대한한방부인과학회지
THE JOURNAL OF ORIENTAL OBSTETRICS & GYNECOLOGY
VOL.18, NO.1 : 045 - 054 (2005)

SKBR3 유방암세포주에 대한 귀전우 메탄올 추출물의 성장억제 및 항산화효과

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ABSTRACT

Growth inhibitory and antioxidative effects of crude methanolic extract from *Euonymus alatus* (Thunb.) Sieb on SKBR3 human breast cancer cell line

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이 연구는 SKBR3 인간 유방암세포주에 대한 귀전우 메탄올추출물(CME)의 증식억제 효과, 세포사 유발 효과 및 항산화 활성을 확인하기 위해 이루어졌다 SKBR3 유방암세포주는 48시간동안 다양한 농도(0～20㎍/㎖)의 CME가 제공된 곳에서 배양되었고, MMT 측정법을 이용하여 세포생존율을 평가하였다. CME의 50%에서 효과를 나타내게 하는 약물농도인 ED_{50} (effective dose 50%)은 6.5±0.3㎍/㎖이며, 투여량이 증가함에 따라 농도에 의존하여 세포증식이 억제되는 것으로 나타났다. 또한 CME의 증식억제 효과는 유방암세포주의 세포사와 관련됨의 세포의 형태학적 변화와 올리고뉴클레오솜 DNA 파편의 확인을 통해 알 수 있었다. 또한 다양한 농도와 배양시간에서 CME가 ROS의 생산을 억제한다는 것을 확인할 수 있었다. 이런 결과들은 귀전우의 메탄올추출물이 SKBR3 인간 유방암세포주에 대해 강력한 증식억제 효과와 강한 항산화 효과를 나타낼 뿐만 아니라 세포사를 유도하는 효능을 가지고 있음을 시사한다. 이러한 효능은 약물에 대한 노출시간과 투여량에 의존하였다. 따라서 귀전우는 다양한 기전에 의해 유방암 세포에 대한 억제효과를 가질 수 있을 것으로 인식할 수 있다.

Author Keywords : Breast cancer; *Euonymus alatus* (Thunb.) Sieb; Antiproliferation; Apoptosis; Antioxidant

Abbreviations : EA, *Euonymus alatus* (Thunb.) Sieb

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Ⅰ. Introduction

Breast cancer is the most common cancer in Korean women. The incidence of breast cancer is around 25 per 100,000 women, and more than 5,500 women are diagnosed as breast cancer annually[1]. Most cases occur during age 45-55. It also occurs in men but is more than 100-fold less frequent than in women[2]. At present, cancer treatment by chemotherapeutic agents, surgery and radiation have not been fully effective against the high incidence or low survival rate of most cancers. The development of new therapeutic approach to breast cancer remains one of the most challenging area in cancer research.

Euonymus alatus (Thunb.) Sieb (EA), known as 'gui jun woo' in Korea, was used in folk medicine to regulate *qi* (bodily energy) and blood circulation, relieve pain, eliminate stagnant blood, and treat dysmenorrhea in oriental countries. It can increase tolerance to oxygen deprivation, and has a significant, albeit temporary, hypotensive effect. It acts as a depressant on the CNS and can lengthen barbiturate-induced sleeping times. Its effects on metabolism include a reduction of blood sugar levels via stimulation of the beta cells of pancreatic islets. Additionally, quercetin has been found to be a good expectorant[3]. The antimetastatic and cytotoxic activity of the crude extract or the isolated compounds, however, have not yet been demonstrated. The stems of EA, commonly known as winged euonymus, have been used in traditional medicine for cancer treatment. Previous phytochemical and biological studies on winged euonymus have resulted in the isolation of cardenolides[4]. Substances isolated from EA have been documented to exhibit antioxidant capabilities, and recent studies also indicated that EA has anti-tumor potential[5]. It was reported that the crude extract of EA markedly prolonged the survival period of cervical carcinoma-bearing mice, and methanol extract from this plant[6]. Methanol and buthanol extracts were also found to have anti-tumor activity in mice[7]. Moreover, there are some reports on the action of EA extract on transformed cells *in vitro*[8]. It was recently found that the methanolic extract of EA exhibited a significant anti-proliferation effect against cultured human cancer cell lines[7]. Our recent findings also suggest that EA is a potent antioxidant in protecting primary hepatocytes from oxidative damage induced by aflatoxin B1, a well recognized hepatocarcinogen [unpublished results]. In our preliminary study, EA inhibited uterine leiomyomal cell(ULMC) proliferation with an increased PKC activity.

