**Ethanol Extract of *Dunaliella salina* Induces Cell Cycle Arrest and Apoptosis in A549 Human Non-small Cell Lung Cancer Cells**

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**Abstract.** The ethanol extract of *Dunaliella salina* (EDS) on proliferation and apoptosis in the A549 human lung cancer cell line and their associated protein expressions were investigated. After 24 and 48 h treatment, MTT assay showed that 25 μg/ml of EDS significantly reduced A549 cell proliferation by 25.2% (p<0.05) and 48.3% (p<0.01), respectively. To explore its molecular mechanisms in regulating cell proliferation, we first showed that EDS markedly reduced A549 proliferation via inhibition of BrdU incorporation at 25 μg/ml by 65.8% (p<0.001). By cytometric analysis, EDS was found to induce apoptosis and cell cycle arrest in the G0/G1 phase. In the DNA gel electrophoresis assay, EDS (25, 50 and 100 μg/ml) induced significant apoptosis at 48 h. Annexin V/Propodium iodide double staining demonstrated that administration of EDS (25 μg/ml) in 12, 24 and 48 h induces apoptosis of 27.7%, 30.7%, and 38.7%. Western blotting assay demonstrated that EDS significantly increased the expression of cyclin-dependent kinase (CDK) inhibitors p53 and p21 and death-receptor proteins Fas and FasL. Bax expression was also elevated by treatment with EDS. Our data suggested that EDS could influence the antiproliferative effects and induce cell cycle G0/G1 arrest and apoptosis of A549 lung cancer cells.

*Lung cancer is the leading cause of cancer mortality in Taiwan (1). Non-small cell lung carcinoma (NSCLC) comprises about 75-85% of all lung cancer cases. Chemoprevention is a significant alternative in reducing lung cancer mortality (2). Consumption of β-carotene or other carotenoid-rich vegetables and fruits has been associated with a lower incidence of diverse types of cancer (3). Supplement action with β-carotene has been found to enhance the cytotoxic activity of NK cells (4). β-Carotene was shown to significantly inhibit the colony-forming efficiency of lung cancer cells and reduce their DNA and RNA synthesis (5). Studies suggest that there is a synergistic effect of all-trans-retinoic acid (ATRA) and arsenic trioxide (As2O3) on growth inhibition and apoptosis in human hepatoma, breast cancer and lung cancer cells (6). A combined supplementation of oxidants (α-carotene, β-carotene and ascorbic acid) was shown to increase the retinoic acid level and inhibit mitogen-activated protein kinase in a ferret lung cancer model (7). Low performance status patients with advanced NSCLC may benefit from a combination of somatostatin, retinoids, melatonin, vitamin D, bromocriptine and cyclophosphamide, in terms of survival and quality of life, with very low side-effects (8).* 

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spontaneous mammary tumours of mice by increasing the homeostatic potential of the host animals, as well as by the well-known antioxidant function of β-carotene (12). *Dunaliella bardawil* promotes the growth of normal mammary gland cells, but inhibits neoplastic transformation (13). It has been implied that the antiperoxidative effect of 9-cis-β-carotene compared with that of the all-trans-isomer is important for its ability to prevent malignant and cardiovascular diseases (14). Recently, Raja *et al.* found that DS from Volvocales, Chlorophyta has a protective effect against experimentally induced fibrosarcoma on Wistar rats (15). Thus, the aim of this study was to investigate the effects of DS on proliferation and apoptosis in the A549 human lung cancer cell line and on cell cycle- and apoptosis-associated protein expressions.

**Materials and Methods**

**Chemicals and reagents.** F-12 was obtained from Gibco BRL (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA, USA). Anti-p53, p21, Fas, FasL and Bax were purchased from Santa Cruz (Santa Cruz, CA, USA).

**DS material and extract preparation.** Spray-dried algae material from DS cultured in outdoor cultivation pools were prepared by GONG BIH Enterprise Co., Ltd. (Doo-Liu City, Taiwan). The composition of the DS produced was analyzed by high performance liquid chromatography (HPLC) and is shown in Table I. Extraction of antioxidant compounds from *Dunaliella salina* microalgae (20 g) was performed using a vacuum evaporator (EYELA N-N SERIALS, Japan). Three different solvents (namely hexane, ethanol and water) were used to obtain extracts with different compositions. Extractions were performed at two different extraction temperatures (40 and 100°C) and extraction times (5, 17.5 and 30 min). The supernatants were collected and concentrated to a volume of 5 ml, and were then stored at −20°C in a freezer (16).

