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 µg/ml)의 CWE를 첨가하면서 배양되었고, 세포의 생존 비율은 MTT 배양을 통해서 평가하였다. 또한 CWE의 증식억제 효과는 유방암 세포주의 세포사와 관련되어 있음을 형태학적인 변화와 유리 고داع률테스트 DNA 분석을 통해 확인하였다.

**Results**: CWE의 50%에서 효과를 나타내게 하는 약물농도인 ED50 (effective dose 50%)은 9.3±2.2µg/ml이며, 약물의 농도에 의존하여 세포의 증식을 억제하였다. 아울러, 다양한 농도와 배양시간에서 CWE가 ROS 생성을 억제하는 것을 알 수 있었다. 따라서 이러한 작용과 항암에 효과는 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
I. Introduction

Breast carcinoma (BC) is the commonest cancer among women and the second highest cause of cancer death\(^1\). 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, the cancer treatment by chemotherapeutic agents, surgery and radiation have not been fully effective against the high incidence or low survival rate of most the 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 eastern Asia 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 butanol 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\(^9\). 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 anti-inflammatory 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 sulfoxide (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). Protease 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 μm 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% CO₂ incubator.

4. Cell proliferation assay
Serial dilutions of CWE (20 μl) were added into each of 96-well plates, then, cells were plated at a density of 1×10⁴ 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% CO₂ incubator. MTT solution was then discarded and 50 μl 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. 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 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.

6. Detection of DNA fragmentation

DNA fragmentation was analysed by agarose gel electrophoresis as described by 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 μl lysis buffer (100 mM TrisHCl pH 8, 100 mM NaCl and 10 mM EDTA). Proteinase K (10 μl 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 phenolchloroformis oamyl 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. 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 H₂O₂ (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 μg/ml for 48 h and then the percentage of cell viability was analysed as described in Materials and Methods. CWE from pericarp of CWE signif
antily 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\(^7\)^,\(^{14}\) and\(^{15}\). CWE at 5.0-50 \(\mu g/ml\) decreased the proliferation of SKBR3 cells by 20-90% and with an \(ED_{50}\) of 9.3 \(\pm\) 2.2 \(\mu g/ml\).

Fig. 1. Effect of CWE on the proliferation of SKBR3 cells. The percentage of cell viability was measured by MTT assay. Data represent the means \(\pm\) S.D. (n=4).

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

After incubation with 40 \(\mu g/ml\) of CWE, 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 CWE for 48 h led to retraction, rounding and some sensitive cells were detached from the surface. Membrane blebbing (Fig. 2C) and apoptotic body (Fig. 2B) were observe
d by phase contrast inverted microscope.

Fig. 2. Morphological alterations of SKBR3 cells following exposure to 40 \(\mu g/ml\) of CWE for 48 h. (A) Control SKBR3 cells were observed by phase contrast inverted microscope. (B) CWE-treated SKBR3 cells were observed by phase contrast inverted microscope. (C) Typical cells showing membrane blebbing. They showed a serial course of normal cells, membrane blebbing, and apoptotic body.

3. Appearance of DNA ladders in CWE-treated cells

The DNA fragment of SKBR3 cells (3 \(\times\) 10\(^6\) cells) was detected on a 2.0% agarose gel electrophoresis after exposing with 0, 5, and 10 \(\mu g/ml\) of CWE for 48 h. At exposure to 10 \(\mu g/ml\) of CWE, fragmented DNA was clearly observed in SKBR3 cells (Fig. 3) whereas control cells did not provide ladders. Thereby, it is possible that CWE from EA causes apoptosis of SKBR3 cells.
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, antihelmintic 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 \( \mu \text{g/ml} \) 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 \( \mu \text{g/ml} \).

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 h 10 \( \mu \text{g/ml} \) 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 \( \mu \text{g/ml} \) 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 \( \mu \text{g/ml} \) 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 \( \mu \text{g/ml} \) 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. Thus, at 20 \( \mu \text{g/ml} \) 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 high (20 μg/ml) dose cause apoptosis whereas at low (2 μg/ml) and medium (5 0-10 μg/ml) 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.

V. Conclusion

In conclusion, the results demonstrated that CWE from pericarp of herbs 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 antioxidant of the 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.

References

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