogenic sarcoma cells. Treat-ment of EEAD in Saos2 cells induced the apoptic cell death in a concentration-dependent manner as determined by MTT assay and DNA fragmentation analysis but not in FOB human normal osteoblast cells. Furthermore, the proteolytic processing and the activity of caspase-3 and -7 were increased by EEAD treatment in Saos2 cells. These results suggest that EEAD can induce the suppression of cell growth and cell apoptosis in Saos2 human osteogenic sarcoma cells, and therefore, it may have potential properties for anticancer drug discovery.**

**Key words:** Human osteogenic sarcoma cell, *Angelica decursiva*, apoptosis, caspases, anti-cancer therapy.

## INTRODUCTION

Recently, there has been a global trend toward the use of natural substances present in fruits, vegetable, oilseeds and herbs as medicine and functional food. Several of these substances, such as Taxol, Oncovin and capto-thecin, are shown to have potential values as cancer chemo-preventive or therapeutic agents within the human body (van Poppel and van den Berg, 1997; Mukherjee et al., 2001; christou et al., 2001; Pezutto, 1997). Chemo-therapeutic drugs are known to induce cytotoxicity in tumor cells through diverse mechanisms, in which sig-naling events play an important role depending upon the cell type and stimulus (Hoshino et al., 1991; Tian et al., 2006). There is a need to find new anticancer drugs that can kill cancerous cells with minimal toxicity. The cyto-static effect of whole plant extracts on cancer cells is often much better than the effect of their particular bio-logically active compounds (Yano et al, 1994; Vickers, 2002).

Apoptosis is a major form of cell death, characterized by series of distinct morphological and biochemical alt-erations. It is characterized by condensation and frag-mentation of nuclear chromatin, campaction of cyto-plasmic organelles, dilatation of the endoplasmic reti-culum, a decrease in cell volume and alterations to the plasma membrane resulting in the recognition and phag-ocytosis of apoptotic cells (Arends and Wyllie, 1991).

*Angelica decursiva*, one of Korean traditional medicine, has been used mainly as a folk remedy for treatment of antitussive, analgesic, antipyretic and cough. However, the anticancer activity of *A. decursiva* was not reported at all. Osteosarcoma is the most common type of malignant bone tumor in which the neoplastic mesenchymal cells

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show evidence of osteoid production (Kim et al., 2006). In this study, we examined the effect of ethanol extract of *A. decursiva* root (EEAD) on cell growth and the mechanism of cell growth suppression in Saos2 human osteogenic sarcoma cells. We report here that the EEAD can induce the suppression of cell growth and cell apoptosis in Saos2 cells.

## MATERIALS AND METHODS

### Materials

The FOB human osteoblast cells and the Saos2 human osteogenic sarcoma cells were provided by the American Type Culture Collection (ATCC; Rockville, MD, USA). 3-[4, 5-dimethylthiazol -2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St Louis, MO, USA). Anti-caspase-3 and anti-caspase-7 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Cell-permeable fluorogenic substrate PhiPhiLux-$G_1D_2$ was purchased from OncoImmunin, Inc. (Gaithersburg, MD, USA).

### Plant material and extract preparation

Dried root parts of *A. decursiva* were purchased from Jeonnam herbal medicine farmer's cooperative, Korea. The botanical identification was made by Prof. Su-In Cho, School of Oriental Medicine, Pusan National University, Korea. The roots were ground with a Wiley mill to pass a 1 mm screen and were extracted with 95% ethyl alcohol (EtOH) at 40°C for 5 h. The extract was then filtered through Advantec No. 1 filter paper. The collected filtrate was dried by evaporation under vacuum at 40°C using a rotary evaporator (N-1000V-W, Eyela, Japan). After evaporation, the concentrated extract was freeze-dried at -40°C for 3 days and stored in a refri-gerator at 2°C until used.

### Cell cultures

The FOB cells were grown in 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's Medium (DMEM) without phenol red supplemented with 2.5 mM L-glutamine, 0.3 mg/ml G418 and 10% FBS (Kim et al., 2006). The Saos2 cells were grown in the DMEM supplemented with 10% FBS (Kim et al., 2006). The FOB and Saos2 cells were maintained as monolayer in plastic culture plates at 37$^{o}$C in the humidified atmosphere containing 5% $CO_2$.

### Inhibition of cell growth (MTT Assay)

The cell viability test was performed according to the previously described method (Lee et al., 2008) with minor modifications. The cells were seeded at a concentration of $5x10^3$ cells / well in 24 well plates. After 24 h growth, the cells were treated with EEAD at various concentrations and incubation times. The cell viability was assessed using MTT assay. Three separate experiments were performed for each concentration/exposure time combination.