**Compositional analysis of β-carotene and EDS by HPLC.** HPLC was conducted to analyze both the standard (all-trans-β-carotene) and EDS. The purity of EDS was more than 95% based on reverse-phase HPLC analysis [instrument: Jasco system; column: Vydac 201 TP54 C18 reversed-phase column with particle size 5 μm (25 cm × 4.6 mm i.d.); mobile phase: methanol:acetonitrile, 9:1 by volume at a flow rate of 1.0 ml/min. It comprised 6% of β-carotene, 0.12% of α-carotene, 0.2% of xanthophyl, 0.3% of zeaxanthin, and scarce amount of lycopene and chlorophyl].

**Cell culture.** A549 cells obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) were grown in F-12 medium (Gibco BRL) containing 10% FBS (Gibco BRL), 10 U/ml of penicillin, 10 μg/ml of streptomycin (Gibco BRL), 2 mM glutamine (Merck, Darmstadt, Germany) and 0.25 μg/ml of amphotericin B (Gibco BRL) at 37°C in a humidified atmosphere comprising 95% air and 5% CO₂. In all of the experiments, the medium was supplemented with 10% (v:v) FBS.

**Total antioxidant status.** Total antioxidant status of the EDS was measured using the 2,2'-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) assay (17). ABTS was dissolved in deionized water to 7 mM concentration, and potassium persulphate was added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12-16 h) in the dark before use. The resulting intensely-coloured ABTS⁺ radical cation was diluted with 0.01 M phosphate-buffered saline (PBS), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. The test compound was diluted 100X with the ABTS solution to a total volume of 1 ml. Absorbance was measured spectrophotometrically at time intervals of 1 min after the addition of each extract. The assay was performed at least in triplicate. Controls containing 990 μl of PBS, to replace ABTS, were used to measure the absorbance of the extract alone. The assay relies on the antioxidant capability of the samples to inhibit the oxidation of ABTS to ABTS⁺ radical cation. The total antioxidant activities were expressed as mM trolox equivalent antioxidant capacity (TEAC).

**Cytotoxicity assay.** MTT assay was performed in the A549 cell lines to measure the cytotoxicity of EDS. In living cells, mitochondrial metabolism of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt into formazan took place, and the amount of formazan produced correlated with the number of viable cells present. A549 was seeded in 96-well plates at 1×10⁶ cells/well in F-12 medium supplemented with 1% FBS. After 24 h, cells were washed with PBS and then exposed to either dimethylsulfoxide (DMSO) alone or to serial dilutions (0, 3.125, 6.25, 12.5 and 25 μg/ml) of EDS in 1% FBS media. After 6, 12, 24 and 48 h, the number of viable cells was determined (18). Briefly, MTT (3 mg/ml in PBS) was added to each well (25 μl/200 ml medium), and the plate was incubated at 37°C for 2 h. Cells were then spun in a centrifuge at 3000g for 5 min and the medium was carefully aspirated. A 50 μl aliquot of DMSO was added and the absorbance at 570 nm was measured for each well on an ELISA reader (Anthos 2001; Anthos Labtech, Austria).

**BrdU incorporation for DNA synthesis.** A549 cells were subcultured at a density of 1×10⁴ cells/well in 96-well plates. Cells were grown in medium containing 1% FBS with different doses of EDS (0, 3.125, 6.25, 12.5 and 25 μg/ml), which was added to the cells for an additional 20-h incubation. Cells were then labeled with 5'-bromo-2'-deoxyuridine (BrdU) for 4 h at 37°C followed by removing the labeling medium to terminate the reaction. Fix-Denat solution (Boehringer Mannheim, Germany) was added to the cells for 30
min at room temperature to fix them. After removing Fix-Denat from the plates, BrdU incorporation into DNA was measured by utilizing a colorimetric reaction with peroxidase-linked anti-BrdU antibody using a cell proliferation ELISA kit according to the manufacturer's instructions (Boehringer Mannheim). The optical density, representing the cell numbers, was measured in an ELISA reader at 370 nm.

**Flow cytometric analysis.** A549 cells were seeded at a density of 2×10^5 cells/well onto a 12-well plate (Falon, Franklin Lakes, NJ, USA) 24 h before treatment with 25 μg/ml of EDS for different time periods (12, 24 and 48 h). The DNA content was assessed using flow cytometry following propidium iodide (PI) staining. Cells were harvested with trypsin-EDTA, washed twice with ice-cold PBS before being fixed in 70% ethanol. For DNA content analysis, cells were centrifuged and resuspended in 0.3 ml of DNA staining solution [100 μg/ml PI, 0.2% NP-40, and 1 mg/ml RNase A (DNase-free) in PBS lacking Ca** and Mg**; at a 1:1:1 ratio by volume]. The cells were suspended and stored on ice in a dark room for a minimum of 30 min and analyzed within 2 h. Cells were analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA). Ten thousand events were acquired and DNA content was determined using the DNA analysis software ModFitLT, version 3.0 (Verity Software, Topsham, ME, USA).

