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鬼箭羽 물 추출물에 의한 유방암 세포주 증식억제에 관한 연구

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ABSTRACT

A study of antiproliferative effect by *Euonymus alatus* (Thunb.) Sieb water-extract on SKBR3 human breast cancer cell line

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Purpose : 이 연구는 SKBR3 인간 유방암 세포주에 대한 *Euonymus alatus* (Thunb.) Sieb 추출물 (CWE) 의 증식억제, 항산화 작용 및 세포사 유발 효과를 검토하기 위해 이루어졌다.

Methods : SKBR3 세포주는 48시간 동안 다양한 농도 (0-40 ㎍/ml)의 CWE를 첨가하면서 배양되었고, 세포의 생존 비율은 MTT 배양을 통해서 평가하였다. 또한 CWE의 증식억제 효과는 유방암 세포주의 세포사와 관련되어 있음을 형태학적인 변화와 올리고뉴클레오솜 DNA 분절을 통해 확인하였다.

Results : CWE의 50%에서 효과를 나타내게 하는 약물농도인 ED_{50} (effective dose 50%)은 9.3+2.2㎍/ml이며, 약물의 농도에 의존하여 세포의 증식을 억제시켰다. 아울러, 다양한 농도와 배양시간에서 CWE가 ROS 생산을 억제하는 것을 밝힐 수 있었다. 따라서 이러한 작용과 항암예방효과는 농도와 노출 시간에 의존하였다.

Conclusion : 이러한 관찰을 통해 *Euonymus alatus* (Thunb.) Sieb의 열수 추출물은 SKBR3 인간 유방암 세포주에 대해 강한 증식억제 효과와 강력한 항산화효과 및 세포사의 유발 효과를 가지는 것으로 인식할 수 있다.

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

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I . Introduction

Breast carcinoma (BC) is the comm onest cancer among women and the se cond highest cause of cancer death[1]. M ost cases occur during age 45-55. It als o occurs in men but is more than 100-f old less frequent than in women[2]. At present, the cancer treatment by chemot herapeutic agents, surgery and radiation have not been fully effective against th e high incidence or low survival rate o f most the cancers. The development of new therapeutic approach to breast can cer remains one of the most challengin g area in cancer research.

Euonymus alatus (Thunb.) Sieb (E A), known as 'gui-jun woo' in Korea, was used in folk medicine to regulate *qi* (bodily energy) and blood circulatio n, relieve pain, eliminate stagnant bloo d, and treat dysmenorrhea in eastern a sia countries. It can increase tolerance t o oxygen deprivation, and has a signifi cant, albeit temporary, hypotensive effec t. It acts as a depressant on the CNS a nd can lengthen barbiturate-induced sle eping times. Its effects on metabolism i nclude a reduction of blood sugar level s via stimulation of the beta cells of pa ncreatic islets. Additionally, quercetin h as been found to be a good expectoran t. [3] The antimetastatic and cytotoxic act ivity of the crude extract or the isolate d compounds, however, have not yet b een demonstrated. The stems of EA, co mmonly known as winged euonymus, have been used in traditional medicine for cancer treatment. Previous phytoche mical and biological studies on winged euonymus have resulted in the isolation of cardenolides[4]. Substances isolated fro m EA have been documented to exhibit antioxidant capabilities, and recent studi es also indicated that EA has anti-tumo r potential[5]. It was reported that the cr ude extract of EA markedly prolonged the survival period of cervical carcinom a-bearing mice, and methanol extract fr om this plant[6]. Methanol and buthanol extracts were also found to have anti-t umor activity in mice[7]. Moreover, there are some reports on the action of EA e xtract on transformed cells in vitro[8]. It was recently found that the methanolic extract of EA exhibited a significant ant i-proliferation effect against cultured hu man cancer cell lines[7]. Our recent findi ngs also suggest that EA is a potent an tioxidant in protecting primary hepatoc ytes from oxidative damage induced by aflatoxin B1, a well recognized hepatoc arcinogen [unpublished results]. In our preliminary study, EA inhibited uterine leiomyomal cell(ULMC) proliferation wi th an increased PKC activity.

From the above traditional usages and later scientific findings suggested t hat the EA is a potential candidate as an anticancer agent. It is very likely th at the traditional uses especially in the treatment of abdominal pain, leucorrhoe a and chronic ulcer are related to the a ntiinflammatory and antioxidant propert

ies 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 water extract (CWE) 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 Eagles 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), 2',7'-dichlorodihydro fluorescein diacetate (DCFH-DA) and 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 water extracts

The stems of *Euonymus alatus* (Thunb.) Sieb was collected in Kyungju city, the Republic of Korea, and the sample and voucher specimen (number 4-99-221) are kept in the herbarium of the College of Oriental Medicine, Dongguk University. The plant samples were extracted three times with water by boiling for 2 hours. The extracts were filtered through a 0.45 ㎛ filter and lyophilized. The w/w yield of the water extracts was about 5.6%.