From the above traditional usages and later scientific findings suggested that the EA is a potential candidate as an anticancer agent. It is very likely that the traditional uses especially in the treatment of abdominal pain, leucorrhoea and chronic ulcer are related to the antiinflammatory and antioxidant properties of EA. Although many benefits of EA have been claimed, only few authentic scientific studies are available. The present investigation was undertaken to evaluate the antiproliferation, apoptosis and antioxidant of crude methanolic extract

(CME) from EA using SKBR3 human breast cancer cell line as a model.

Ⅱ. Materials and methods

1. Reagents

Fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Jeil Biotech, Inc. (Daegu, Korea). Dulbecco's Modified Eagle's Medium (DMEM), glutamine, dimethyl sulphoxide (DMSO) and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma Chem. Co. (St. Louis, USA). RPMI 1640 medium and foetal calf serum (FCS) were obtained from Biochrom (Berlin, Germany). Hanks' balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-di phenyl tetrazolium bromide (MTT), propidium iodide (PI), Benzimidazole Hoechst 33342 (Ho33342), 2′,7′-dichlorodihydro fluorescein diacetate (DCFH-DA) and α-tocopherol (Vitamin E) were purchased from Sigma (St. Louis, MO). Proteinase K was purchased from Promega (Madison, WI) and RNase A was from Amresco (Buckinghamshire, UK).

2. Plant material of methanol extracts

The plant samples were extracted three times with methanol at 70℃ for 5 hours. The extracts were filtered through a 0.45 ㎛ filter and lyophilized. The w/w yield of the methanol extracts was about 2.25%[7]. The extracts were filtered and concentrated to remove the solvent at 75℃ for 4 h and 100 g of CME was yielded eventually. The CME was kept at 4℃ and dissolved with 10% DMSO in RPMI 1640 medium containing 10% FCS for further experiment. The stems of EA (Thunb.) Sieb were collected in Kyungju city, Korea, and the sample and voucher specimen (number 4-99-221) are kept in the herbarium of the College of Oriental Medicine, Dongguk University.

3. Cell culture

SKBR3 cell line was obtained from the American Type Culture Collection (Rockville, MD) and was cultured in RPMI 1640 or DMEM medium supplemented with 10% (v/v) FSC, 100 mg/l of streptomycin and 100,000 U/l of penicillin G at 37℃ in 5% CO_2 incubator.

4. Cell proliferation assay

Serial dilutions of CME (20㎕) were added into each of 96-well plates, then, cells were plated at a density of 1×10^4 cells/well and incubated for 48 h. After incubation, the medium was removed and cells in each well were incubated with HBSS contained 1 mg/㎖ MTT for 2 h at 37℃ in 5% CO_2 incubator. MTT solution was then discarded and 50㎕ of isopropanol was added into each well to dissolve insoluble formazan crystal. Plates were then kept agitation for 5 min at room temperature for complete solubilization. The level of colored formazan derivative was analysed on a microplate reader (Molecular Devices, CA) at a wavelength of 590 nm[7]. The percentage of cell viability was calculated according to the following equation.

5. Observation of cells by phase contrast microscope

Cells (2×10^5 cells/well) were incubated for

48 h in the absence or presence of CME in 24-well plates. After incubation, the medium was removed and cells in wells were washed once with HBSS. They were observed by phase contrast inverted microscope (Zeiss, Germany) at 400×magnification[9].

