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Antiproliferative activity of sulfated polysaccharide isolated from an enzymatic digest of *Ecklonia cava* on the U-937 cell line

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Abstract A sulfated polysaccharide purified from a brown alga *Ecklonia cava*, having high anticoagulant activity was investigated for its antiproliferative effect on murine colon carcinoma (CT-26), human leukemic monocyte lymphoma (U-937), human promyelocytic leukemia (HL-60), and mouse melanoma (B-16) cell lines. The sulfated polysaccharide isolated and purified from an enzymatic extract of *E. cava* had a good selective tumor cell growth inhibition effect; its effect on HL-60 and U-937 was especially promising. The IC_{50} value for the sulfated polysaccharide from *E. cava* (ECSP) on U-937 was 43.9 μg mL^{-1}. The presence of the sample in the cell culture media stimulated the induction of apoptosis, revealed by nuclear staining with Hoechst 33342. The apoptosis induction was confirmed by the cell cycle analysis, while pronounced sub-G1 phase arrests of 9.5% and 13.8% were also clearly observed when the cells were treated at 15 and 30 μg mL^{-1} of ECSP in the U-937 cell line, respectively. After a 24-h incubation period, ECSP dose-dependently enhanced the DNA fragmentation on the U-937 cell line as observed in the agarose gel electrophoresis assay. To rule out the action mechanism of ECSP for its anticancer activity, some western blot analyses were conducted with several antibodies (caspase-7, caspase-8, Bax, Bcl-xL, and PARP) and ECSP had a clear effect on the caspase -7 and 8 which cleave protein substrates, including PARP, an inducer of apoptosis responsible for DNA cleavage. Moreover, ECSP controlled the cellular transmembrane molecules like Bax and Bcl-xL. Taken together, the above results demonstrate that the apoptosis for antiproliferative effect of ECSP was clearly induced on U-937 cells.

Keywords *Ecklonia cava* · Sulfated polysaccharide · Apoptosis

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Introduction

Marine algae have been reported to contain diverse classes of biologically active compounds which are useful in the pharmaceutical industry. Antitumor activity is one of the important biological activities in marine algae. Some of the marine algae and their metabolites have shown promising activities and hence they are important sources to investigate for new antitumor drugs.

Most natural anticancer compounds are able to manipulate the growth of cancer cells with reduced side effects. Therefore, novel natural anticancer drugs have been commercially developed to successfully treat cancer patients (Mans et al. 2000). As has been reported, around 60% of the current commercial anticancer drugs have originated from natural sources. It is noteworthy to highlight that, during the last decade, about 2,500 new marine-derived metabolites with antiproliferative activity have been discovered; however, the modes of action for their activity remain to be elucidated.

Sulfated polysaccharides isolated from marine brown algae are also promising compounds with multiple biological activities. Extracts of edible algae have been investigated for their protective effect against the L-1210 leukemia cell line in vivo. Of the tested species, the crude fucoidan separated from a sample of *Eisenia bicyclis*, a brown seaweed, showed considerable activity (Yamamoto et al. 1984). The polysaccharide fraction of *Capsosiphon fulvescens* (Chlorophyta), consisting of xylose (19.1%), fucose (15.3%), mannose (4.2%), and galactose (8%), successfully inhibited sarcoma-180 growth and exhibited immunostimulating activity in vitro (Park et al. 2006). Due to numerous health benefits, the compound is currently being investigated both in vivo and in vitro to utilize it as a therapeutic drug. At the moment, there are several nutrient supplements containing fucoidans (sulfated polysaccharides) on the market.

Ecklonia cava, a brown seaweed, is plentifully produced in Jeju Island in Korea (30,000 t $year^{-1}$) and, although not available in Europe, is popular in Korea and Japan where this valuable brown alga has been utilized in the field of food ingredients, animal feed, fertilizers, and medicine. In addition, *E. cava* contains xanthopophyll pigment, fucoxathin, phlorotannins, fucoidans, and especially is a good source of alginates, which can be used as viscosifiers and thickeners in a wide variety of products (Ahn et al. 2007).