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 °C in 5% CO_2 incubator.

4. Cell proliferation assay

Serial dilutions of CWE (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/ml MTT for 2 h at 37 °C 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 (2×10^5cells/well) were incubated for 48 h in the absence or presence of CWE 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 °C in 100 ㎕ lysis buffer (100 mM TrisHCl pH 8, 100 mM NaCl and 10 mM EDTA). Proteinase K (10 ㎕ of 20 mg/ml) was added and further incubated for 30 min at 50 °C. RNase (3 ul of 10 mg/ml) was then added and incubated for 2 h at 50 °C. The DNA was extracted with phenolchloroformisoamyl 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 CWE-treated and control cells using DCFH-DA[13]. Briefly, 2×10^5 cells/well were exposed to CWE 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 ml PBS containing 10 μM DCFH-DA at 37 °C for 30 min and then incubated with 4 mM H_2O_2 (as inducer for ROS production) at 37 °C 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 and *P* values at a level of 95% confidence limit.

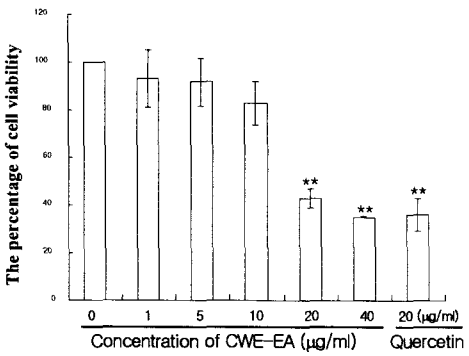
Ⅲ. Results

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

The relationship between concentration of CWE and their cytotoxic effect on SKBR3 cells was investigated by MTT assay. Cells were treated with CWE at concentrations ranging from 0 to 40 ㎍/ml for 48 h and then the percentage of cell viability was analysed as described in Materials and Methods. CWE from pericarp of CWE signific

antly inhibited the proliferation of SKB R3 cells in a dose-dependent manner (Fig. 1). Similar result was observed w hen quercetin was served as a positive control[7,14] and[15]. CWE at 5.0-50 ㎍/ml decreased the proliferation of SKBR3 c ells by 20-90% and with an ED_{50} of 9. 3±2.2 ㎍/ml.

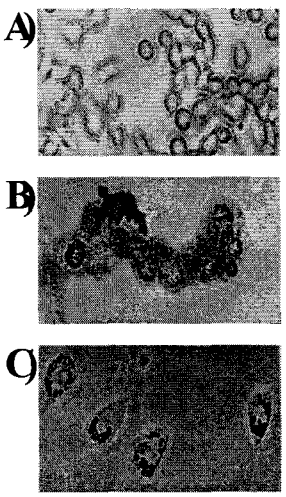
Fig. 1. Effect of CWE from CWE on the proliferation of SKBR3 cells. The percentage of cell viability was m easured by MTT assay. Data repr esent the means±S.D. (*n*=4).



2. Effect of CWE on the morphologi cal changes of SKBR3 human breast cancer cell line

After incubation with 40 ㎍/ml of CWE, morphological alterations in SKB R3 cells were illustrated (Fig 2B) comp aring with control cells (Fig. 2A). Untr eated or control cells were cuboid and polygonal in normal shape. Exposure of SKBR3 cells to CWE for 48 h led t o retraction, rounding and some sensit ive cells were detached from the surfa ce. Membrane blebbing (Fig. 2C) and apoptotic body (Fig. 2B) were observe d by phase contrast inverted microsco pe.

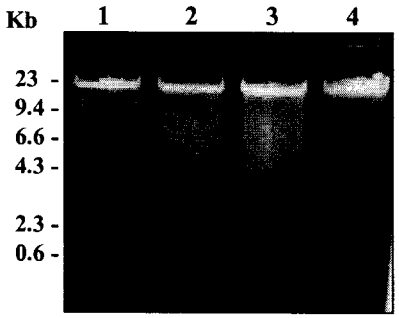
Fig. 2. Morphological alterations of SKB R3 cells following expose to 40 ㎍/ml of CWE for 48 h. (A) Control SKBR3 cells wer e observed by phase contrast inverted micro scope. (B) CWE-treated SKBR3 cells were o bserved by phase contrast inverted microsco pe. (C) Typical cells showin membrane blebbi ng. They showed a serial course of normal cells, membrane blebbing, and apoptotic bod y.



3. Appearance of DNA ladders in C WE-treated cells

The DNA fragment of SKBR3 cell s (3 x 10^6 cells) was detected on a 2. 0% agarose gel electrophoresis after ex posing with 0, 5, and 10 ㎍/ml of C WE for 48 h. At exposure to 10 ㎍/m l of CWE, fragmented DNA was clearl y observed in SKBR3 cells (Fig. 3) wh ereas control cells did not provide lad ders. Thereby, it is possible that CWE from EA causes apoptosis of SKBR3 c ells.