**DNA gel electrophoresis assay.** A549 cells were plated in 10-cm dishes at a density of 5×10^6 cells and grown for 24 h. Different concentrations of EDS (25, 50 and 100 μg/ml) were then added as described above while only adding DMSO for the control regimen, and the cells grown at 37°C, in a humidified atmosphere 5% CO₂ and 95% air for 48 h for DNA fragmentation electrophoresis assay as described below. For this, 5×10^6 cells were harvested in PBS and lysed overnight in a digestion buffer containing 0.5% sarkosyl, 0.5 mg/ml proteinase K, 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA at 55°C. Subsequently cells were treated with 0.5 μg/ml RNase A for 2 h at 37°C. The genomic DNA was extracted by phenol-chloroform-isoamyl alcohol extraction (24:25:1) and analyzed by gel electrophoresis at 50 Volts for 90 min using 2% agarose. Approximately 20 μg of genomic DNA was loaded in each well, visualized under ultraviolet (UV) light and photographed.

**Annexin V/PI double staining.** Apoptosis was detected using Annexin V plus PI for apoptosis detection according to the manufacturer’s instructions (Biosource, Camarillo, CA, USA). In brief, exponentially growing A549 cells were trypsinized and harvested in suspension. Cells were then seeded into 25 cm² flasks at a density of 2×10^5 cells, 5 mL per flask. Twenty-four hours later, 25 μg/ml EDS was added. After being incubated for 0, 12, 24 and 48 h, cells were harvested by trypsinization and rinsed with cold PBS twice. After centrifugation (4°C, 1000×g) for 10 min, cells were suspended by 200 mL binding buffer and then treated with 10 mL Annexin V-FITC and 5 mL PI for 15 min at room temperature (19). Flow cytometric analysis of cells was performed with a FACS Calibur using CellQuest software as per manufacturer instructions (Becton Dickinson). Cytometric analysis was repeated three times.

**Western blotting.** A549 cells cultured in 6-well plates were incubated with 25 μg/ml of EDS in F-12 containing 1% FBS for 48 h. The cells were collected, then lysed in a RIPA solution (2% SDS, 50 mM DTT, 62.5 mM Tris-HCl, pH 6.8) followed by incubation at 95°C for 5 min. Total proteins were separated using SDS-PAGE before being transferred to PVDF membranes, blocked with 5% non-fat dry milk in PBS-Tween and probed with the desired antibody (anti-p53, anti-p21, anti-Bax, and anti-Fas; Santa Cruz, Santa Cruz, CA, USA) overnight at 4°C. The blots were then incubated with horseradish peroxidase-linked secondary antibody for 1 h followed by development with the electrochemiluminescence (ECL) reagent and exposure to Hyperfilm (Amersham, Arlington Height, IL, USA).
data were analyzed by Gel-Logic 200 Imaging Systems, Molecular Imaging Software.

Data analysis. Values are presented as mean±SD relative to those of the control. Statistically significant differences from the control group were identified by Student’s t-test for the data. P<0.05 was considered significant for all tests.

Results

HPLC was conducted to analyze both the standard (all-trans-β-carotene) and EDS. The HPLC fingerprint of EDS is shown in Figure 1. Both the standard and EDS showed a similar peak at the retention time around 24 min. The chromatogram indicated that EDS did contain the active ingredient all-trans-β-carotene. However, we found that the 9-cis-β-carotene was also evidently shown in the fingerprint (Table I).

The antioxidant capacity of crude extract, hexane, ethanol and water soluble fractions of DS were evaluated according to the ABTS decoloration method. The results are shown in Figure 2. The ethanol fraction displayed the highest total antioxidant activity (21.58±2.15 mM TEAC) (Figure 2). Total antioxidant capacity of hexane and water fractions of DS were evaluated as 13.47±1.82 mM and 8.62±1.29 mM, respectively. The ethanol fraction had the highest total antioxidant capacity.

MTT assay was performed in the A549 cell line to measure the cytotoxicity of EDS. As shown in Figure 3, EDS induced cytotoxicity in A549 in dose- and time-dependent manners. The inhibitory effect of 25 μg/ml of EDS on cell proliferation was significant at 24 h (p<0.05; 25.2%) and 48 h (p<0.01; 48.3%).