In our previous study (Athukorala et al. 2006), we isolated a highly sulfated (0.92 sulfate/total sugar) polysaccharide from an enzymatic hydrolysate of *E. cava* (ECSP) which was mainly composed of fucose (82%), galactose (14%), and small amounts of xylose and mannose. The ECSP showed high anticoagulant activity and strongly interfered with coagulation cascade by inhibiting biological activity of the activated blood coagulation FII, FX and FVII as serine proteases. Also, sulfated polysaccharide from *E. cava* strongly and selectively (FVII, FX, and FII) enhanced ATIII-mediated coagulation factor inhibition in both the extrinsic and common coagulation pathways (Jung et al. 2007).

In this study, we investigated antiproliferative activity of the ECSP on several cancer cell lines, including murine colon carcinoma (CT-26), human leukemic monocyte lymphoma (U-937), human promyelocytic leukemia (HL-60), and mouse melanoma (B-16) cell lines. Additionally, its effect on apoptotic related protein expression was investigated by western blot analysis.

Materials and methods

Marine brown algae used in this study were collected close to the shores of Jeju Island in Korea during January to April 2006. Salt, sand, and epiphytes were removed using tap water. Finally, seaweed samples were rinsed carefully with fresh water and freeze-dried at −20°C for further experiments. AMG 300 L (an exo1, 4-alpha-d-glucosidase) was from Navo (Novozyme Nordisk, Denmark). RPMI-1640, DMEM medium and fetal bovine serum (FBS) were from Gibco/BRL (Canada). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and Hoechst 33342 were from Sigma (St. Louis, USA). The other chemicals and reagents used were of analytical grade.

Purification of the sulfated polysaccharide from *E. cava* (ECSP)

The purification of ECSP was as previously reported (Athukorala et al. 2006). The dried alga sample was ground (MFC SI mill; Janke and Kunkel Ika, Germany) and sieved through a 50 standard testing sieve. A sample of 100 g alga was homogenized with water (2 L), and then 1 mL of AMG 300 L was mixed. The enzymatic digestion was performed for 12 h to achieve optimum degree of the hydrolysis. Before the digestion, the pH of the homogenate was adjusted to its optimal value, and after digestion the digest was boiled for 10 min at 100°C to inactive the enzyme. Sample was clarified by centrifugation (3,000 *g*, 20 min, 4°C) to remove the residue. The enzymatic digest was (240 mL) mixed well with 480 mL 99.5% ethanol. Then, the mixture was allowed to stand for 30 min at room temperature and crude polysaccharides were collected by centrifugation at 10,000 *g* for 20 min at 4°C (Kuda et al. 2002; Matsubara 2004). Freeze-dried crude polysaccharide from the AMG digest was further purified on a new DEAE-cellulose column. Thereafter, the sample was applied into a gel permeation chromatography on Sepharose-4B to purify the sample according to its molecular weight. The purity of the sample was confirmed by agarose gel electrophoresis, and the molecular weight of the sample was determined by GFC system. The ECSP (0.92 sulfate/total sugar) showed 1,381 kDa molecular weight and was comprised mainly of fucose and a small amount of galactose.

Assessment of cell viability

Cell viability was estimated via an MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazoliumbromide) assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mossman 1983). The cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells mL^{-1} and treated with the sulfated polysaccharides at different concentrations. After 72 h, MTT stock solution (50 mL; 2 mg mL^{-1}) was applied to each of the wells, to a total reaction volume of 200 mL. After 4 h incubation, the plates were centrifuged for 5 min at 800 *g*, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 mL of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was a sign of apoptosis (Gschwind and Huber 1995; Lizard et al. 1995). The tumor cells were placed in 24-well plates at a concentration of 1.0×10^5 cells mL^{-1}, and then different concentrations of ECSP were added. After 24 h, 1.5 mL of Hoechst 33342 (stock 10 mg mL^{-1}) and a DNA-specific fluorescent dye were added to each well (1.5 mL), followed by 10 min incubation at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera in order to examine the degree of nuclear condensation.

Flow cytometry analysis

Flow cytometry analyses were conducted in order to determine the proportion of apoptotic sub-G1 hypodiploid cells (Nicoletti et al. 1991). The tumor cells were placed in 6-well plates at a concentration of 1.0×10^5 cells mL^{-1}, with different concentrations of ECSP. After 24 h, the treated cells were harvested at the indicated time, and fixed for 30 min in 1 mL of 70% ethanol at 4°C. The cells were then washed twice with PBS (phosphate buffer saline) and incubated for 30 min in darkness in 1 mL of PBS containing 100 mg PI (propidium iodide) and 100 mg Rnase A, at 37°C. Flow cytometric analysis was conducted using a FACSCalibur flow cytometer (Becton Dickinson, USA). Effects on the cell cycle were determined by measuring changes in the percentage of cell distribution at each phase of the cell cycle, and were assessed by histograms generated by the Cell Quest and Mod-Fit computer programs (Wang et al. 1999).

