# Algal sulfated carrageenan inhibits proliferation of MDA-MB-231 cells via apoptosis regulatory genes

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**Abstract.** Marine algae are prolific sources of sulfated polysaccharides, which may explain the low incidence of certain cancers in countries that traditionally consume marine food. Breast cancer is one of the most common types of non-skin cancer in females. In this study, extracted sulfated carrageenan (ESC), predominantly consisting of ι-carrageenan extracted from the red alga *Laurencia papillosa,* was characterized using Fourier transform infrared spectrometry. The biological effects of the identified extract were investigated and its potential cytotoxic activity was tested against the MDA-MB-231 cancer cell line. The biological biometer of the inhibitory concentration of the polysaccharide-treated MDA-MB-231 cells was determined as 50 $\mu$M. Treatment with 50 $\mu$M ESC inhibited cell proliferation and promptly induced cell death through nuclear condensation and DNA fragmentation. Characterization of polysaccharide-treated MDA-MB-231 cell death revealed that induction of apoptosis occurred via the activation of the extrinsic apoptotic caspase-8 gene. The apoptotic signaling pathway was regulated through caspase-3, caspase-9, p53, Bax and Bcl-2 genes. These findings suggest that ESC may serve as a potential therapeutic agent to target breast cancer via prompting apoptosis.

## Introduction

Naturally occurring sulfated polysaccharides (SPs) are commonly found in three major groups of marine algae (1). SPs are historically known to exhibit a number of therapeutic and biological effects, including antioxidant (2), anti-proliferative, anti-viral (3), anti-coagulant (4) and anti-tumor/cancer therapy (5-7) abilities. Previous studies have shown that numerous algal bioactive molecules, SPs included, may be implicated in the low incidences of cancer in countries that traditionally consume high levels of marine food (8,9).

In 2011, breast cancer was the most frequently diagnosed type of cancer and the leading cause of cancer mortality among females in the United States, accounting for 23% of the total number of cancer cases and 14% of cancer-related mortalities (10). Several epidemiological studies have provided evidence that marine algae consumption correlates with lower breast cancer rates in East Asia (11-16). Furthermore, intake of seaweed in the diet has been associated with a low risk of developing breast cancer (11). The potential anti-cancer effects of marine algae are partially attributed to polysaccharide compounds, particularly those which are sulfated, including carrageenans (17,18). Carrageenans are a family of linear SPs which are divided into three categories (κ, ι or λ) depending on their degree of sulfonation, solubility and gelling properties (19). A number of studies concerning novel anti-cancer drugs have determined that the modulation of signal-transduction pathways, inhibition of cell proliferation, induction of apoptosis, inhibition of tumor metastasis and inhibition of angiogenesis are all mechanisms which are involved in the control of carcinogenesis (17,18). Therefore, increasing the levels of apoptosis in cancer cells may be an effective method of chemopreventative and chemotherapeutic intervention in numerous types of cancer (17,18).

In the present study, the therapeutic effects of *Laurencia papillosa* against human breast cancer cells were investigated. The identified extracted sulfated carrageenan (ESC) was evaluated for its effects on the viability and proliferation of MDA-MB-231 human breast cancer cells.

## Materials and methods

*Plant material and extract preparation*. The red alga *L. papillosa* was collected from Syrian coastal waters and processed at the Marine Biology Laboratory of Tishreen University (Lattakia, Syria). The collected algal biomass was washed and air-dried at 60˚C to a constant weight, followed by heating in water (1.5% w/v) for 12 h with mechanical stirring. The carrageenan extract was dissolved in Milli-Q water (Millipore, Billerica, MA, USA), filtered and immediately mixed with three volumes of ethanol (95%) (Sigma-Aldrich, St. Louis,

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MO, USA) which caused precipitation of the carrageenan. The extract was collected and oven dried at 50-60˚C to a constant weight.

