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ScienceDirect
Food Chemistry 104 (2007) 196–201
Food Chemistry
www.elsevier.com/locate/foodchem

Induction of apoptosis in HeLa cells by ethanolic extract of *Corallina pilulifera*

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Received 12 January 2006; received in revised form 5 September 2006; accepted 10 November 2006

Abstract

Marine organisms are rich sources of new, biologically active compounds. Seaweeds have traditionally been used as food, but have also been used as folk medicine, particularly by coastal peoples. Recently, much attention has been paid to the anticancer activity of seaweed. Thus, we have screened organic extracts of seaweeds for their anticancer activity against human cell lines, and selected *Corallina pilulifera* as a candidate for use in treatment. The ethanolic extracts of *Corallina pilulifera* (EECP) showed cytotoxic activity against human cervical adenocarcinoma cell line, HeLa. The IC_{50} of EECP against the HeLa cells was 250 μg/ml. Treatment of HeLa cells with various concentrations of EECP resulted in growth inhibition and induction of apoptosis in a dose-dependent manner. In Western blot analysis, apoptosis in the HeLa cells was associated with the release of cytochrome *C* from mitochondria into the cytosol, activation of caspase-3 and caspase-8, and proteolytic cleavage of PARP (poly (ADP-ribose) polymerase). These results strongly suggest that EECP may be a potential candidate in the field of anticancer drug discovery.
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Keywords: *Corallina pilulifera*; Seaweed; Antiproliferative activity; Hela cell line; Anticancer; Apoptosis

1. Introduction

Due to their tremendous biodiversity, marine organisms are attractive sources of novel, biologically-active compounds. Over the past two decades, about 3000 new compounds have been discovered and isolated from various marine organisms; some of these compounds have been employed in clinical therapies (Cragg, Newman, & Weiss, 1997).

Seaweeds have traditionally been used as food in Asia, including Korea. Seaweeds have also been used as folk medicine for curing helminth infections, gout, and eczema, particularly by coastal peoples (Michanek, 1979). Many compounds derived from seaweeds, which possess biological activities for medicinal use, have been reported (Da Rocha, Lopes, & Schwartsmann, 2001; Faulkner, 2000a, 2000b; Moore, 1978; Schwartsmann, Da Rocha, Berlinck, & Jimeno, 2001). Recently, much attention has been paid to the anticancer activity of seaweed constituents. Several investigations have reported that crude seaweeds or their organic extracts have antiproliferative activity in human cancer cell lines in vitro, as well as inhibitive activity in tumours growing in mice (Furusawa & Furusawa, 1985; Nagumo et al., 1988; Noda, Amano, Arashima, Hashimoto, & Nishizawa, 1989). In recent studies, the antiproliferative activity of ethanolic and aqueous extracts from Spirulina and Chlorella seaweeds (Wu, Ho, Shieh, & Lu, 2005) and also from *Turbinaria ornata* (Deslandes et al.,

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doi:10.1016/j.foodchem.2006.11.031

2000) against human cancer cells were reported. Nevertheless, the opportunities to discover new anticancer agents in seaweeds remain great. Thus, we have surveyed organic extracts of seaweeds for anticancer activity against human cell lines; we selected *Corallina pilulifera* collected from the coastline of Korea as a candidate for anticancer drugs. *C. pilulifera* is calcareous red algae that belongs to the family Corallinaceae (Rhodophyta) and is widely distributed in tidal pools, as well as intertidal zones.

Induction of apoptosis is a useful approach in cancer therapies. Apoptosis, a major process of programmed cell death, plays an important role in the regulation of tissue development and homeostasis (Green & Reed, 1998; Hengartner, 2000; Kaufmann & Hengartner, 2001). Cancer is a disease state caused by the disruption of cellular homeostasis between cell death and cell proliferation (Thompson, 1995). As compounds which can induce apoptosis are considered to have potential as antitumour agents (Frankfurt & Krishan, 2003), many efforts have been made to discover new drugs through the isolation of apoptosis-inducing agents from natural products.

In this work, we evaluated the anticancer activity of the crude ethanolic extracts of *C. pilulifera* (EECP) against human cervical adenocarcinoma cell line, (HeLa line) via induction of apoptosis.