6. Detection of DNA fragmentation

DNA fragmentation was analysed by agarose gel electrophoresis as described by [12] with slight modifications. Cells (3×10^6 cells) were exposed to the extract for 48 h and were gently scraped and harvested by centrifugation. The cell pellets were incubated for 60 min at 50℃ in 100㎕ lysis buffer (100 mM Tris-HCl pH 8, 100 mM NaCl and 10 mM EDTA). Proteinase K (10㎕ of 20 mg/㎖) was added and further incubated for 30 min at 50℃. RNase (3㎕ of 10 mg/㎖) was then added and incubated for 2 h at 50 ℃. The DNA was extracted with phenol-chloroform-isoamyl alcohol, subjected to 2.0% of agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light transilluminator (Fotodyne, WI, USA).

7. Measurement of ROS production

Intracellular reactive oxygen species (ROS) production was measured in both CME-treated and control cells using DCFH-DA[13]. Briefly, 2×10^5cells/well were exposed to CME with various concentrations and different incubation times. After incubation, cells were detached with trypsin-EDTA and washed once with PBS. Treated and control cells were resuspended in 0.5 ㎖ PBS containing 10μM DCFH-DA at 37℃ for 30 min and then incubated with 4 mM H_2O_2 (as inducer for ROS production) at 37℃ for 30 min. ROS production of cells were subjected to evaluate by luminescence spectrophotometer (Perkin-Elmer, MA).

8. Statistical analysis

The experiments were repeated three to four times and the results were expressed as mean±S.D. Statistical analysis was done using two-tailed Student's *t* test.

Ⅲ. Results

1. Effect of CME on the proliferation of SKBR3 human breast cancer cell line

The relationship between concentration of CME and their cytotoxic effect on SKBR3 cells was investigated by MTT assay. Cells were treated with CME at concentrations ranging from 0 to 20 ㎍/㎖ for 48 h and then the percentage of cell viability was analysed as described in Materials and Methods. CME from EA significantly inhibited the proliferation of SKBR3 cells in a dose-dependent manner [Fig. 1]. Similar result was observed when quercetin was served as a positive control[7,14,15]. CME at 5.0-20㎍/㎖ decreased the proliferation of SKBR3 cells by 20-90% and with an ED50 of 6.5±0.5 ㎍/㎖.

2. Effect of CME on the morphological changes of SKBR3 human breast cancer cell line

After incubation with 20㎍/㎖ of CME, morphological alterations in SKBR3 cells were illustrated (Fig 2B) comparing with control cells (Fig. 2A). Untreated or control

cells were cuboid and polygonal in normal shape. Exposure of SKBR3 cells to CME for 48 h led to retraction, rounding and some sensitive cells were detached from the surface. Membrane blebbing (Fig. 2B), arrow No. 2) and apoptotic body (Fig. 2B), arrow No. 3) were observed by phase contrast inverted microscope.

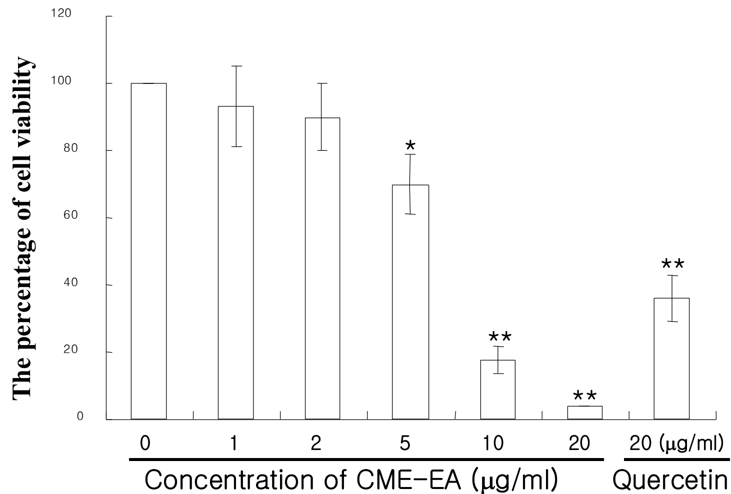

Fig. 1. Effect of CME from EA on the proliferation of SKBR3 cells. The percentage of cell viability was measured by MTT assay. Data represent the means±S.D. (n=4). *, $p<0.05$; **, $p<0.01$ versus control (0).