To further support the inhibitory effect of EDS on A549 proliferation, DNA synthesis affected by EDS was determined. A549 cells were treated with 0, 3.125, 6.25, 12.5
and 25 μg/ml of EDS for 48 h to evaluate its effect on DNA synthesis. DNA synthesis was evaluated using BrdU incorporation into the deoxynucleotide backbone. Figure 4 shows that 25 μg/ml DS significantly decreased \( p < 0.001 \) BrdU incorporation by 65.8% as compared to the control.

Effects of EDS on the cell cycle were measured using flow cytometry in A549 cancer cells treated with 25 μg/ml of EDS for different time periods (12, 24 and 48 h). Figure 5 shows that 25 μg/ml EDS caused a significant accumulation of cells in G0/G1 \( p < 0.05 \); 14.9%, 19.9% ) phase at 24 and 48 h, respectively. Moreover, 25 μg/ml EDS caused a significant increase in the population of sub-G1 cells, making up 8.89% and 14.62% of all cells at 24 and 48 h, respectively.
DNA laddering provides evidence for apoptosis. As seen in Figure 6, our data showed that A549 cells can be induced for DNA fragmentation at 25, 50 and 100 μg/ml of EDS at 48 h. This finding suggests that EDS induced concentration-dependent apoptosis by DNA fragmentation.

Figure 7 (B, C, and D) demonstrated that EDS (25 μg/ml) was able to induce early and late apoptosis from 12, 24 and 48 h. The Annexin V/PI method demonstrated that EDS (25 μg/ml) induced cell apoptosis of 27.7% (p<0.01), 30.7% (p<0.01) and 38.7% (p<0.001), respectively (Figure 7E).

To investigate how the various cell cycle regulation protein expressions affected the G0/G1 arrest of A549 cells, 25 μg/ml of EDS were administrated to A549 for various times (6, 12, 24 and 48 h) for Western blotting. Our results showed that the expression of p53 was markedly higher at 12, 24 and 48 h on EDS treatment compared to the control (Figure 8A).

To determine the role of p21 in the effect of EDS and whether the expression of p21 is p53-dependent, we detected the expression of p21 in A549 cell using the Western blotting
assay. In Figure 8B, our results demonstrated that EDS-treated A549 cells exhibited an increase in p21 protein at 12, 24 and 48 h. We suggested that EDS-mediated apoptosis and cell cycle arrest at G0/G1 phase might be through p21 activation in a p53-dependent event in A549 cells.

Since Fas/FasL play a trigger role in mediating apoptosis, we investigated whether they were involved in the cell death response induced by EDS. Our results showed that in EDS-treated A549 cells, Fas and FasL proteins were expressed at significantly higher levels after 12, 24 and 48 h (Figure 9A, B). The expression of the pro-apoptotic factor Bax significantly increased after 12, 24 and 48 h (Figure 9C) incubation with 25 μg/ml EDS treatment in the A549 cancer cell lines.

Discussion

DS is a well known source of β-carotene. It has been shown that DS is safe and can be a potential source of food supplement (20). In recent research, Raja et al. found that DS from Volvocales, Chlorophyta has a protective effect against experimentally induced fibrosarcoma on Wistar rats (15). In this study, we demonstrated that EDS not only inhibited A549 cell proliferation (Figure 3) but also induced apoptosis and cell cycle G0/G1 arrest (Figure 5). We first demonstrated that 25 μg/ml EDS could significantly attenuate the proliferation and decrease of DNA synthesis in A549 cells, which is consistent with a previous study (5).

Expression of ras oncogene was found to be inhibited by β-carotene because the product of ras p21 protein of cancer cells was decreased after treatment with β-carotene (5). DS induced G0/G1 arrest in the investigated A549 cancer cells (Figure 5). Our results demonstrated that induction of p21 and p53 protein expression (Figure 8) may be responsible for EDS-induced G0/G1 arrest. p21 was significantly induced by EDS in A549 cells (Figure 8B). The induction of p21 suggests that p21\textsuperscript{WAF1/CIP1} may be one of the EDS response genes. p21 is a strong inhibitor of cdk complexes and is induced in response to DNA damage (21). Recent evidence indicates that p21\textsuperscript{WAF1/CIP1} arrests the cell cycle in G0/G1 by preventing the phosphorylation of critical cdk substrates required for cell cycle progression (21). p53 is known to be responsible for apoptosis triggered by some anticancer drugs (22). We found that the induction of p21 by EDS is mediated by a p53-dependent process (Figure 8A, 8B). Our flow-cytometric analysis revealed that EDS arrested A549 cells in the G0/G1 phase (Figure 5E). This inhibition of cell cycle progression might be associated with an altered expression of cell cycle relevant regulator, including p21 and its upstream molecule, p53 (23).