DNA gel electrophoresis (DNA laddering)

DNA laddering is an indicator of the programmed cell death, and the intensity of a drug to induce DNA degradation of tumor cells can be simply evaluated through agarose gel electrophoresis. In this study, tumor cells (5×10^5 cells/60-mm culture dish) were incubated with the presence of ECSP at different concentrations (30, 60, 120 μg mL^{-1}) and investigated by agarose gel electrophoresis for the effect of the ECSP on internucleosomal DNA cleavage of the tumor cells. Cell DNA was separated and purified according to the manufacturer's guidelines. Four μL of the DNA was applied into a 1.5% agarose gel containing ethidium bromide, and the DNA fragmentation was observed by observation under ultraviolet illumination.

Western blot analysis

Tumor cells (2×10^5 cell mL^{-1}) were treated with ECSP, incubated for 24 h and harvested. The cell lysates were prepared with lysis buffer (50 mm L^{-1} Tris-HCl (pH. 7.4), 150 mm L^{-1} NaCl, 1% Triton X-100, 0.1% SDS and 1 mm L^{-1} EDTA). Cell lysate was washed in PBS and centrifuged, and then the protein content of the supernatant was determined by BCA™ protein assay kit. The lysate containing 40 μg of protein was subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane (Bio-Rad). The membranes were incubated with primary antibody against Bax, Bcl-xL, cleaved caspase-3, carpase-7, and β-actin (Cell Signaling Technology, USA) in tween-tris buffered saline (TTBS; 25 mm L^{-1} Tris-HCl, 137 mm L^{-1} NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% nonfat dry milk for 1 h. Membranes were washed with TTBS and incubated with secondary antibodies. Signals were developed using an ECL (enhanced chemiluminesence) western blotting detection kit and exposed to X-ray films.

Results and discussion

Algal polysaccharides are renowned for their unique mode of action which has long made them attractive for use in the food and pharmaceutical industries. The most popular algal polysaccharides, including agar, carrageenans, alginates, laminaran, rhamnan sulfate, and fucoidan, are commercially produced and used in the food, agriculture and other related industries. Recently, as an abundant, renewable natural source with multiple biological activities, fucoidan has been playing an important key role in life science research. Owing to its special structural features, fucoidan isolated from marine algae is known to exert anticoagulant, anticancer, antimitogenic, anticompliment, antiviral, anti-adhesive, anti-inflammatory, and antioxidant activities. The composition of the fucoidan or other polysaccharides can vary according to the algal species, extraction procedure, harvesting season, and the local climate conditions. In our previous study, a sulfated polysaccharide with promising anticoagulant activity was isolated from the enzymatic digests of *E. cava* and was revealed as a kind of fucoidan by determination of its sugar composition. The present work was devoted to investigating the anticancer activities of the isolated polysaccharide in vitro with several important cancer cell lines, including CT-26, B-16, HL-60, and U-937.

Interestingly, the sulfated polysaccharide purified from *E. cava* (ECSP), selectively and dose-dependently suppressed the proliferation of all the cancer cell lines in vitro. ECSP showed a strong anticancer activity on the murine colon carcinoma cell (CT-26) line (Fig. 1). The growth inhibition rate of CT-26 cells increased consistently with the sample concentration, in which the highest activity (around 40%) was recorded at 100 μg mL^{-1} sample concentration. In the previous study, *Codium contractum* and *C. fragile* collected from Jeju Island exhibited remarkable cytotoxic activities against HL-60 or CT-26 cells (Kim et al. 2006a). Moreover, in the same study, *Sagassum coreanum* and *S. siliquastrum* aqueous extracts obtained at a low temperature exhibited strong cytotoxic activities against U-937, HL-60, and HeLa cells. In this study, the presence of the sulfated polysaccharide dose-dependently delayed the growth on the U-937 cell line, and the IC_{50} of ECSP on human leukemic monocyte lymphoma cell line was 43.9 μg mL^{-1} (Fig. 1). A sulfated polysaccharide (B-1) isolated and purified from the culture filtrate of marine *Pseudomonas* sp. induced apoptotic changes in the morphology of U-937 cells at concentrations greater than 0.1 μg mL^{-1} (Matsuda et al. 2003).