2. Materials and methods

2.1. Preparation of C. pilulifera extract

C. pilulifera was collected from the coast of the Busan area of the Republic of Korea from April to May 2004; the organism was identified by Prof. C.H. Shon of the Department of Aquaculture, Pukyong National University. A voucher specimen (No. 200405) has been deposited in the laboratory of the author (H.J. Kwon). Samples were collected and washed using tap water for the removal of salt and sand. After complete drying for 7 days at room temperature, the samples were extracted three times with ethanol at room temperature and were then extracted twice further with ethanol at 70 °C under reflux. All extracts were mixed, and the mixture was filtered and concentrated using a rotary evaporator and then evaporated to dryness. The extract (EECP) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at 4 °C.

2.2. Cell line and cell culture

Human cervical adenocarcinoma cell line, HeLa, used in this work was purchased from the American type culture collection (ATCC, Rockville, MD). HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 0.1% gentamycin, in a humidified incubator containing 5% CO_2 at 37 °C. When necessary, 0.5% DMSO, containing an appropriate concentration of EECP was added.

2.3. Cytotoxicity and antiproliferative activity

Cytotoxicity was determined by the MTT assay, as previously described (Mosmann, 1983). In brief, cells were plated in 96-well plates at an initial density of 1×10^4 cells per well. After incubation for 24 h at 37 °C, cells were treated with various concentrations of EECP and incubated for 24 h. MTT solution was added to each well and further incubated for 4 h at 37 °C. The optical density was read with an ELISA reader at 550 nm.

Antiproliferative activity was determined by a trypan blue exclusion assay (Jones & Senft, 1985). For the trypan blue exclusion assay, cells were cultured in a 35 mm dish and exposed to various concentrations of EECP for 7 days. The cells were trypsinised, washed with phosphate buffered saline (PBS), and trypan blue dye solution was then added to the cell suspension. Viable cells were counted with a haemocytometer.

2.4. Flow cytometry analysis

Cells were plated at a density of 5×10^5 cells in a 35 mm dish. After 48 h of exposure to EECP, cells were collected by trypsinisation, washed with cold PBS, and resuspended in PBS. DNA staining was carried out using the Cycle-TEST™ PLUS kit (Becton Dickinson, Heidelberg). Propidium iodide-stained nuclear fractions were obtained according to the kit protocol. Fluorescence intensity was determined using a FACScan flow cytometer and analysed by CellQuest software (Becton Dickinson).

2.5. DAPI staining

Cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The cells were washed twice with PBS and analysed under a fluorescence microscope.

2.6. TUNEL assay

TUNEL assay was also used for determination of apoptosis through the detection of DNA fragmentation. TUNEL assay was performed using the Deadend™ Colorimetric Apoptosis Detection System (Promega KK, Tokyo, Japan). In brief, cells were plated on poly-L-lysine-coated slides. The cells were then air-dried in a tissue culture hood for 1 h. After two washes with PBS, the cells were fixed with 4% (w/v) paraformaldehyde in PBS in a Coplin jar for 25 min at room temperature and rinsed twice with PBS. Then, the cells were immersed in 0.2% (v/v) Triton X-100 solution for 5 min and rinsed with PBS. Control and positive control cells were treated with DMSO and DNase I, respectively. The cells were equilibrated with equilibration buffer at room temperature for 5 min. TdT (Terminal deoxynucleotidyl transferase) enzyme reaction

mixture and biotinylated nucleotide mixture were subsequently added to the cells, and the cells were then covered with cover slips and incubated for 1 h at 37 °C. The reaction was terminated by immersion of the slides in 2 × SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 15 min. The slides were washed with PBS and treated with 0.3% (v/v) hydrogen peroxide for 5 min. The slides were then incubated with streptavidin HRP solution in PBS for 30 min, rinsed with PBS, and finally incubated with DAB components until a light brown background developed. The stained cells were immediately examined under a light microscope.