Fig. 3. Effect of CWE on DNA fragmentation of SKBR3 cells and ladders were detected by 2.0% agarose gel electrophoresis. Lanes 1 and 2, untreated cells; 3, treated cells with 5 μg/ml CME; 4, treated cells with 10 μg/ml CME.

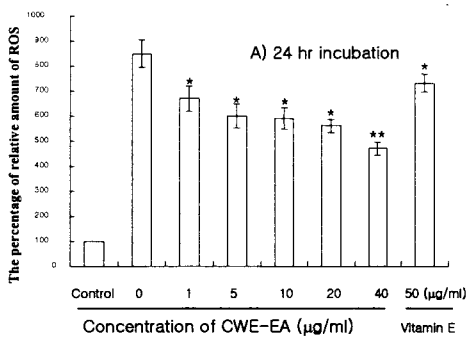


4. Effect of CWE on the ROS production of SKBR3 human breast cancer cell line

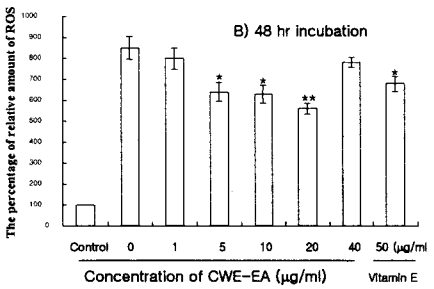
To investigate possible correlation between time and concentration of CWE on ROS production, SKBR3 cells were incubated with CWE at concentrations ranging from 0 to 40 μg/ml for 24, 48 and 72 h using Vitamin E as a positive control. Intracellular ROS was measured in terms of fluorescence by DCFH-DA. CWE from EA could significantly suppressed the intracellular ROS production of SKBR3 cells in a dose-dependent manner (Fig. 4). Notably, at 20 μg/ml of CWE 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.

Fig. 4. Effect of CWE from EA on ROS production of SKBR3 cells by using DCFH-DA as fluorescence probe. Data represent the means±S.D. (n=3).

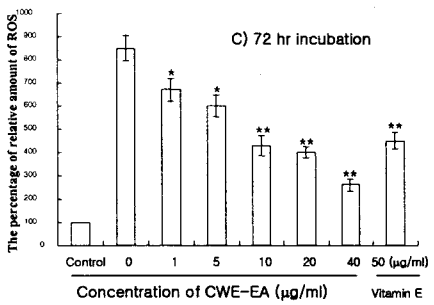
A) Incubation time 24 h



B) Incubation time 48 h



C) Incubation time 72 h



Ⅳ. Discussion

Although EA has long been served as a traditional medicine, 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, antihelminth 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 CWE from EA on human breast cancer cell line. We found that CWE 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 ㎍/ml of CWE was sufficient to effectively inhibit the cell proliferation. Thus, CWE displayed the strong antiproliferative activity on breast cancer cells with an ED_{50} of 6.5±0.3 ㎍/ml.

To investigate whether apoptosis is involved in the cell death caused by CWE on SKBR3 breast cancer cells, we assessed morphological changes and DNA ladder patterns on agarose gel electrophoresis (Fig.2) after treating cells with 10 ㎍/ml of CWE 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 10 ㎍/ml of CWE (Fig. 3). These hallmark features of morphological changes suggested that CWE from EA caused apoptosis of SKBR3 breast cancer cells.

In this study, we found that CWE 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 ㎍/ml of CWE at 48 h incubation time and mostly decreased by the same concentration at 72 h incubation time. It was possible that CWE at a concentration of 20 ㎍/ml 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 ㎍/ml of CWE and 72 h incubation time, ROS level was dramatically and decreased since only cell debris rema

ins in well. It appeared that CWE at hi gh (20 μg/ml) dose cause apoptosis wh ereas at low (2 μg/ml) and medium (5 0-10 μg/ml) doses show antioxidative e ffects on breast cancer cells. On the oth er hand, it has been proposed that the excessive production of ROS is not inv olved in cancer cell proliferation but it is purposed to apoptosis of cells.

V. Conclusion

In conclusion, the results demonstrate d that CWE from pericarp of herbs h ave a powerful antiproliferation by ind ucing apoptotic cell death and a potent antioxidation by inhibiting the intracell ular ROS production significantly. More over, we assume that determination of ROS level not only measure antioxidati on of the extract on cells but also mea sure its induction of apoptosis on cells. These probable properties of EA provid e scope of further detail evaluation. So me constituents from EA may serve as a novel powerful antitumour agent and free radical scavenger after further deta iled investigation. Moreover, other biolo gical activities and on different cell line s which are correlated to traditional tre atments of EA should be investigated a s well such as gastrointestinal tract dis order and chronic infections.

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