When the human promyelocytic leukemia cell (HL-60) line was treated with different concentrations of the polysaccharide sample, a promising antiproliferative effect was observed (Fig. 1). Around 50% cell growth inhibition was recorded when the sample was treated at 100 μg mL^{-1} concentration, while the inhibition rate gradually decreased with the decreasing sample concentrations. The sample ability to control the growth of mouse melanoma cell line (B-16) was less, but with an almost similar pattern to that of the HL-60 cell line (Fig. 1). As reported previously, *Polysiphonia japonica* showed interesting cytotoxic activity against U-937, HL-60 and B-16 cells. In that study, moreover, the extracts of *Scinaia okamurae* and *Chondrus crispus* showed cell growth inhibitory activity of more than 50% against HL-60 and B-16 cells. And, especially, the former sample showed over 80% growth inhibition activity against B-16 cells (Kim et al. 2006b)

Taken together, ECSP showed a promising antiproliferative effect on all the cancer cell lines tested. It is worth

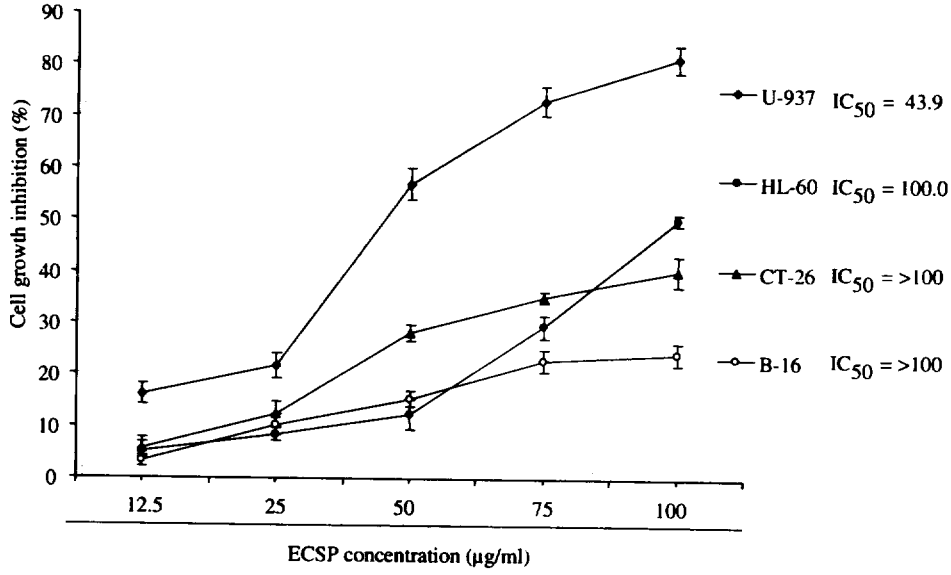

Fig. 1 The effect of sulfated polysaccharide purified from *Ecklonia cava* (ECSP) on the growth of several cancer cell lines. Cells were seeded on 96-well plate at 1×10^3 cells/well and were treated with the sulfated polysaccharides at different concentrations. Percentage of cell viability was determined by MTT assay after 72 h of the treatment. The cell growth inhibition was calculated as function of the control. Bars=standard error, $n=3$

mentioning that the sample had no cytotoxic effect on an endothelial cell line (Jung et al. 2007). Furthermore, the tested polysaccharide showed no cytotoxic effect on Chinese hamster fibroblast cell line/normal cell line (unpublished data). In a cell when the mitochondrial reductase enzymes are active, the presence of MTT converts yellow-colored formazan into purple-colored; hence the technique could be applied in cancer research to investigate the anticancer activity of unknown samples. The depicted results of this study clearly demonstrated the ability of ECSP to suppress the growth of cancer cells in vitro. ECSP showed selective inhibition against four cancer cell lines, and showed its growth inhibitory efficacy in a decreasing order of U-937 > HL-60 > CT-26 and B-16. Hence, the further experiments were carried out on the U-937 cell line to investigate mechanism for the antiproliferative activity of ECSP.