### DNA fragmentation analysis

Following treatment with 0.3 μg/ml EEAD for 8 and 24 h, approximately $5x10^6$ cells were collected and transferred to lyses buffer containing 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% SDS and 0.5 mg/ml proteinase K and incubated at 65$^{o}$C. DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v) and precipitated with ethanol. The DNA was resuspended in Tris-EDTA buffer, pH 8. 0 containing 5μg/ml DNase- free RNase and incubated at 37$^{o}$C for 1 h. The DNA was visualized on 1.5% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

### Immunoblotting

The cells were treated with 0.3 μg/ml EEAD for 8 and 24 h. Immunoblotting was performed according to the previously described method (Kim et al., 2006; Kim et al., 2008)) with minor modifications. The anti-caspase-3 or anti-caspase-7 antibody (1:1000 dilutions, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the primary antibody.

### Determination of caspase activation

The activity of caspase-3/7 was determined using the cell-permeable fluorogenic substrate PhiPhiLux-$G_1D_2$ (OncoImmunin, Inc. Gaithersburg, MD, USA), which was used according to the manufacturer's instructions. The cells were treated with 0.3 μg/ml EEAD for 24 h and incubated with PhiPhiLux-$G_1D_2$. The activity of cas-pase-3/7 was visualized by fluorescence microscopy (Ix71, Olympus, Japan).

### Data analysis

All experiments were performed in triplicate. Results are presented as mean ± S.E.M. Statistical significance was analyzed by using Student's t- test for two groups and one way analysis of variance for multi-group comparisons. $P<0.05$ is considered statistically significant.

## RESULTS AND DISCUSSION

To analyze the effect of EEAD on the viability of cells, the cells were treated with EEAD at various concentrations for 24 h, and then the MTT assay was performed. Treatment of EEAD (0.01 - 30 μg/ml) inhibited the growth of Saos2 human osteogenic sarcoma cells in a concentration-dependent manner with an $IC_{50}$ value of 1.4 ± 0.2 μg/ml (mean ± S.E.M. of three separate experiments) (Figure 1). In contrast, EEAD did not inhibit the prolixferation of FOB human osteoblast cells (Figure 1), suggesting the EEAD has a specific effect for inhibition of tumor cell growth. Recently, scientific attentions increas-ed to oriental medicine for the discovery of novel drugs including anticancer agents (Cheng et al., 2005; Hu et al., 2002; Lee et al., 2002; Park et al., 2005; Tan et al., 2005). The *A. decursiva* has been used as a material for medical treatment in Korean traditional medicine. How-ever, its anticancer effect has been unknown. The main goal of this study was to investigate the effect of EEAD on cell growth and the mechanism of growth suppression in Saos2 human osteogenic sarcoma cells.

In MTT assay, EEAD inhibited growth of Saos2 cells in a concentration-dependent manner (Figure 1). This corresponds with the results of several extracts (Echinacea root, Toona sinensis, Willow bark) (Chicca et al., 2007; Yang et al., 2006; Hostanska et al., 2007) that have

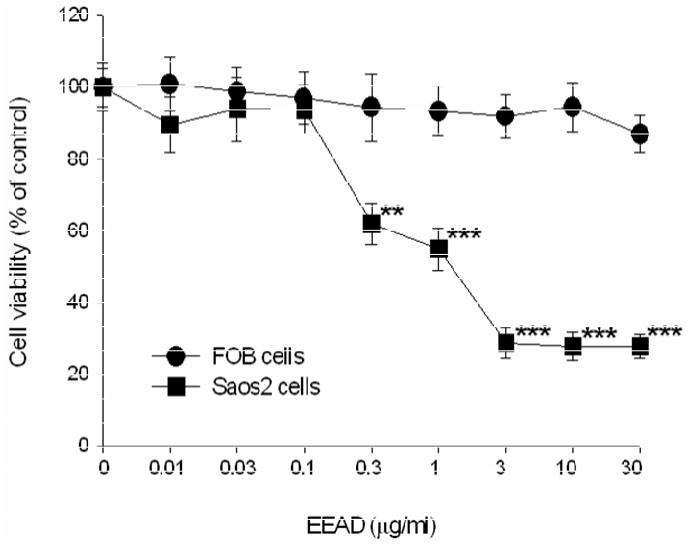


**Figure 1.** Cytotoxic effects of EEAD in FOB and Saos2 cells. The FOB (filled circle) and Saos2 (filled square) cells were treated with 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30 μg/ml EEAD for 24 h. Cell viabilities were determined by the MTT assay. The percentage of cell viability was calculated as a ratio of A570 nm of EEAD treated cells and untreated control cells. Each data point represents the mean ± SEM for three experiments. **P<0.01 vs. control and ***P<0.001 vs. control (the control cells were measured in the absence of EEAD treatment).

