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Induction of Apoptosis by *Angelica decursiva* Extract in Glioma Cells

조선대학교 대학원

치의생명공학과

조 선 호

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Angelica decursiva 추출물에 의한
신경교종 세포의 Apoptosis 유도

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이 논문을 이학 석사학위신청 논문으로 제출함.

2009년 10월 일

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ABSTRACT

Induction of Apoptosis by *Angelica decursiva* Extract in Glioma Cells

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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, it has been completely unknown its anti-tumor activity. In this study, therefore, we have examined the cytotoxic activity of ethanol extract of *Angelica decursiva* root (EEAD), and the mechanism of cell death exhibited by EEAD in C6 rat glioma cells.

The cytotoxic effect of EEAD on cell growth inhibition in C6 cells was examined using MTT assay. And the cell death mechanism by EEAD was

examined using DNA fragmentation analysis, immunoblotting and caspase activation measurement.

Treatment of EEAD in C6 cells induced the apoptotic cell death in a concentration- and a time-dependent manner as determined by MTT assay and DNA fragmentation analysis. The proteolytic processing of caspase-3, -7 and -9 was increased by EEAD treatment in C6 cells. In addition, activation of caspase-3 and -7 was detected in living C6 cells by fluorescence microscopy.

These results suggest that the EEAD can induce the suppression of cell growth and cell apoptosis in C6 rat glioma cells, and that it may have potential properties for anti-tumor drug discovery.

KEY WORDS: anti-tumor therapy, apoptosis, cell death, EEAD, glioma cells

I. INTRODUCTION

In recent years, 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 captothecin, are shown to have potential values as tumor chemo-preventive or therapeutic agents within the human body.[1-4] Most of these bioactive substances exert their tumor chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death.[1-4] Therefore, induction of apoptosis in tumor cells has become an important indicator of the tumor treatment response in employing a plant-bioactive substance to reduce and control human mortality due to tumor.[5,6]

Apoptosis, which is a major way of programmed cell death, plays an important role in the regulation of tissue development and homeostasis in eukaryotes.[7-9] Apoptosis may occur via a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway and apoptosis is induced by treatment of chemotherapeutic agents.[10,11]

There are several medicinal plants that are considered to possess significant anti-tumor activity.[12-16] *Angelica decursiva*, one of Korean traditional medicine, has been used mainly as a folk remedy for treatment of antitussive, analgesic, antipyretic and a cough. However, the anti-tumor activity of *Angelica decursiva* was not reported at all.

Glioma is the most common type of malignant brain tumors and accounts for 40 - 50% of primary brain neoplasms.[17] Despite advances in microsurgical

techniques, radiotherapy and chemotherapy, there has been little improvement in the clinical outcome of patients suffering from these kinds of tumors.[18] In this study, therefore, we examined the effect of ethanol extract of *Angelica decursiva* root (EEAD) on cell growth and the mechanism of cell death elicited by EEAD in C6 rat glioma cells.

II. MATERIALS AND METHODS

1. Materials

The C6 rat glioma cells were provided by 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, anti-caspase-7 and anti-caspase-9 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Cell-permeable fluorogenic substrate PhiPhiLux-G1D2 was purchased from OncoImmunin, Inc. (Gaithersburg, MD, USA).

2. Plant material and extract preparation

Dried root parts of Angelica decursiva were purchased from Jeonnam herbal medicine farmer's cooperative, Republic of Korea. The botanical identification was made by Prof. Su-In Cho, School of Oriental Medicine, Pusan National University, Republic of 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℃ for 5 hours. The extract was then filtered through a Advantec No. 1 filter paper. The collected filtrate was dried by evaporation under vacuum at 40℃ using a rotary evaporator (N-1000V-W, Eyela, Japan). After evaporation, the concentrated extract was freeze-dried at -40℃ for 3 days and stored in a refrigerator at 2℃ until used.

3. Cell cultures

The C6 rat glioma cells were grown in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and the appropriate concentrations of antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).[19] The C6 cells were maintained as monolayers in plastic culture plates at 37°C in the humidified atmosphere containing 5% CO_2.

4. Inhibition of cell growth (MTT assay)

The cell viability test was performed according to the previously described method with minor modifications.[20,21] The cells were seeded at a concentration of 5 X 10^3 cells/well in 24-well plates. After 24 hours 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.

5. DNA fragmentation analysis

Following treatment with 1 μg/ml EEAD for 0, 1 and 2 days, approximately 5 X 10^6 cells were collected and transferred to lysis 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°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 5 ug/ml DNase-free RNase and incubated at 37°C for 1 hr. The DNA was visualized on 1.5% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

6. Immunoblotting

The cells were treated with 1 μg/ml EEAD for 0, 1 and 2 days. Immunoblotting was performed according to the previously described method with minor modifications.[22] The anti-caspase-3, anti-caspase-7 or anti-caspase-9 antibody (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the primary antibody.

