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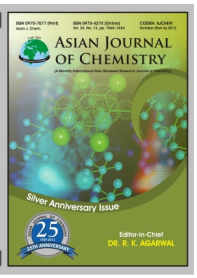

Anticancer Activities of Extract from *Amaranthus viridis* L.

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Amaranthus viridis L. is a traditional medicine in Asia. In this study, the ethyl ether fraction of *A. viridis* L. (EA) was tested *in vitro* for anticancer activity against human colon cancer HT-29 cells. HT-29 cells were treated with different concentrations of EA. Cell proliferation was detected by MTT assay. The gene expression of Bax, Bcl-2, caspase-3, c-myc, p53 was determined by RT-PCR. The generation of reactive oxygen species (ROS) and cell cycle assay was monitored by flow cytometry. Results indicated the EA significantly inhibited human colon cancer HT-29 cell growth in a dose-dependent manner by inducing G0/G1 phase arrest and apoptosis. The inhibition was associated with intracellular reactive oxygen species generation. Cell death induced by EA displayed features characteristic of apoptosis and was associated with generation of reactive oxygen species and enhanced expression of caspase-3 and the Bax/Bcl-2 ratio in HT-29 cells treated with EA. These findings suggest that *A. viridis* L. have anticancer properties and may be valuable for application in pharmaceutical industry.

Key Words: *Amaranths viridis* L, Anticancer activity, Cell cycle, Reactive oxygen species, HT-29 cell.

INTRODUCTION

Cancer is one of the major causes of human suffering. There are more than 12.6 million new cancer cases occurred in 2008 which will be increased to 21.3 million in 2030[1]. Currently, surgery, chemotherapy and radiation therapy have been considered to be the main tools for cancer treatment[2,3]. Herbal products and natural agents can boost the actions and reduce the toxicity of conventional chemotherapeutic drugs. Apoptosis is a key regulator of tissue homeostasis and imbalance between cell death and proliferation may result in tumor formation[4]. Therefore, the identification of activators of apoptosis may help in providing more effective strategies for cancer therapy.

It is well established that reactive oxygen species (ROS) are mediators of intracellular signaling cascades. Oxidative stress can down-regulate the level of the anti-apoptotic protein[5], which is an inhibitor of caspase. Excessive generation of ROS can induce redox-signaling pathways, including oxidative stress, apoptosis, loss of cell function and cell cycle arrest[6]. The objective of application of anticancer agents is to induce apoptosis-related signaling, disrupt cell proliferation in cancer cells. Although there have been several ethnopharmacological studies on the anti-cytotoxic effects of the plant[7], the underlying issue is about its mode of action which remains largely unexplained and requires further investigation.

Amaranthus plants (Amaranthaceae) are spread throughout the world, grow under a wide range of climatic conditions and they are known to produce grains and leafy edible vegetables[8]. The leaves of amaranth constitute an inexpensive and rich source of protein, carotenoids, vitamin C and dietary fibre[9], minerals like calcium, iron, zinc, phosphorus[10,11] and magnesium[9]. The limited studies have been reported on the biological activity of Amaranthus extracts.

Amaranthus viridis L. (Amaranthaceae) has been used in Chinese traditional system[12]. Traditionally, the plant parts of *A. viridis* L. were used in the treatment of dysentery, enteritis, hemorrhoids and kidney diseases. Other traditional application of *A. viridis* L. are anti-inflammatory, in venereal diseases, vermifuge, diuretic, antirheumatic, antiulcer, analgesic, antiemetic, laxative, improving appetite, antileprotic, respiratory problems, eye treatment and for asthma, antidiabetic, antihyperlipedemic and antioxidant activities[13-22]. A novel extract including an antifungal and antiproliferative lectin, a β-carotene and a ribosome inactivating protein was isolated from *A. viridis* that possess antiviral activity[23-26]. The main objective

of this study was to evaluate the ethyl ether fraction of *A. viridis* L. (EA) on its cytotoxic properties and the possible mechanism of cell death elicited by EA on colonic cancer cell.