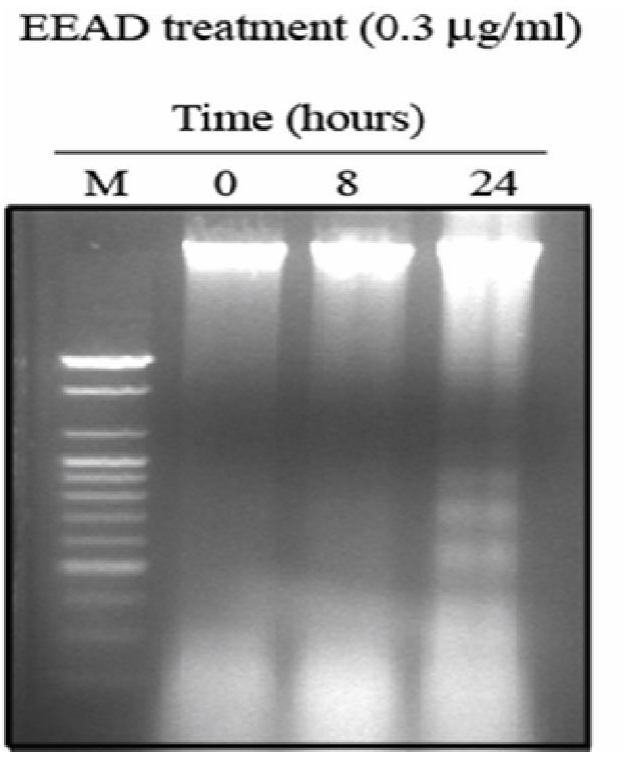


**Figure 2.** Fragmentation of internucleosomal DNA by EEAD in Saos2 cells. The cells were treated with 0.3 μg/ml EEAD for 0, 8 and 24 h and nuclear DNA was subjected to agarose gel electrophoresis

anticancer effects via the suppression of cancer cell growth in a concentration-dependent manner. The relatively lower concentration (1 μg/ml) of EEAD was enough to inhibit the Saos2 cell growth compared with other extracts (Chicca et al., 2007; Yang et al., 2006; Hostanska et al., 2007). In addition, even in high concentration (30 μg/ml), the EEAD did not inhibit the prolixferation of FOB human osteoblast cells as a control normal cell line compared with Saos2 cells. These results speculated that EEAD has specific cytotocity for only cancer cells and potential value for anticancer drug discovery.

Increased cellular apoptosis is only one among several possible mechanisms involved in reduced cell prolixferation. To determine if apoptosis is indeed the underlying mechanism for the reduced cell proliferation we had observed, the Saos2 cells treated with EEAD were subjected to DNA fragmentation. As shown in Figure 2, the formation of DNA ladder in the Saos2 cells treated with 0.3 μg/ml EEAD was observed in a time-dependent manner. Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death (Green and Reed, 1998; Hengartner, 2000; Kaufmann and Hengartner, 2001). And, the induction of apoptosis in cancer cells is one useful strategy for anticancer drug development (Hu and Kavanagh, 2003).

In this study, treatment with EEAD induced internucleosomal DNA fragmentation in Saos2 cells, suggesting apoptotic cell death (Figure 2). These results indicated that EEAD inhibits the growth of these cells by activating cell apoptosis. The levels of procaspase- 3 and 7 were examined by immunoblotting and detected by fluorescence microscopy using a selective fluorogenic substrate since caspase-3 and 7 are effectors caspases of apoptotic cell death. Treatment with 0.3 μg/ml EEAD significantly promote proteolytic cleavages of procaspase-3 and 7 in the Saos2 cells, with the decreases in the amount of procaspase-3 and 7 (Figure 3). In addition, activation of caspase-3 and 7 in EEAD treated Saos2 cells were confirmed by fluorescence microscopy using fluorogenic substrate. As shown in Figure 4, EEAD treatment led to the activation of the caspase-3 and 7 in the Saos2 cells.

The activation of a family of intracellular cysteine proteases, called caspases, is known to play an important role in the initiation and execution of apoptosis induced by various stimuli (Datta et al., 1997; Liu et al., 1997). Among the caspases identified in mammalian cells, caspase- 3 and 7 may serve as effectors caspases of apoptotic cell death (Datta et al., 1997; Liu et al., 1997; Cohen, 1997). Caspase-3 and 7 are synthesized as in-active proenzymes (of sizes 32 and 35 kDa, respectively), which require proteolytic activation (Datta et al., 1997; Liu et al., 1997; Cohen, 1997). Our results show that high levels of procapase-3 and 7 were present in EEAD untreated cancer cells, and the amount of procaspase-3 and 7 was decreased after EEAD treatment in the Saos2