Many compounds used for the treatment of malignant tumors are cytotoxic drugs can induce tumor cell death by apoptosis. A key regulator of apoptosis is the Fas
receptor/Fas ligand (Fas/FasL) system. Fas belongs to the family of tumor necrosis factor agents, and the FasL induces apoptosis through Fas (CD95) including caspase cascade activation, first caspase 8 followed by caspase 3 (24). Our results demonstrated that Fas and FasL were up-regulated in A549 cells after incubation with 25 μg/ml EDS (Figure 9A, 9B). This could be associated with p53, which peaked at 24 h incubation. Up-regulation of Fas receptor might be a major cause of apoptosis in chemotherapy (25). The action of p53 and its effect on the Fas/FasL system has demonstrated that p53 induces the expression of Fas (23).

The Bcl-2 family is composed of anti-apoptotic (Bcl-2 and Bcl-xL) and pro-apoptotic (Bax, Bas, and Bak) factors (25). Bcl-2 and its homologous proteins, such as Bax, modulate the apoptotic process. These molecules interact with each other to form homo- and heterodimers. Once Bcl-2 interact with Bax, Bcl-2/Bax heterodimer inhibits the apoptotic effect of Bax (27). Some Bcl-2 family members are located on the mitochondria membranes and could alter the permeability of the mitochondrial membrane, which triggers the release of apoptotic proteins, cytochrome c (26) and activates the post-mitochondrial caspase cascade leading to apoptotic cell death. The Bcl-2 family is the key regulator of apoptosis. Wild-type p53 is known as an upstream regulator of the Bax gene promoter that contains p53-binding sites and can be directly activated by wild-type p53 (23). The highest increase of Bax expression in A549 after 48 h incubation of EDS may be the result of the increase of p53 accumulation. Microarray expression analysis identifies Bax as a mediator of β-carotene effects on apoptosis (28). It has been reported that the apoptosis-inducing effect of p53 was in part due to its activation of Bax (29). In our study, Bax protein expression was increased by EDS in the A549 cancer cell lines (Figure 9C). Hence, p53 may have played a role in the modulation of EDS-induced apoptosis in A549 cancer cells.

DS contains α-carotene, β-carotene, lutein, cryptoxanthin and Zeaxanthin (Table I). In vivo antioxidant activity of carotenoids from DS has been shown to be greater than those of synthetic carotene (9, 30). Combined antioxidant supplementation could be a useful chemopreventive strategy.
against oxidative damage and carcinogenesis (7). There are strong in vitro interactions among β-carotene, ascorbic acid and α-tocopherol in terms of mutual beneficial protection against oxidative damage (7). α-Tocopherol and ascorbic acid could regenerate β-carotene from its radical cation (31), therefore preventing β-carotene from being further oxidized. Moreover, β-tocopherol could regenerate α-tocopherol from its radical cation (32). Pre-treatment of human lung cells with both vitamin E and β-carotene has also been found to provide protection against DNA strand breaks induced by tobacco-specific nitrosamines (33). In our study, ethanol extract of DS demonstrated significant antiproliferative and apoptotic effects (Figures 3 and 5). This suggests that the combined ingredients from EDS could be necessary to treat the A549 lung cancer cells.

The unicellular algae DS is known to contain a high concentration of β-carotene, composed of approximately equal amounts of all-trans and 9-cis isomers, which could have different physicochemical features and antioxidant activities. 9-cis isomer exhibits a higher antioxidant activity than all-trans-β-carotene (34). In vivo study has indicated that the stereoisomer mixture in Dunaliella bardawil protects against oxygen toxicity in the central nervous system, whereas no protection is observed with all-trans-β-carotene or with oxidized natural β-carotene (35). It has been implied that the antiperoxidative effect of 9-cis-β-carotene compared with that of all-trans-isomer is important for the prevention of malignant and cardiovascular diseases (14). Other reports on 9-cis-β-carotene extracted from a commercial extract of DS (Betatene) showed that it was less active than all-trans β-carotene in reducing proliferation and in up-regulating expression of connexin43 in 10T1/2 cells. However, it had comparable ability to suppress carcinogen-induced neoplastic transformation (36).

In conclusion, EDS can induce apoptosis and G0/G1 arrest in the A549 cancer cell line. We demonstrated that EDS induced apoptosis in A549 possibly via p53 and p21 promoting the protein expression of Fas and Fasl. Therefore, DS might act as a potential chemopreventive agent.

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