7. 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 1 μg/ml EEAD for 24 hours and incubated with PhiPhiLux-G_1D_2. The activity of caspase-3/-7 was visualized by fluorescence microscopy (IX71, Olympus, Japan).

8. 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.

Ⅲ. RESULTS

1. Cytotoxic effect of EEAD in C6 cells

To analyze the effect of EEAD on the viability of cells, the cells were treated with EEAD at various concentrations for 1, 2 and 3 days, and then the MTT assay was performed. From 0.01 to 300 ㎍/ml treatment of EEAD, the inhibition of C6 cell growth depended on the EEAD treatment time (Fig. 1). When the cells were treated with EEAD (0.01 - 300 ㎍/ml), EEAD inhibited the proliferation of C6 cells in a dose-dependent manner (Fig. 1). The IC_{50} values of EEAD on the cell viability are shown in TABLE 1.

TABLE 1. ANTIPROLIFERATIVE EFFECT OF EEAD IN C6 CELLS

days	IC_{50} (㎍/ml)
1 day	> 300
2 days	1.05 ± 0.113
3 days	0.19 ± 0.026

The IC_{50} values represent the mean ± S.E.M. for three experiments.

2. DNA fragmentation

Increased cellular apoptosis is only one among several possible mechanisms involved in reduced cell proliferation. To determine if apoptosis is indeed the underlying mechanism for the reduced cell proliferation we had observed, the C6 cells treated with EEAD were subjected to DNA fragmentation. As shown in Fig. 2, the formation of DNA ladder in the C6 cells treated with 1 μg/ml EEAD was observed in a time-dependent manner.

3. Activation of caspases by EEAD

The levels of procaspase-3, procaspase-7 and procaspase-9 were examined by immunoblotting and the levels of procaspase-3 and procaspase-7 were detected by fluorescence microscopy using a selective fluorogenic substrate since caspase-3, caspase-7 and caspase-9 are effector caspases of apoptotic cell death. Treatment with 1 μg/ml EEAD significantly promoted proteolytic cleavages of procaspase-3, procaspase-7 and procaspase-9 in the C6 cells, with the decreases in the amount of procaspase-3, procaspase-7 and procaspase-9 (Fig. 3). In addition, activation of caspase-3/-7 in EEAD treated C6 cells was confirmed by fluorescence microscopy using fluorogenic substrate. As shown in Fig. 4, EEAD treatment led to activate the caspase-3/-7 in the C6 cells.

Ⅳ. DISCUSSION

Recently, scientific attentions increased to oriental medicine for the discovery of novel drugs including anti-tumor agents.[12-16] Chemotherapeutic drugs are known to induce cytotoxicity in tumor cells through diverse mechanisms, in which signaling events play an important role depending upon the cell type and stimulus.[23,24] There is a need to find new anti-tumor drugs that can kill cancerous cells with minimal toxicity. The *Angelica decursiva* has been used as a material for medical treatment in Korean traditional medicine. However, its anti-tumor effect has been unknown at all. The main goal of this study was to investigate the effect of EEAD on cell growth and the cell death mechanism by EEAD in C6 rat glioma cells.

In MTT assay, EEAD inhibited growth of C6 cells in a concentration- and a time-dependent manner (Fig. 1). This corresponded with the results of several extracts (*Echinacea* root, *Toona sinensis*, *Willow bark*) that have anti-tumor effects via the suppression of tumor cell growth in a concentration-dependent manner.[25-27] The relatively lower concentration (1 ㎍/ml) of EEAD was enough to inhibit the C6 cell growth compared with other extracts.[25-27] These results speculated that EEAD has specific cytotocity for tumor cells and potential value for anti-tumor drug discovery.

Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death.[7-9] And, the induction of apoptosis in tumor cells is one of

useful strategies for anti-tumor drug development.[28] So, many studies were performed for screening of apoptosis including extracts from plants. In this study, treatment with EEAD induced internucleosomal DNA fragmentation in C6 cells, suggesting apoptotic cell death (Fig. 2). These results indicated that EEAD inhibits the growth of these cells by activating cell apoptosis.