EXPERIMENTAL

A. viridis L. were collected from Yangzhou and identified by Professor H.Y. Huai (College of bioscience and biotechnology, Yangzhou University, China). 2,7-Dichlorofluorescin diacetate (DCFH-DA), propidium iodide (PI) and 1-(4,5-dimethyl-thiazol-2-yl)-3,5-diphenylformazan (MTT), obtained from Sigma (St, Louis, MO, USA). Human colon cancer (HT-29) and human embryonic kidney (HEK293) cell lines were obtained from the Korean Cell Line Bank (Seoul Korea). RPMI medium 1640, Dulbecco's modified Eagle's medium (DMEM) and feral bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other reagents were used analytical grade.

Preparation of extracts: 250 g dried sample of the powder was subjected to a methanol extraction process for 3 days, produce a crude extract of 16.6 g. The crude extract was extracted sequentially with petroleum ether, ethyl ether, ethyl acetate and *n*-butanol. The final yields of petroleum ether fraction, ethyl ether fraction, ethyl acetate fraction, *n*-butanol fraction and water fraction were 28.9, 3.1, 9.0, 6.0 and 53.6 %, respectively.

Cancer cell culture and MTT assay for measurement of cell proliferation: HEK293 cells were maintained in DMEM; HT-29 cells were maintained in RPMI 1640 medium (10 % FBS and 100 U/mL penicillin and 100 µg/mL streptomycin).

The cytotoxic effects of sample were determined by the MTT assay. Viable cell numbers were estimated 24 h after the cells were exposed to ethyl ether fraction of *A. viridis* L. (EA) using the MTT assay, as described by Hu *et al.*[27].

Light microscopic examination: The HT-29 cells were observed after 48 h of treatment with 0, 50, 100 µg/mL EA and 10 µg/mL paclitaxel. Photographs of HT-29 cells were observed by a light microscope (Olympus, Tokyo, Japan).

Measurement of cellular reactive oxygen species (ROS) level: Dichlorofluorescin diacetate (DCFH-DA) was used to detect cellular ROS levels in HT-29 cells after 24 h of treatment with 25, 50, 100, 200 µg/mL EA. The cells were labeled with 20 µM of DCFH-DA for 0.5 h and ROS was measured by a FACScan flow cytometer.

RT-PCR analysis of EA-treated cells: The HT-29 cells were treated with various concentrations of EA for 24 h. Total RNA was isolated using a Trizol reagent (Life Technologyies). For cDNA synthesis, 1 µg of RNA was reverse transcribed with moloney murine leukemia virus reverse transcriptase (Invitrgen). The primers used were as follows: β-actin, 5'-TCACCCTGAAGTACCCCATC-3' and 5'-CCATCTCT TGCTGCAAGTCC-3', capspase-3, 5'-TCACAGCAAAAGG AGCAGTTT-3' and, 5'-CGTCAAAGGAAAAGGACTCAA-3' , c-myc, 5'-GAACAAGAAGATGAGGAAGA-3' and 5'-AGTTTGTGTTTCAACTGTTC-3' , p53, 5'-TGTGGAG TATTTGGATGACA-3' and 5'-GAACATGAGTTTTT TATGGC-3' , Bax, 5'-TCCACCAAGAAGCTGAGCGA-3'and 5'-GTCCAGCCCATGATGGTTCT-3', Bcl-2, 5'-TGTGG CCTTCTTTGAGTTCG-3' and 5'- TCACTTGTGGCTCAG ATAGG-3'. The amplified fragments were separated by electrophoresis using 1.0 % agarose gel and stained with ethidium bromide.

Cell cycle analysis: Flow cytometric analysis of cell cycle progression after 24 h treatment with differing concentrations of EA. The Cells were stained with 10 µg/mL of PI for 1 h and cell cycle was measured by a FACScan flow cytometer.

Statistical analyses: Statistical analyses were processed using Statistical Analysis System software (SAS Institute, Cary, NC, USA). The data were expressed as the mean ± standard derivation (SD). Statistical significance differences among the means of groups were tested at the $p < 0.05$ using Duncan's multiple range test.