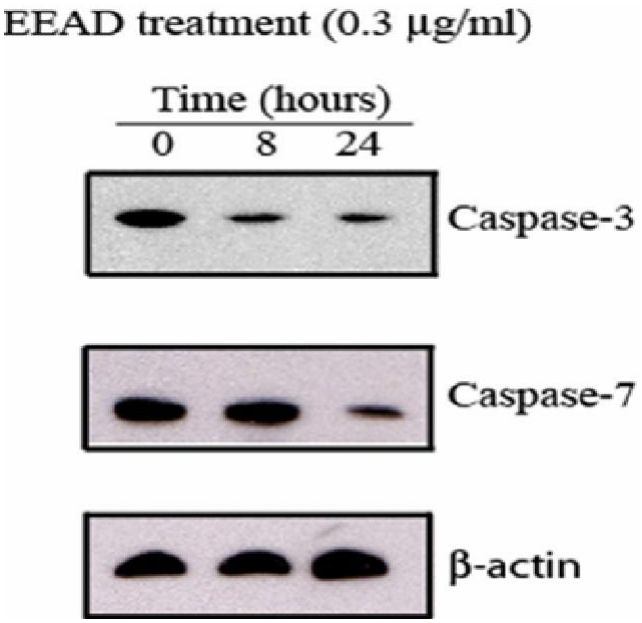


**Figure 3.** Proteolytic cleavage of procaspase-3 and 7 by EEAD treatment in Saos2 cells. Activity of procaspase-3 and procaspase-7 by EEAD was measured in Saos2 cells. The cells were treated with 0.3 g/ml EEAD for 0, 8 and 24 h. The cell lysate was prepared and analyzed by western blot analysis as described in "Materials and Methods".

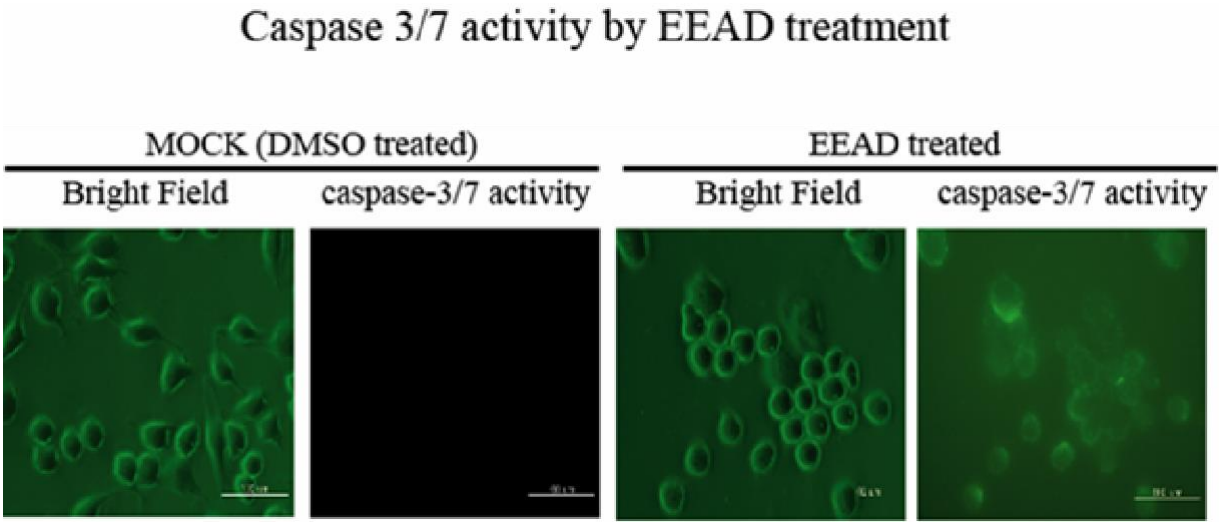


**Figure 4.** Activation of caspase-3 and 7 by EEAD treatment in living Saos2 cells. The cells were treated with 0.3 g/ml EEAD for 24 h and added specific cell-permeable substrate Phiphilux $G_1D_2$. Active of caspase-3/7 was visualized by fluorescence microscopy.

cells (Figure 3). In addition, the activity of caspase-3and 7 was increased by EEAD treatment in Saos2 cells compared with DMSO treatment as a control (Figure 4). These results suggested that EEAD induces apoptotic cell death through caspase-3 and 7 dependent processing in the Saos2 cells. The mechanisms of apoptosis induced by EEAD are not yet completely under-stood. Further studies will reveal the precise cellular and molecular mechanisms of apoptosis induced by EEAD.

In conclusion, we found that the EEAD highly inhibits cell proliferation and induces apoptosis in Saos2 human osteogenic sarcoma cells, but not inhibit the proliferation

of FOB human osteoblast cells. Moreover, these results suggest that the EEAD could be a new agent of chemo-therapeutic for the inhibition of osteogenic sarcoma cell growth. However, to elaborate this nascent possibility, further investigation of its activity including in vivo and purification of bioactive compounds is now in progress.

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