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.[29,30] Among the caspases identified in mammalian cells, caspase-3, caspase-7 and caspase-9 may serve as effector caspases of apoptotic cell death.[29-31] Caspase-3, caspase-7 and caspase-9 are synthesized as inactive proenzymes (of sizes 32 kDa, 35 kDa and 47 kDa, respectively), which require proteolytic activation.[29-31] Our results show that high levels of procapase-3, procapase-7 and procapase-9 were present in EEAD-untreated C6 cells, and the amount of procaspase-3, procapase-7 and procaspase-9 was decreased after EEAD treatment in the C6 cells (Fig. 3). In addition, the activity of caspase-3/-7 was increased by EEAD treatment in C6 cells compared with DMSO treatment as a control (Fig. 4). These results suggested that EEAD induces apoptotic cell death through caspase-3-, caspase-7- and caspase-9-dependent processing in the C6 cells. The mechanisms of apoptosis induced by EEAD are not yet completely understood. 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 C6 rat glioma cells. Moreover, these results suggest that the EEAD could be a new agent of chemotherapeutic for the inhibition of glioma 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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Ⅵ. FIGURE LEGENDS

Fig. 1. Cytotoxic effects of EEAD in C6 cells.

The C6 cells were treated with 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 ㎍/ml EEAD for 1 day (filled circle), 2 days (filled square) and 3 days (filled triangle). 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).

Fig. 2. Fragmentation of internucleosomal DNA by EEAD in C6 cells.

The cells were treated with 1 ㎍/ml EEAD for 0, 1 and 2 days and nuclear DNA was subjected to agarose gel electrophoresis.

Fig. 3. Proteolytic cleavage of procaspase-3, procaspase-7 and procaspase-9 by EEAD treatment in C6 cells.

Activity of procaspase-3, procaspase-7 and procaspase-9 by EEAD was measured in C6 cells. The cells were treated with 1 ㎍/ml EEAD for 0, 1

and 2 days. The cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS".

Fig. 4. Activation of caspase-3/-7 by EEAD treatment in living C6 cells.

The cells were treated with 1 μg/ml EEAD for 24 hours and added specific cell-permeable substrate Phiphilux G_1D_2. Active of caspase-3/-7 was visualized by fluorescence microscopy.

Ⅶ. FIGURES

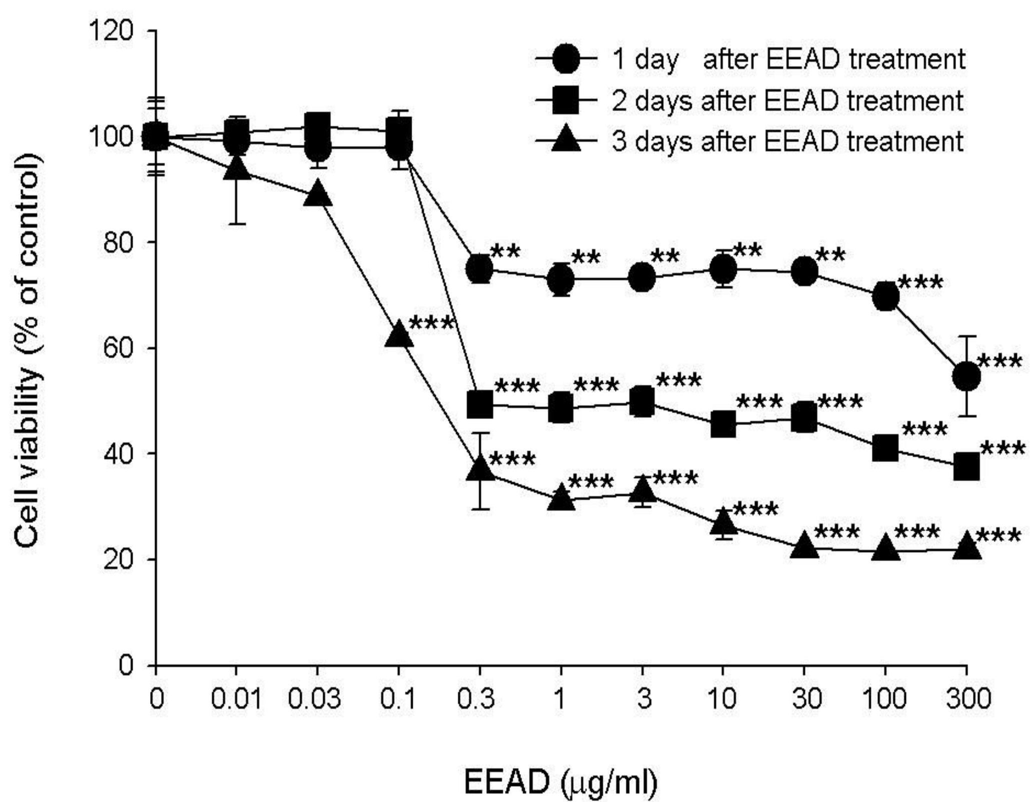

Fig. 1.