RESULTS AND DISCUSSION

Cytotoxicity and antiproliferation assay: Cancer cell line HT-29 cells were used to examine the anti-proliferation activity and cytotoxicity of the ethyl ether fraction of *A. viridis* L. (EA) extract using the MTT based assay. In HT-29 cells, extract of EA inhibited cell growth in a dose-dependent manner and showing inhibition of growth rate on 24 h basis by 16.4, 24.2, 33.0, 48.3 and 65.3 % at concentrations of 25, 50, 100, 200 and 400 µg/mL, respectively (Fig. 1). Comparatively, pretreatment with 20 µg/mL FU-5 revealed that the lower concentration (200 µg/mL) of EA had less impact than higher concentrations on HT-29 cells at 24 h. The effect of EA on the viability of normal cell line HEK 293 cells were also investigated. At these concentrations, EA had minimal effects on the proliferation of normal cell lines on basis of MTT assays. Based on the previous research, using MTT assays to compare the effect of differing fractions of *A. viridis* L. on HT-29 cancer cells, cancer cells were most sensitive to the EA compared to other fractions. In addition, with comparison of inhibited growth on a series of cancer lines, EA has shown to be an obvious anti-proliferative effect on HT-29 cancer cells.

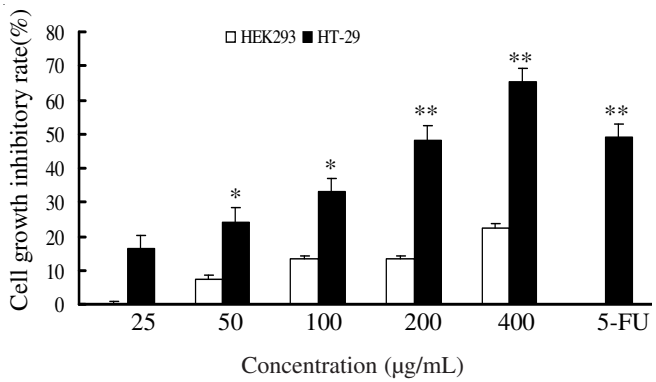

Fig. 1. Effects of EA on cell proliferation of HT-29 cancer cells at 24 h. Cytotoxicity was measured by MTT assay. These cells were treated with EA for 24 h at 0-400 µg/mL followed by cell death measurement. 5-FU (20 µg/mL) was used as a positive control

To examine how EA affects cellular morphology during cell death, HT-29 cells were treated with 0, 50 and 100 µg/mL of EA or paclitaxel 10 µg/mL (positive control) for 48 h and then analyzed under a light microscope (Fig. 2). The untreated cells displayed normal, healthy shape with a distinct cytoskeleton.

After incubation with EA, the cellular morphology of HT-29 cells was severely distorted and some cells turned round in shape. When the dose was increased to 50 μg/mL, the apoptosis activity from the anticancer drug at different levels was comparable to the positive control, paclitaxel.

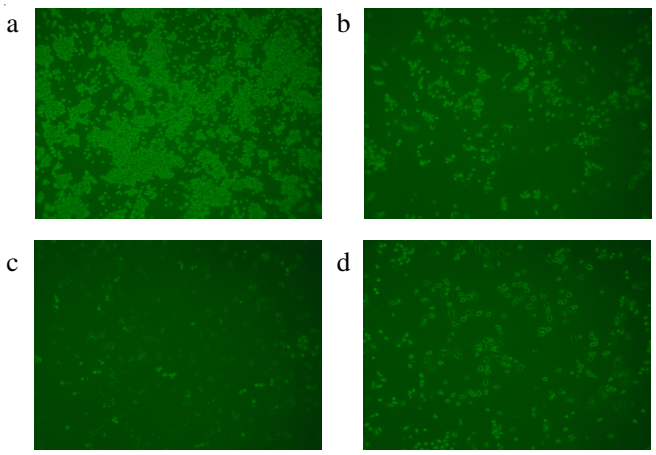

Fig. 2. EA-induced apoptosis in HT-29 cancer cells. HT-29 cells were incubated with EA or paclitaxel for 48 h. (a), control (DMSO); (b), 50 μg/mL; (c), 100 μg/mL; (d), paclitaxel (10 μg/mL)

Detection of apoptotic-related gene expression in HT-29 cells: Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Excessive apoptosis causes atrophy, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer. The activation of caspase-3 has been regarded as a primary mechanism of apoptosis[28,29]. Reverse transcription PCR was used to examine in Bcl-2, Bax, p53, caspase-3, c-myc and β-actin expression. As shown in Fig. 3, EA up-regulated the expression of Bax and down-regulated the expression of Bcl-2 in HT-29 cells. In order to test the other apoptosis-related genes, treating HT-29 cells with 0, 50, 100 and 200 μg/mL of EA for 24 h, the expression of caspase-3 was up-regulated but c-myc and p53 were not observed. Our results indicated that treatment with EA induced apoptosis in HT-29 cells accompanied by dose-dependent up-regulation of Bax/Bcl-2 ratio and caspase-3. Increased Bax/Bcl-2 ratio observed in our study was in agreement with earlier findings demonstrating[30].