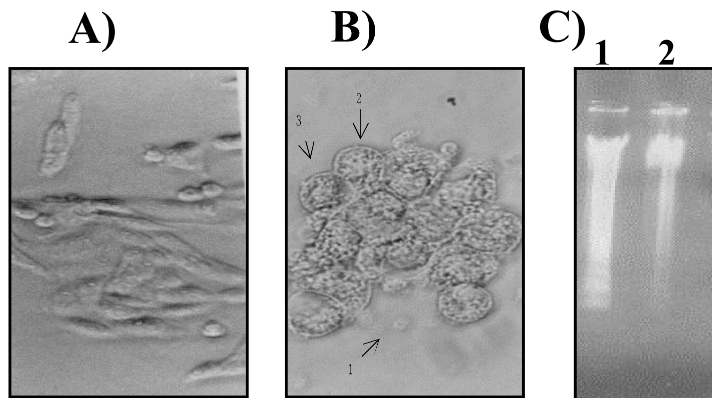

Fig. 2. Morphological alterations of SKBR3 cells following expose to 20㎍/㎖ of CME for 48 h.

(A) Control SKBR3 cells were observed by phase contrast inverted microscope. (B) CME-treated SKBR3 cells were observed by phase contrast inverted microscope. 1: normal cells; 2: membrane blebbing; 3: apoptotic body. (C) Effect of CME on DNA fragmentation of SKBR3 cells and ladders were detected by 1.2% agarose gel electrophoresis. 1: EA treated cells; 2: controls

3. Appearance of DNA ladders in CME-treated cells

The DNA fragmentation of SKBR3 cells (3×10^6 cells) were detected on a 2.0% agarose gel electrophoresis after exposing with 0, 10, 20 and 50㎍/㎖ of CME for 48 h. At exposure to 50㎍/㎖ of CME, fragmented DNA was clearly observed in SKBR3 cells (Fig. 2C) whereas control cells did not provide ladders. Thereby, it is possible that CME from EA causes apoptosis of SKBR3 cells.

4. Effect of CME on the ROS production of SKBR3 human breast cancer cell line

To investigate possible correlation between time and concentration of CME on ROS production, SKBR3 cells were incubated with CME at concentrations ranging from 0 to 20 ㎍/㎖ for 24, 48 and 72 h using Vitamin E as a positive control. Intracellular ROS was measured in terms of fluorescence by DCFH-DA. CME from EA could significantly suppressed the intracellular ROS production of SKBR3 cells in a dose-dependent manner (Fig. 3).

Notably, at 20㎍/㎖ of CME and incubation time for 48 h, treated cells showed a remarkably increase of ROS level. This case presumably revealed that most cells were induced early apoptosis which caused by oxidative stress. Such condition led to oxidative injury of cells that eventually resulted in cellular component damage and late apoptosis.