Ⅶ. FIGURES

EEAD treatment (1 ㎍/㎖)

Time (days)

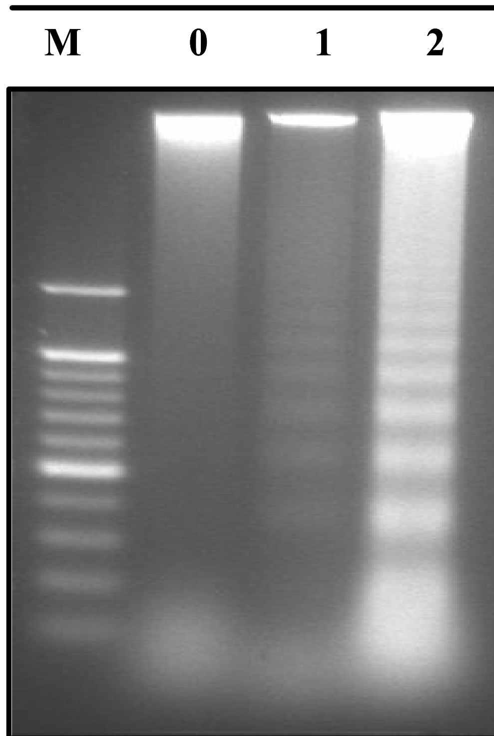

Fig. 2.

Ⅶ. FIGURES

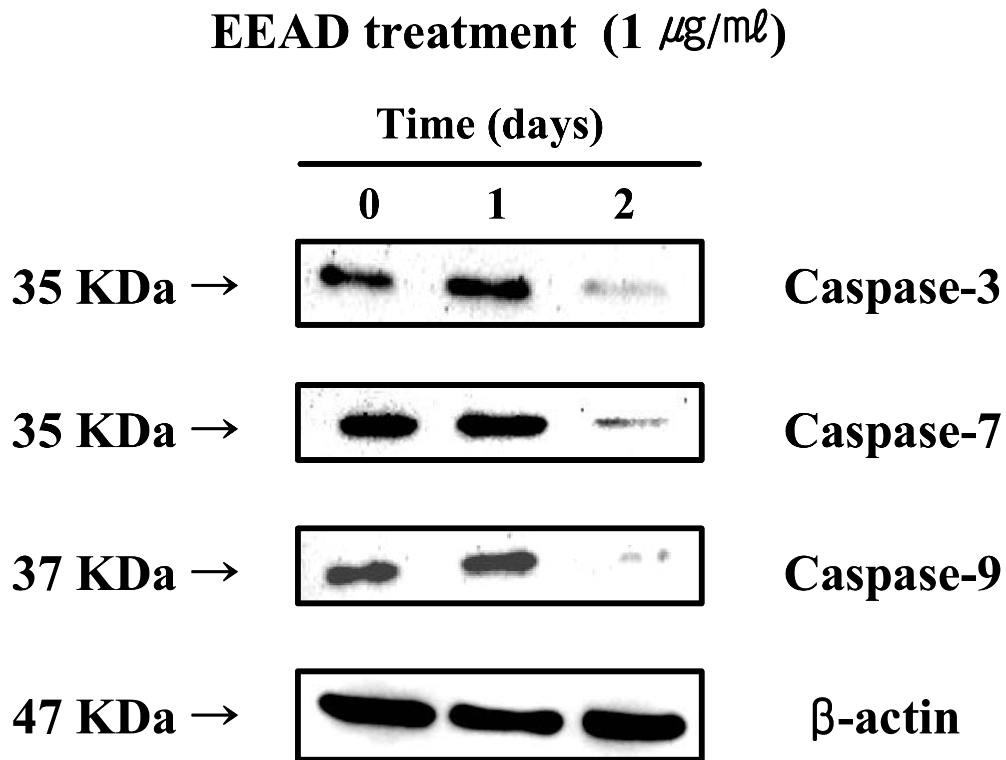

Fig. 3.