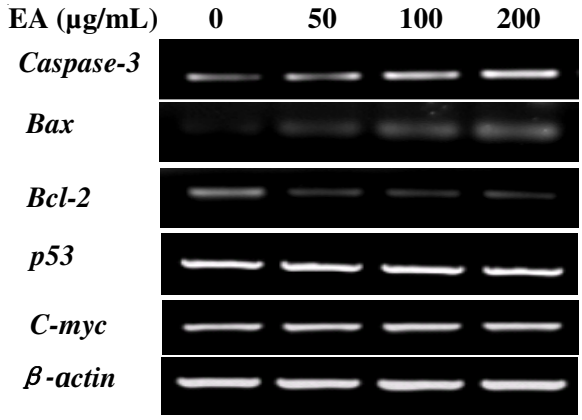

Fig. 3. RT-PCR analysis of caspase-3, Bcl-2, Bax, p53 and c-myc mRNA expression on HT-29 cells. HT-29 cells were treated with EA (0, 50, 100 and 200 μg/mL) for 24 h

Ethyl ether fraction of *A. viridis* L. (EA) enhances ROS accumulation in HT-29 cells: ROS has been associated with cancer. Normal cells have low basal levels of ROS[31] with a diminished reliance on the ROS stress-response pathway, whereas cancer cells have high levels of ROS and might therefore be expected to have a strong reliance on the ROS stress-response pathway[32]. Some small molecules have been reported to promote ROS in cancer cells[33]. We determined the effect of EA on cellular ROS levels in HT-29 cells through flow cytometry using the fluorescent probe DCF-DA. Treatment with 25, 50, 100, 200 μg/mL EA caused a marked increase ROS levels in a dose-dependent manne in this cancer cells (Fig. 4). These results indicate that EA induce apoptosis in HT-29 cells partly by inducing the formation of reactive oxygen species.

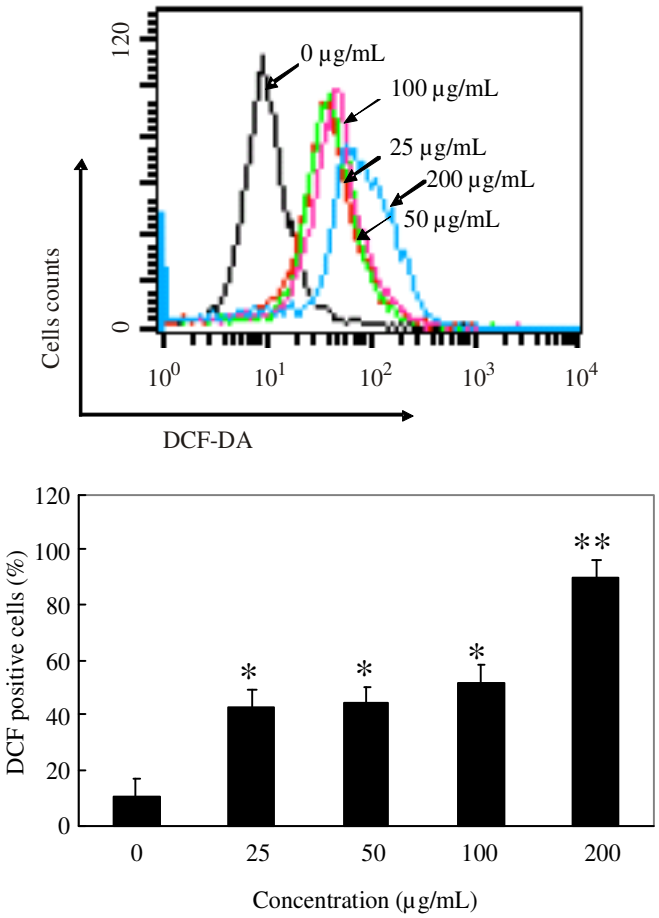

Fig. 4. EA-induced ROS elevation in cancer cells were measured by flow cytometry using DCF-DA. HT-29 cells were treated with EA (25, 50, 100 and 200 μg/mL) for 24 h