Since there is a big demand for cancer chemotheraphy, the anticancer action mode of ECSP is worth studying. Hence, in order to dissect the mechanism of action for its high anticancer activity, following the sample treatment, cells were stained with Hoechest 33342. The latter dye is able to migrate into the cells and stain the DNA in a quantitative manner. Through a florescent microscope, the apoptotic cells can be easily distinguished. Normally, living cells appear with normal nuclei, with blue/green pale chromatin having an organized structure. Apoptotic cells (especially early apoptotic cells) can be identified by the presence of chromatin condensation within the nucleus and intact nuclear boundaries, bright blue chromatin that is highly condensed, and marinated (late apoptotic cells) exhibiting nuclear fragmentation into smaller nuclear bodies within an intact cytoplasmic membrane (Cavas et al. 2006). The morphological evaluations of chromatin condensation and/or fragmentation induced with the sulfated polysaccharides on U-937 cells are shown in Fig. 2. ECSP successfully induced apoptotic bodies in the U-937 cells compared with the non-treated control group.

Therefore, apoptosis induction of ECSP may probably be the reason for its anticancer activity. The polysaccharide isolated from *Misgurnus anguillicaudatus* showed a high antiproliferative effect associated with apoptosis on the HL-60 cell line; apart from that the polysaccharide enhanced the content of nitric oxide (NO) and activity of lactate dehydrogenase (LDH). Therefore, sometimes, multiple mechanism of actions are combined with the anticancer activity of sulfated polysaccharides (Zhang and Huang 2005). The ability of a compound to induce apoptosis in which cells are programmed to die is an interesting and therapeutically useful way for an anticancer agent to govern the growth of cancer cells. The unique morphological, biochemical, and cellular changes associated with the apoptotic cells are helpful in distinguishing them from the

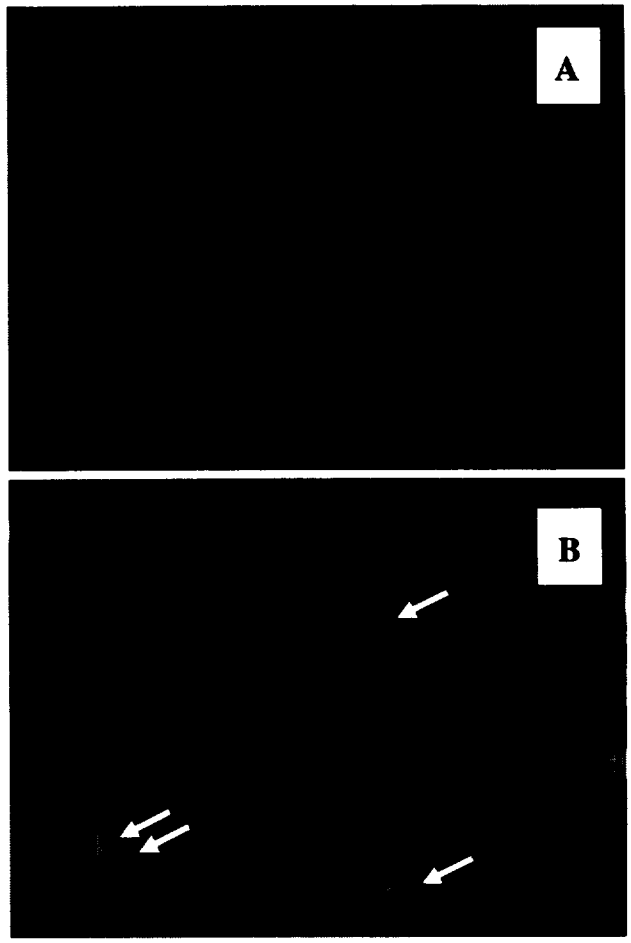

Fig. 2 Effect of *E. cava* sulfated polysaccharide on morphological changes in the U-937 cell line. Cells were treated in the absence of (**A**) or in the presence of (**B**) 75 μg/ml of sulfated polysaccharide for 24 h, stained with Hoechst 33342, and observed by fluorescence microscopy. Arrows in (**B**) indicate a typical apoptotic cell with apoptotic body. Apoptotic nuclei appear brighter and more fragmented than healthy nuclei, which are less bright and intact