Ⅶ. FIGURES

Caspase-3/-7 activity by EEAD treatment

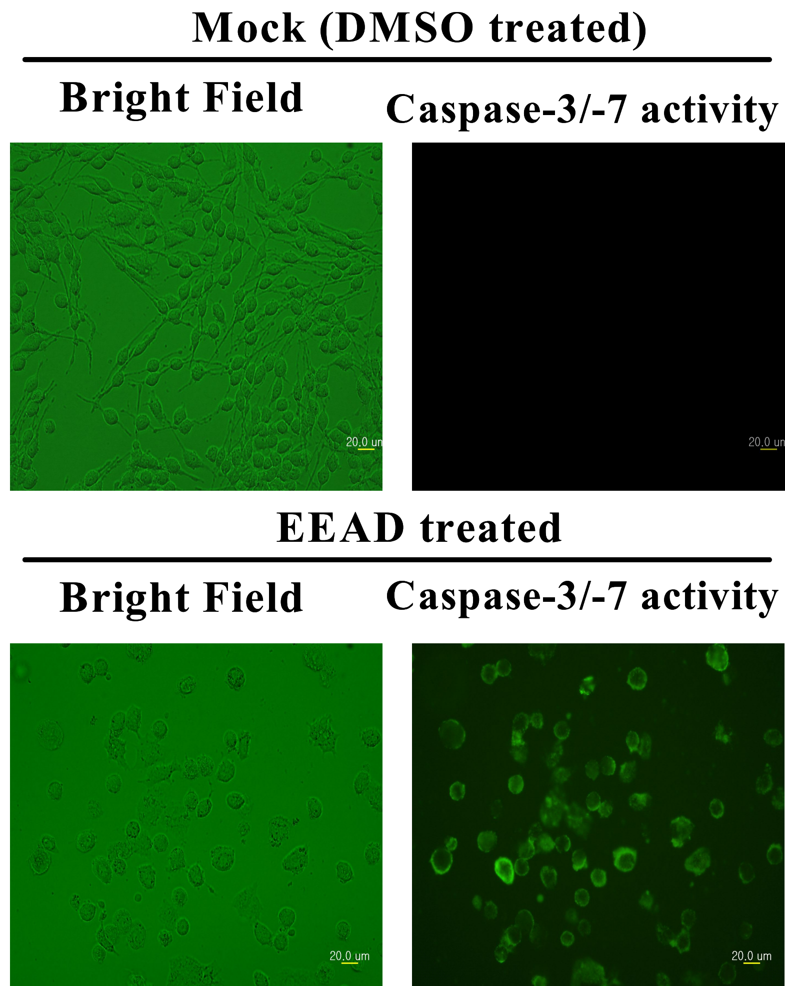

Fig. 4.

ABSTRACT in KOREAN

Angelica decursiva 추출물에 의한 신경교종 세포의 Apoptosis 유도

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(지도교수: 김 도 경)

우리나라에서 오래전부터 의약품으로 사용되어져 온 전통 약제 중 하나인 *Angelica decursiva*는 민간요법으로 진해제, 진통제, 해열제 또는 기침약 등으로 사용되어져 왔다. 그러나 *Angelica decursiva*의 항암효과에 관한 자료는 거의 없다. 본 연구는 흰쥐 신경교종 세포 C6 세포주를 이용하여 *Angelica decursiva*의 암세포 성장억제에 미치는 효과와 세포성장 억제기전을 밝히기 위해, C6 세포주에서 *Angelica decursiva*의 에탄올 추출물(EEAD)을 이용하여 MTT 분석, DNA fragmentation 분석 및 immunoblotting 등을 시행하였다.

EEAD는 C6 세포의 성장을 시간과 농도에 의존적으로 억제하였다. EEAD를 처리한 C6 세포주 실험군에서 DNA fragmentation 현상을 확인할 수 있었다. C6 세포에

EEAD를 처리한 실험군에서 procaspase-3, procasapse-7 및 procaspase-9의 proteolytic cleavage 현상을 확인할 수 있었다. C6 세포에 EEAD를 처리한 실험군에서 caspase-3/-7의 활성화를 확인할 수 있었다.

본 연구의 결과로 *Angelica decursiva* 에탄올 뿌리 추출물 EEAD는 흰쥐 신경교종 세포 C6 세포주의 apoptosis를 유도하여 암세포 성장을 억제시키는 것으로 사료된다. 또한 본 연구의 결과로, EEAD를 이용한 암세포의 성장억제에 관한 하나의 방향을 제시할 수 있을 것으로 사료된다.

저작물 이용 허락서

<table>
<tr><td>학 과</td><td>치의생명공학과</td><td>학 번</td><td>20087280</td><td>과 정</td><td>석사</td></tr>
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<tr><td>논문제목</td><td colspan="5">한글: Angelica decursiva 추출물에 의한 신경교종 세포의 Apoptosis 유도

영문: Induction of Apoptosis by Angelica decursiva Extract in Glioma Cells</td></tr>
</table>

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

\- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의(O) 반대( )

2010년 2월 일

저작자: 조 선 호 (서명 또는 인)

조선대학교 총장 귀하