2.7. Western blot analysis

Cells were plated at a density of 5×10^5 cells in a 60 mm dish and incubated 24 h. After 48 h of exposure to EECP at designated concentration (50–200 μg/ml), cells were collected by trypsinisation and washed with cold PBS. Collected cells were lysed in CSK buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride) containing 0.1% Triton X-100, 1 mM ATP, and proteinase inhibitors (Pharminogen, San Diego, CA). The lysates were centrifuged at 20,000*g* for 30 min; the protein concentration in the supernatant was determined with a BCA protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by SDS-PAGE (8% PARP, 15% caspase-3, caspase-8, cytochrome *C*, actin) and transferred to a PVDF membrane (Pall Corporation, Ann Arbor, MI). The membrane was incubated with primary antibodies in a blocking solution (Blockace™, Dai-Nippon, Osaka, Japan) at 37 °C for 1 h. After being washed with Tris buffered saline (TBS; 50 mM Tris/HCl, pH 7.5, and 0.15 M NaCl) containing 0.1% Triton X-100, the membrane was incubated with peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL). The immunoreacted proteins were detected using a chemiluminescence system (Super-Signal West Femto Maximum Sensitivity Substrate, Pierce), and the level of reactivity was quantified (Fluorchem™ 5500, Alpha Innotech, San Leandro, CA). Antibodies to actin, PARP, caspase-8, and were purchased from Santa Cruz Biotechnology Inc., and antibodies to caspase-3 and cytochrome *C* were purchased from BD Biosciences Pharminogen.

3. Results

3.1. Cytotoxic activity of EECP on HeLa cells

The cytotoxic effect of EECP on HeLa cells was determined. Cells were exposed to various concentrations of EECP (0–500 μg/ml) for 24 h. Cells treated with 0.1% DMSO were used as control. The IC_{50} value of EECP on HeLa cells was 250 μg/ml after treatment for 24 h.

To investigate the effect of EECP on growth inhibition of HeLa cells, cells were treated with EECP (50, 100, 150 or 200 μg/ml) for 7 days and counted at 2 day intervals by the Trypan blue exclusion method. Compared to control cells, cells treated with EECP were significantly inhibited by $30.3 \pm 4.0\%$ at 50 μg/ml EECP concentration for 1 day of incubation. When the EECP concentration was increased to 50, 100, 150 and 200 μg/ml, the inhibitory rate was increased to $38.2 \pm 3.7\%$, $73.8 \pm 4.3\%$, $93.4 \pm 2.0\%$ and $95.5 \pm 1.6\%$, respectively, after 7 days incubation. These dose- and time-dependent effects of EECP on growth inhibition of HeLa cells are shown in Fig. 1.

3.2. Induction of apoptosis by EECP on HeLa cells

In order to determine whether the growth inhibitory effect of EECP was due to apoptosis, HeLa cells were treated with EECP for 48 h, and nuclear DAPI staining was performed. As shown in Fig. 2, nuclei with condensed chromatin and apoptotic bodies, typical characteristics of apoptosis, were observed in HeLa cells incubated with EECP, and the number of apoptotic cells increased as the EECP concentration increased (data not shown). Because DNA fragmentation is another characteristic of apoptosis (Parrish et al., 2001), DNA fragmentation assay was also carried out by a TUNEL assay using the Deadend™ Colorimetric TUNEL System (Promega). As shown in Fig. 3, the nuclei of cells treated with EECP were stained dark brown, but no stained nucleus was observed in control cells.

3.3. Cell cycle analysis

The apoptotic morphological change described above was also confirmed with a flow cytometric analysis. After

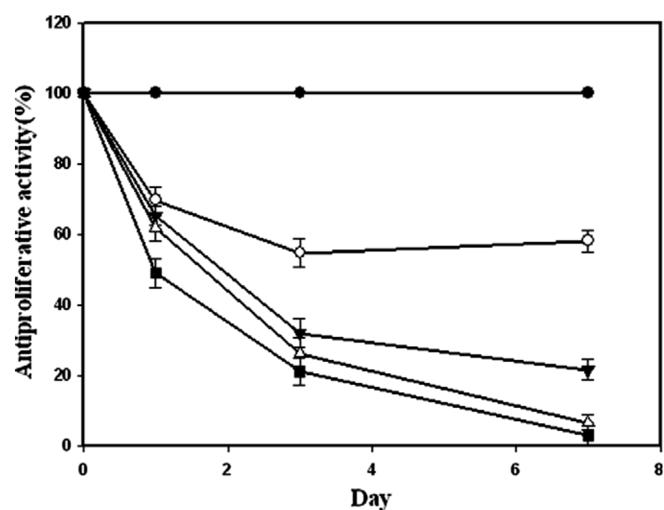

Fig. 1. The growth inhibition effects of EECP on HeLa cells. Cells were treated with EECP ranging in dose from 0 μg/ml to 200 μg/ml for 7 days. The growth inhibition effects were determined by Trypan blue exclusion assay. Cells treated with 0.1% DMSO were used as control. Legend: 0 μg/ml (●), 50 μg/ml (○), 100 μg/ml (▼), 150 μg/ml(△) and 200 μg/ml (■) EECP. All experiments were conducted in triplicate, and the values and bars represent mean and SD, respectively.