normal cells. Flow cytometry technique is a rapid technique to analyze the cell cycle. After anticancer treatment, as a result of the apoptosis, DNA fragmentation can take place. The small fragments of the DNA are able to elute by washing with PBS. The remaining DNA quantitatively binds with the binding dye (propidium iodide). Cells that have lost DNA take up less stain and will appear to the left of the G1 peak. Hence, the size of G1 peak of the histogram is directly proportional to the inducted apoptosis of a given treatment. In this study, after the sample treatment, cells were harvested, fixed, and processed for DNA fragmentation by flow cytometry to elucidate their efficacy for apoptosis induction. The effect of the sulfated polysaccharides on the cell cycle of U-937 was investigated. The treatment of ECSP showed around 9% and 13% cell cycle arrest on U-937 cells at 15 and 30 μg mL^{-1} sample

concentrations tested, respectively (Fig. 3). The sample effect on the other growth phases of the cell cycle was very low or less, (Fig. 4); therefore, this phenomenon clearly showed that the ECSP introduced apoptosis formation. Of

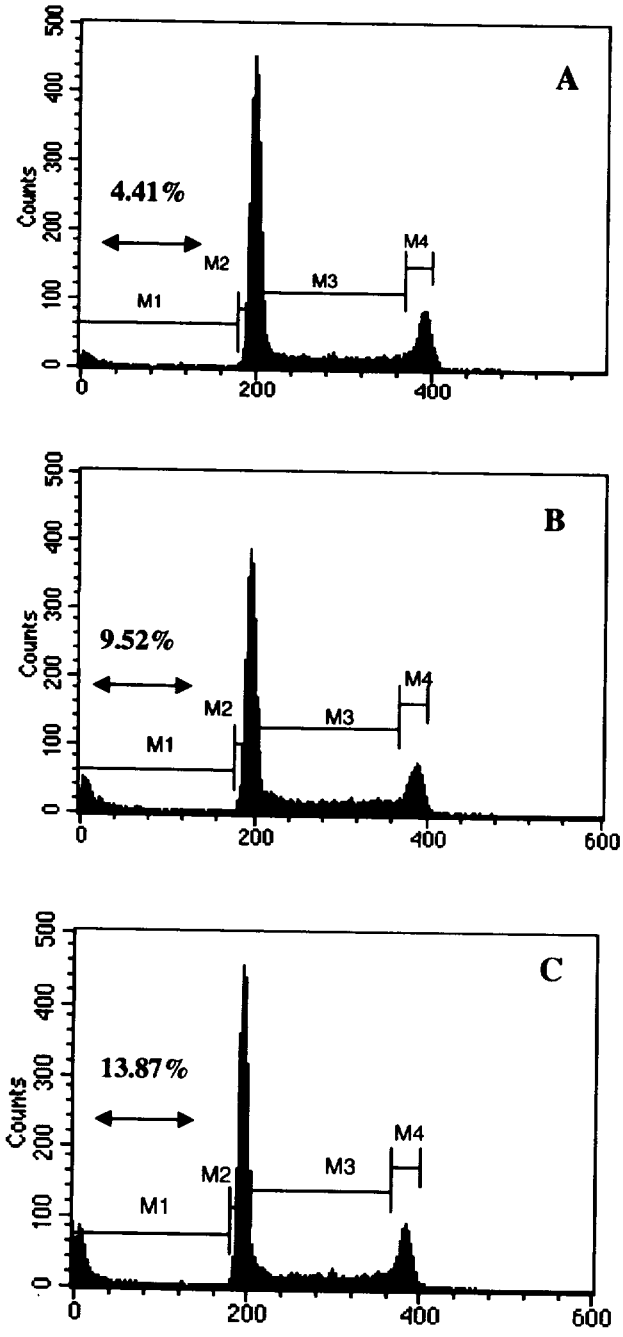

Fig. 3 The effect of *E. cava* sulfated polysaccharide on the cell cycle distribution of human leukemic U-937. The analysis of cell cycle distribution was performed on an equal number of cells (1×10^5) by flow cytometry after the staining of DNA by propidium iodide. **A** The control group where there is no sample, **B** the effect on the cell cycle after 15 $\mu g \cdot mL^{-1}$ of sample, **C** the effect on the cell cycle after 30 $\mu g \cdot mL^{-1}$ of sample