A) **Incubation time 24 h**

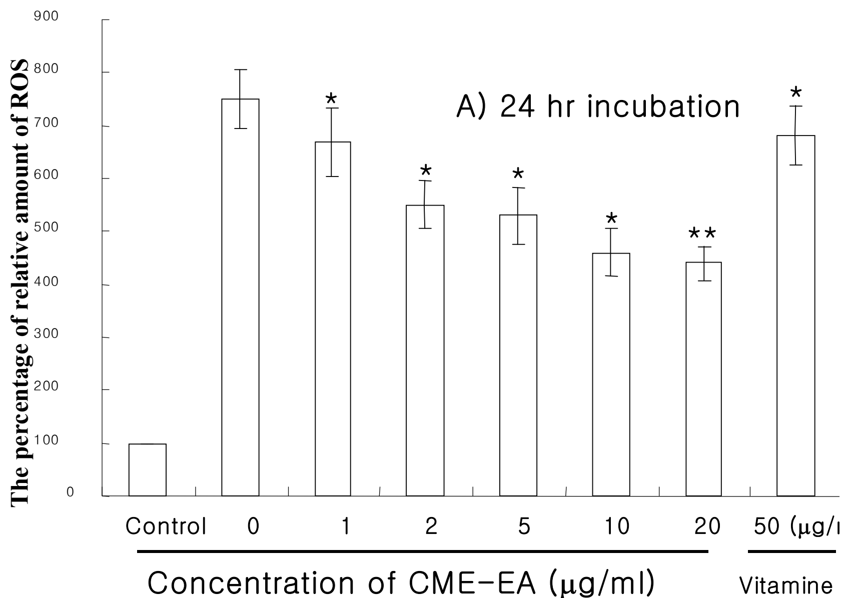

Fig. 3-1. **Effect of CME from EA on ROS production of SKBR3 cells by using DCFH-DA as fluorescence probe.** Data represent the means±S.D. (*n*=7). *, $p<0.05$; **, $p<0.01$ versus control (0). Controls cells without H2O2.

B) **Incubation time 48 h**

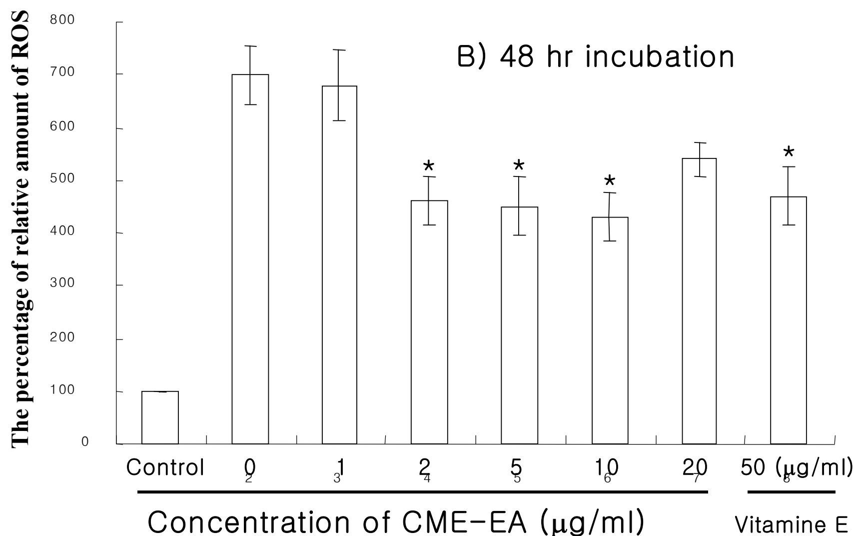

Fig. 3-2. **Effect of CME from EA on ROS production of SKBR3 cells by using DCFH-DA as fluorescence probe.** Data represent the means±S.D. (n=7). *, $p<0.05$; **, $p<0.01$ versus control (0). Controls cells without H2O2.

C) **Incubation time 72 h**

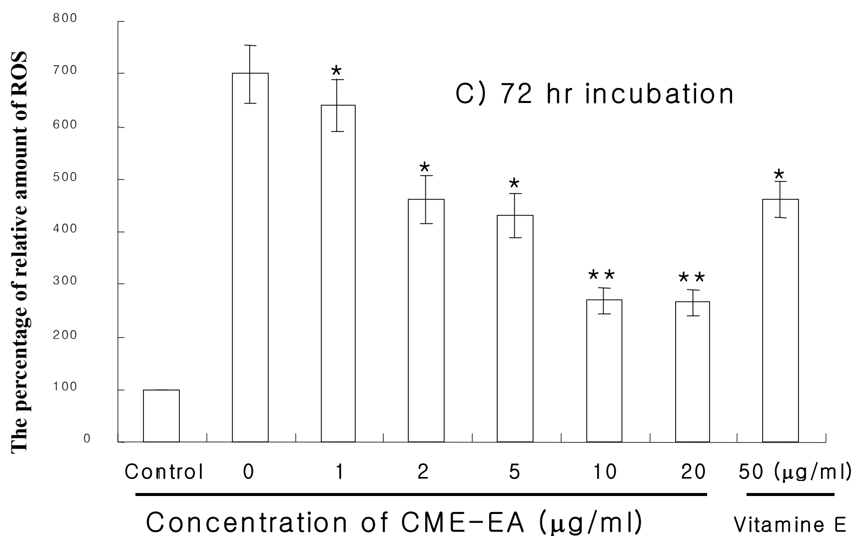

Fig. 3-3. **Effect of CME from EA on ROS production of SKBR3 cells by using DCFH-DA as fluorescence probe.** Data represent the means±S.D. (n=7). *, $p<0.05$; **, $p<0.01$ versus control (0). Controls cells without H2O2.