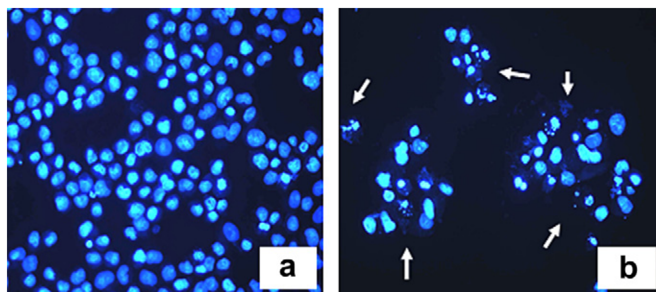

Fig. 2. Morphological changes of HeLa cells after EECP treatment for 48 h followed by DAPI staining. (a) Fluorescence microscope photographs of control cells treated with 0.1% DMSO and (b) cells treated with 150 μg/ml EECP. Arrows indicate apoptotic bodies of nuclear fragmentation. Magnification × 100.

the HeLa cells were treated with EECP at designated concentrations (50 and 200 μg/ml) for 48 h, they were harvested and stained with propidium iodide, and the cell populations of each phase were counted by flow cytometry. As shown in Fig. 4, the sub-G1 population, which indicated apoptotic cells, increased in dose dependent manner from 6.3% at 0 μg/ml (control) to 9.1% at 50 μg/ml, 10.6% at 100 μg/ml, 16.3% at 150 μg/ml, 30.0% at 200 μg/ml, after exposure to EECP for 48 h. Although the G1 population decreased along with an increase of sub-G1, the other portion of non-apoptotic cells did not show a significant change. These results suggested that EECP can induce apoptosis in HeLa cells without cell cycle arrest.

3.4. Effects of EECP on expression of apoptosis-related proteins

Activation of a caspase cascade occurs in apoptotic events. As an initiator of apoptosis, procaspase-8 is cleaved to create an active form of caspase-8. Once activated, cas-

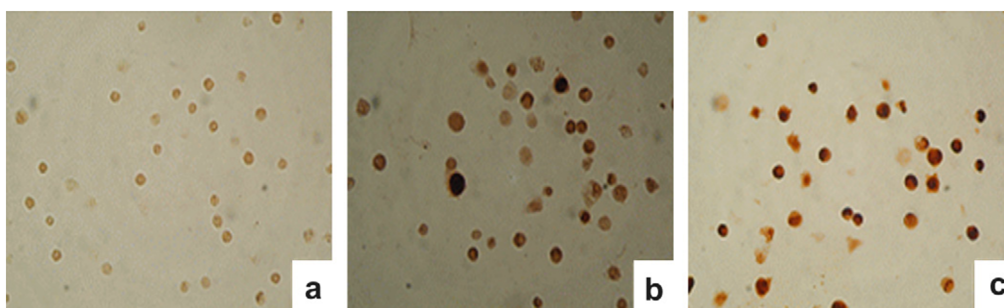

Fig. 3. TUNEL assay of HeLa cells after treatment with 0.1% DMSO (a – negative control), EECP (b – 150 μg/ml), or DNase I (c – positive control) for 48 h. Dark brown staining of the nuclei of the HeLa cells was observed after cells were treated with EECP and DNase I, whereas no staining of the nuclei of DMSO-treated control cells was detected. a, b, c: Magnification ×100.