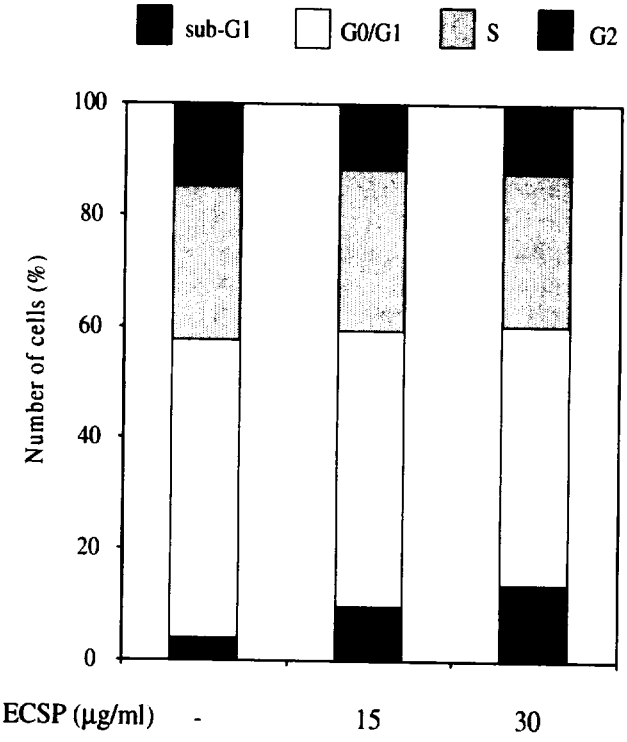

Fig. 4 The cell cycle pattern of U-937 after treatment of sulfated polysaccharides purified from *E. cava*. Percentages of cells in the *sub-G1*, *G0/G1*, *S*, and *G2/M* phases were determined using established CellFIT DNA analysis software

the five phases of a normal cell cycle (mitosis, M; gap1, G1; resting, G0; synthesis, S; and gap2, G2), the G1 phase is important, and most anticancer drugs target this phase to control the growth of the cancer cells. A commercial fucoidan sample purified from *Fucus vesiculosus* enhanced sub-G1 phase arrest in a time-dependent manner (0 h, 1.1%; 24 h, 4.0%; 36 h, 28.7%; 48 h, 89.0%) after stimulation with 100 mg mL^{-1} fucoidan. Moreover, the results indicate that the fucoidan induced apoptosis through caspase and mitochondrial pathways (Asia et al. 2005).

Taken together, the results of this study clearly demonstrate the ability of ECSP to control proliferation of tumor cells. The apoptosis induction of ECSP is assumed to be the reason for its anticancer effect. In order to clarify the apoptosis formation of the cultured cells by ECSP, the DNA fragmentation effect of the treated cells was investigated by agarose gel electrophoresis. DNA fragmentation is a hallmark of the programmed cell death (apoptosis). In this study, ECSP-treated cells were harvested after 24 h incubation and the cell DNA was extracted. On the agarose gel, a ladder of small fragments of double-stranded DNA was dose-dependently observed, and the damage was enhanced with the increasing concentrations (Fig. 5). The control sample in which there is no treatment clearly indicated very low DNA damage. In contrast, the cells treated with ECSP

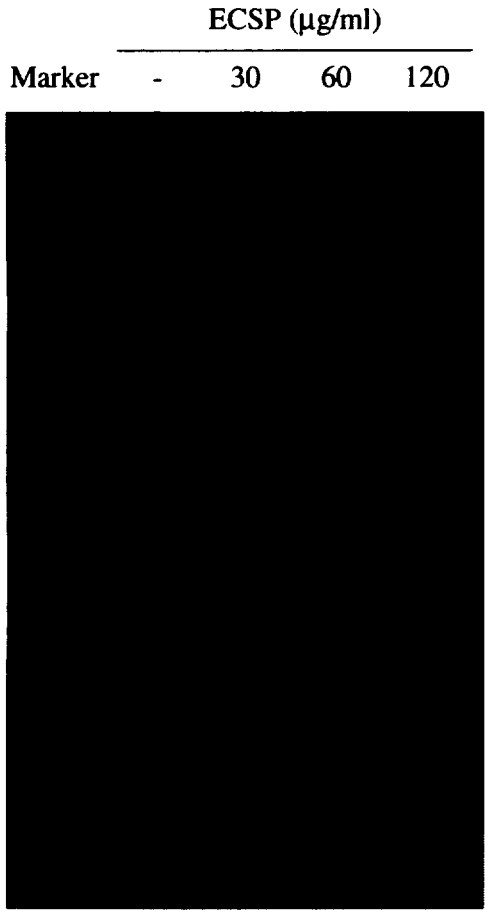

Fig. 5 The dose-dependant (30, 60, and 120 $\mu g \cdot mL^{-1}$) effect of the sulfated polysaccharide isolated from *E. cava* on DNA fragmentation of U-973 cells after 24 h incubation period