Ⅳ. Discussion and conclusion

Although EA has long been served as traditional medicines, very few authentic scientific studies in field of cancer therapy are available. Recent in vitro studies have shown that many constituents from EA have a wide range of biological actions including antibacterial, antifungal, antihelmith and insecticidal activities. Although the possible mechanism involved in the inhibition of proliferation is unknown, the effects of EA on cellular growth were investigated. In this study, we investigated the antiproliferation, antioxidation and induction of apoptosis by CME from EA on human breast cancer cell line. We found that CME significantly inhibited the proliferation of breast cancer cells after an incubation period of 48 h and the antiproliferative effect was evaluated by MTT reduction assays. The results presented here showed a concentration-dependent decrease in the percentage of cell viability and at a concentration of 6.5-20㎍/㎖ of CME was sufficient to effectively inhibit the cell proliferation. Thus, CME displayed the strong antiproliferative activity on breast cancer cells with an ED_{50} of 6.5±0.3㎍/㎖.

To investigate whether apoptosis is involved in the cell death caused by CME on SKBR3 breast cancer cells, we assessed morphological changes and DNA ladder patterns on agarose gel electrophoresis. Morphological analysis of cells with Ho33342 and PI staining strikingly displayed nuclear shrinking, DNA condensation and fragmentation (Fig.2) after treating cells with 10㎍/㎖ of CME for 48 h. Moreover, morphological changes were also observed by phase contrast microscope which exhibited cytoplasmic membrane shrinkage, loss of contact with neighboring cells, membrane blebbing and apoptotic body (Fig.2). In addition, oligonucleosomal DNA fragments (ladders) from cells were exhibited by 2.0% agarose gel electrophoresis after incubation with 50㎍/㎖ of CME (Fig. 3). These hallmark features of morphological changes suggested that CME from EA caused apoptosis of SKBR3 breast cancer cells.

In this study, we found that CME significantly decrease intracellular ROS production on SKBR3 cells in dose-and time-dependent manner during 24 and 72 h. Although the ROS level was increased by 20㎍/㎖ of CME at 48 h incubation time and mostly decreased by the same concentration at 72 h incubation time. It was possible that CME at a concentration of 20㎍/㎖ and with 48 h incubation time, early apoptosis could have been induced in cells. This phenomenon is possible, since the accumulation of intracellular ROS is one of the important processes leading to early apoptosis. Such condition of oxidative stress causes the damage of various cellular component (protein, DNA and other organelles) and finally results in programmed cell death or apoptosis[16]. Thus, at 20㎍/㎖ of CME and 72 h incubation time, ROS level was dramatically and decreased since only cell debris remains in well. It appeared that CME at high (20㎍/㎖) dose cause apoptosis whereas at low (2㎍/㎖) and medium (5-10 ㎍/㎖) doses show antioxidative effects on

breast cancer cells. On the other hand, it has been proposed that the excessive production of ROS is not involved in cancer cell proliferation but it is purposed to apoptosis of cells.

In conclusion, the results demonstrated that CME from pericarp of EA have a powerful antiproliferation by inducing apoptotic cell death and a potent antioxidation by inhibiting the intracellular ROS production significantly. Moreover, we assume that determination of ROS level not only measure antioxidation of extract on cells but also measure its induction of apoptosis on cells. These probable properties of EA provide scope of further detail evaluation. Some constituents from EA may serve as a novel powerful antitumour agent and free radical scavenger after further detailed investigation. Moreover, other biological activities and on different cell lines which are correlated to traditional treatments of EA should be investigated as well such as gastrointestinal tract disorder and chronic infections.

□ 투 고 일 : 2004년 07월 28일
□ 심 사 일 : 2004년 08월 02일
□ 심사완료일 : 2004년 08월 07일

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