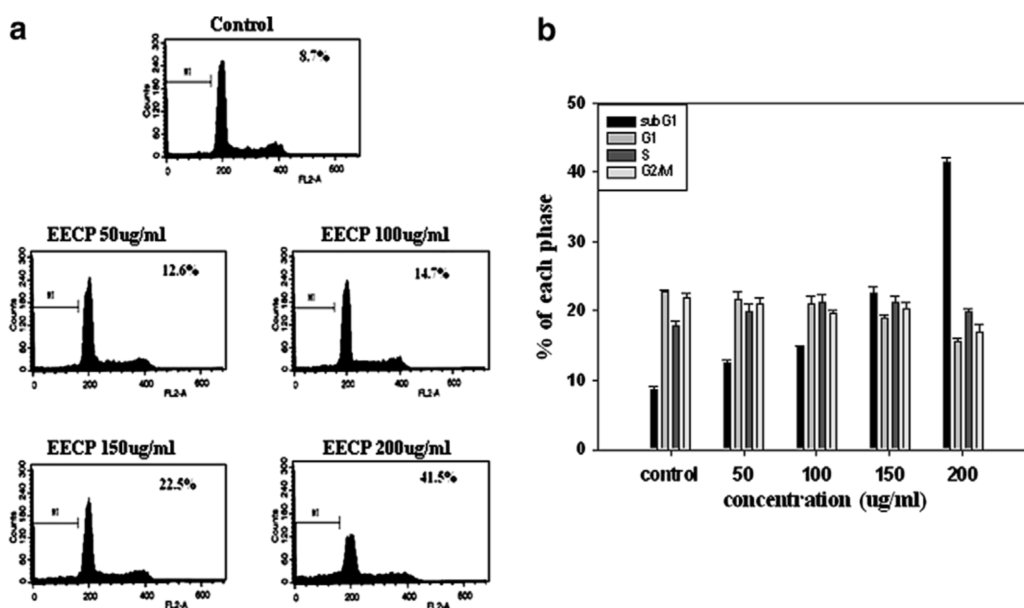

Fig. 4. Cell cycle analysis of HeLa cells treated with EECP by flow cytometry. (a) HeLa cells were incubated with various concentration of EECP as indicated in each graph for 48 h. The cells were then stained with PI and analysed by flow cytometry. (b) The percentage of each phase analysed by Cell Quest software.

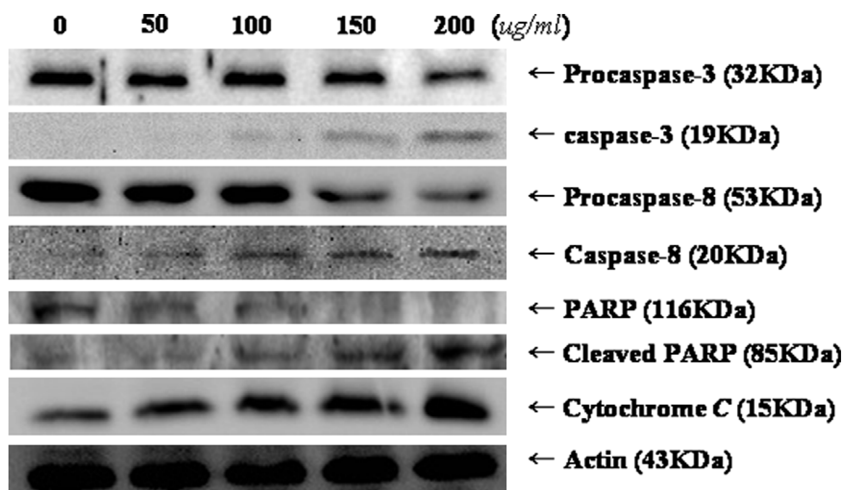

Fig. 5. Western blot analysis of caspase-3, caspase-8, cytochrome *C*, and PARP in HeLa cells treated with EECP. Cells were treated with EECP (50–200 μg/ml). Cytosolic proteins (40 μg), except PARP (total proteins), were resolved by SDS-PAGE, and Western blot was performed. β-Actin was used as internal control.

pase-8 cleaves procaspase-3 to create an active dimeric form of caspase-3 for the execution of apoptosis. To certify whether EECP induces the activation of these caspases, HeLa cells were treated with EECP, and expression levels of caspase-3, and -8 were determined by Western blot analysis. As shown in Fig. 5, EECP activated procaspase-8 (53 kDa) and procaspase-3 (32 kDa) into caspase-8 (20 kDa) and caspase-3 (17 kDa), each after respective exposure to EECP (50–200 μg/ml) for 48 h. In a mitochondria-dependent intrinsic apoptotic pathway, cytochrome *C* is released from mitochondria into the cytosol. As shown in Fig. 5, cytosolic cytochrome *C* gradually increased in relation to the increase in EECP concentration. The cleavage of 116 kDa PARP proteins into 85 kDa fragments by active caspase-3 is a characteristic marker of the execution of apoptosis. As shown in Fig. 5, PARP is cleaved into an 85 kDa fragment after treatment with EECP. These results suggest that EECP could induce apoptosis on HeLa cells via a mitochondria-dependent intrinsic pathway.