dose-dependently showed pronounced double-stranded DNA damage. As has been reported, fucoidan extract derived from *Cladosiphon novae-caledoniae* effectively reduced both intracellular and released H_2O_2 of HT1080 cells, which might lead to the suppression of MMP-2/9 expression and subsequent inhibition of the invasive ability. Moreover, the scientists examining seaweed polysaccharides predicted the ability of fucoidan extract to stimulate antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Ye et al. 2005). Therefore, several factors may probably be associated with the anticancer activity of the sulfated polysaccharide.

Apoptosis induction is followed by the activation of several proteins. In order to evaluate the roles of the ECSP on the expression of cytosolic proteins of U-937 cells, some western blot studies were conducted. In the results shown in Fig. 6, the presence of ECSP had a clear effect on the expression of apoptosis-related proteins, and this may explain the molecular mechanism of the sample for its anticancer effect. PARP (ADP-ribose) is an important compound in maintaining the health of the cells. Upon stress or anticancer activity, the latter compound can break down into small molecular weight compounds and thereby directly stimulate apoptosis. In this study, when the U-937 cells were treated with the sulfated polysaccharide, a clear PARP breakdown (89 KD) was observed. It is believed that the cleaved PARP can take place due to the activation of cleaved caspase-7 (20 KD). According to western blot results of this study, after 24 h incubation period the cleaved caspase-7 was clearly observed in 60 and 120 μg mL^{-1} sample treated groups indicating caspase pathway involved in the PARP cleavage. Also, in this study, caspase-8 of the U-937 was cleaved by the sulfated polysaccharide treated; the cleaved caspase-8 is able to activate downstream caspase like caspase-7. As has been observed previously, the caspase pathway associated with PARP was regulated by the highly sulfated polysaccharide of *Cladosiphon okamuranus* (Teruya et al. 2007). Moreover, in this study, Bax, a key component of apoptosis, was highly expressed after treatment of the sample, and Bcl-xL, which prevented apoptosis, was clearly down regulated. Taken together, the western blot result of this study clearly shows a signaling pathway of apoptosis stimulated by ECSP on U-937 cells. However, additional signaling pathways which are related to apoptosis need to be mentioned in order to have a clear picture of the sulfated

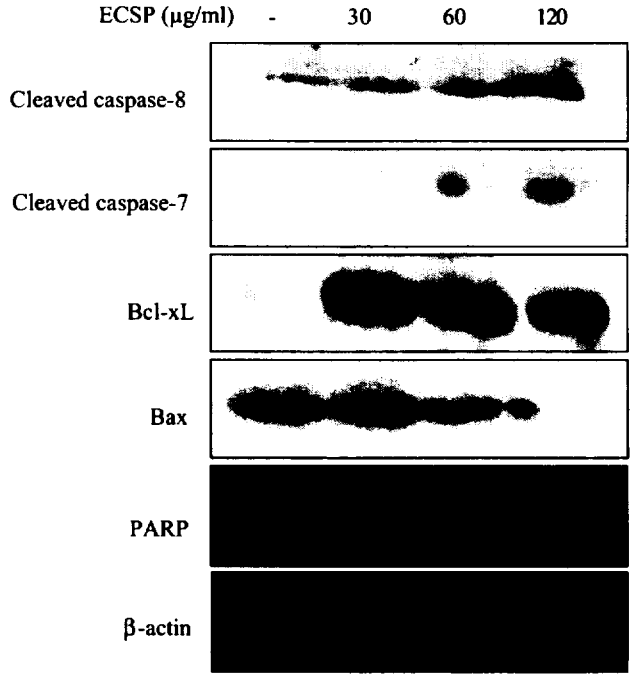

Fig. 6 The effect of the sulfated polysaccharide isolated from *E. cava* on the expressions of *caspase-7*, *caspase-8*, *Bax*, *Bcl-xL*, *PARP* and *β-actin* in U-937 cells treated with various sample concentrations (30, 60 and 120 $\mu g \cdot mL^{-1}$) after 24 h of incubation

polysaccharide isolated from *E. cava* for its anticancer activity.

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