Cell cycle analysis: The cell cycle consists of four distinct phases: G1 phase, S phase (synthesis), G2 phase (collectively known as interphase) and M phase (mitosis). Although the duration of cell cycle in tumor cells is equal to or longer than that of normal cell cycle, the proportion of cells that are in active cell division (*versus* quiescent cells in G_0 phase) in tumors is much higher than that in normal tissue[34]. In order to assess the effect of EA on cell cycle progression, we performed FACS analysis of cells after PI staining of cellular DNA. The cell cycle profiles of HT-29 changed significantly after treatment

for 24 h. Treatment with 0, 50, 100 and 200 µg/mL EA for 24 h significantly decreased G0/G1 populations from 58.05-43.94, 34.98 and 29.40 %, respectively (Fig. 5). On the other hand, EA arrested the cell cycle of HT-29 cells in a dose-dependent manner and the percentage of cells in sub-G1 increased from 4.45-11.53, 14.52 and 33.70 %, after treatment with 0, 50, 100 and 200 µg/mL EA for 24 h, respectively (Fig. 5).

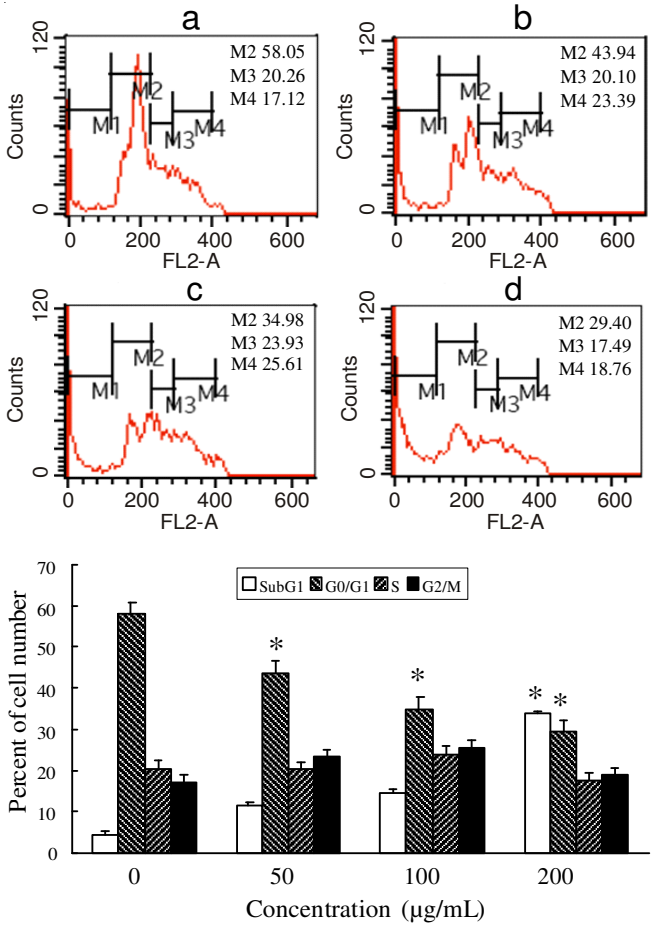

Fig. 5. Flow cytometric analysis of cell cycle progression after EA treatment. Cell phase distribution of HT-29 after a 24 h treatment with 0 (a), 50 µg/mL (b), 100 µg/mL (c) and 200 µg/mL (d) of EA. Areas M1, M2, M3 and M4 represent the Sub-G1, G0/G1, S and G2/M phases, respectively

Conclusion

This study demonstrated that ethyl ether fraction of *A. viridis* L. (EA) has an anti-proliferative effect by reducing cell viability, ROS generation, regulation of the caspase-3 gene, activation of Bax and Bcl-2 and cell cycle arrest. EA increases the level of ROS and apoptotic cell death in HT-29 cancer cells. Our results demonstrate that the cell apoptosis induced by EA induces G0/G1 cell cycle arrest and was associated with generation of ROS and enhanced expression of caspase-3 and the Bax/Bcl-2 ratio.

ACKNOWLEDGEMENTS

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