4. Discussion

Recently, much attention has been paid to marine organisms for the screening of biologically active compounds (Cragg et al., 1997). Among these marine organisms, seaweeds are considered to be very attractive sources, due to their huge biodiversity and safety, as they have long been used in traditional Asian foods (Michanek, 1979). Although several reports have suggested that crude seaweed extracts have antiproliferative activity in cancer cell lines, most studies focused on antioxidant activity. Water-soluble polysaccharides, such as laminarans and fucoidans, are representative anticancer substances extracted from seaweeds (Furusawa & Furusawa, 1985; Nagumo et al., 1988; Noda et al., 1989; Koyabagi, Tanigawa, Nakgawa, Soeda, & Shimeno, 2003; Le Tutour et al., 1998).

In this work, we found that the crude ethanolic extracts of *C. pilulifera* (EECP) had antiproliferative activity on human cervical adenocarcinoma cell line. *C. pilulifera* is calcareous red algae and is distributed in tidal pools and intertidal zones worldwide. Jeong et al. reported that the methanolic extracts of *C. pilulifera* have algicidal activity against the toxic microalga, *Cochlodinium polykrikoides* (Jeong, Jin, Sohn, Suh, & Hong, 2000).

Induction of apoptosis is a useful approach in cancer therapies. Apoptosis, a major process of programmed cell death, plays an important role in maintaining cellular homeostasis (Green & Reed, 1998; Hengartner, 2000; Kaufmann & Hengartner, 2001). In apoptotic cells, several cellular and molecular biological features, such as cell shrinkage, DNA fragmentations, and activation of the caspase cascade, are exhibited (Germain et al., 1999). In our results, we also observed cell shrinkage and DNA fragmentation, as determined by DAPI staining and TUNEL assay, respectively. Activation of the caspase cascade is a well-known molecular mechanism for the induction of apoptosis. In the early stage of activation of the caspase cascade, inactive initiator caspases, such as procaspase-8 and -9, are turned to active caspase-8 and -9 by self-processing. Activated initiator caspases then cleave inactive executioner procaspase-3 into active caspase-3, which acts downstream in the cleavage of specific intracellular substrates, such as poly (ADP-ribose) polymerase (PARP). Mitochondria also play an important role in the induction of apoptosis. The release of cytochrome *C* from the mitochondria into the cytosol is a characteristic of a mitochondria-dependent pathway (Kelekar & Thompson, 1998). We reported here that EECP can activate caspase-8 and -3, cleave PARP, and mediate the release of cytochrome *C*. These results suggest that EECP-induced apoptosis may be mediated via a mitochondria-dependent pathway.

Regulation of the cancer cell cycle is one strategy in the development of anticancer drugs (Camero, 2002). The

result of cell cycle analysis determined by flow cytometry analysis also showed that EECP can induce apoptosis in HeLa cells. In DNA microarray analysis, EECP can down-regulate DNA topoisomerase IIα gene expression (data not shown). DNA topoisomerase IIα is an enzyme that is essential for the maintenance of chromatin structure, replication of DNA, and the repair and transcription of DNA and the cell cycle. Inhibition of DNA topoisomerase is also regarded as a novel target for the discovery of anticancer drugs (Li & Liu, 2001).

In conclusion, this study showed that EECP could inhibit the growth of cancer cells and could induce apoptosis via a mitochondria-dependent pathway in human cervical adenocarcinoma HeLa cells. According to these results, it is suggested that the ethanolic extracts of *C. pilulifera* are valuable for the development of anticancer drugs. Further investigations to determine its bioactive compounds are currently in progress.

Acknowledgements

This research was supported by a Grant (B-2004-03) from the Marine Bioprocess Research Center of the Marine Bio 21 Center, funded by the Ministry of Maritime Affairs & Fisheries of Republic of Korea.

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