*Fourier transform infrared (FT-IR) analysis*. The IR spectra of the extracted polysaccharide were determined using a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA). The polysaccharide was ground with spectroscopic-grade potassium bromide (KBr) powder, dispersed in a KBr disk and pressed into 1-mm pellets for FT-IR measurement in the wave-number range of 600-4,000 $cm^{-1}$ using 16 scans.

*Cell culture*. MDA-MB-231 cells [provided by Professor P. Bécuwe from the Cancer Research Unit (EA SIGRETO), Nancy, France] were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 50 U/ml penicillin/streptomycin and 2 mM L-glutamine. The cells were cultured at 37˚C in 5% $CO_2$. All materials used in the cell culture were supplied by Gibco-BRL (Carlsbad, CA, USA).

*XTT assay*. Cells were seeded at a density of $2x10^3$ in a 96-well plate. Following a 24-h culture the cells were treated with ESC (1, 10, 50 or 100 $\mu$M) and incubated for 24, 48 or 72 h. Cell viability was measured using an XTT assay kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The number of living cells was quantified by measuring absorbance at a wavelength of 490 nm using a microplate reader (Multiskan EX Microplate Readers; Thermo Scientific) and the absorption of the controls was set to 100%. A graph of cell viability percentage against ESC concentration was produced from the mean absorbance values, by calculating the percentage growth of the ESC-treated cells compared with the growth of the untreated cells. Treatment with each ESC concentration was conducted in triplicate.

*Cell bioimaging*. Cells ($2x10^3$) were seeded into a slide chamber (Nalge Nunc International, Penfield, NY, USA) and cultured for 24 h, followed by treatment with ESCs at the desired concentration. Formaldehyde-fixed cells (4% formaldehyde) were inspected using a x10 objective lens of an Olympus inverted microscope (Olympus CK2; Olympus Corporation, Tokyo, Japan). Images were then captured using a microscope-branched Olympus DP70 camera (Olympus Corporation).

*DAPI staining*. Cells ($2x10^5$) were first cultured in a slide chamber. Following treatment with 50 $\mu$M ESC, the cells were fixed using 4% formaldehyde. The cells were washed in phosphate-buffered saline and incubated in 1 $\mu$g/ml DAPI (Sigma-Aldrich) (dissolved in methanol) for 5 min in the dark. Slides were mounted and observed using a Nikon ECLIPSE 80i fluorescence microscope (Nikon, Tokyo, Japan). Images were captured using a microscope-branched Nikon DS-Ri1 camera (Nikon).

*DNA fragmentation assay*. Cells were treated with 50 $\mu$M ESC for 12, 16 and 24 h prior to harvesting. Genomic DNA was extracted using previously described methods (20). The DNA samples were separated using a 1.5% agarose gel and DNA fragments were visualized by UV transillumination following ethidium bromdie staining. Fluorescence intensity was quantified using a Gel Doc 2000 system (Bio-Rad, Hercules, CA, USA) in order to determine the amount of DNA that was degraded upon treatment with 50 $\mu$M ESC.

*RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis*. Cells were seeded at a density of $1x10^6$ cells per 75 $cm^2$ flask and cultured for 24 h. The cells were treated with 50 $\mu$M ESC for 6, 18, 24 and 48 h prior to being harvested. Total RNA was extracted using a RNeasy kit (Qiagen, Valencia, CA, USA). The cDNA was directly prepared from total RNA using thr M-MLV RT First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA, USA) and an oligo (dT) 12-18 primer (Invitrogen Life Technologies) according to the manufacturer's instructions. Transcript levels of caspase-8, caspase-3, caspase-9, p53, Bcl-2, Bax and a GAPDH reference gene were determined using transcript-specific primers (16). qPCR was performed with a StepOne/Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions for relative expression ($\Delta\Delta$Ct method). Thus, the expression levels were expressed as relative fold differences compared with the expression levels of the reference gene.

*Statistical analysis*. The results were expressed as the mean value ± the standard error of the mean of individual experiments. Comparisons of the means were conducted using a one-way analysis of variance followed by Bonferroni's post hoc test (Prism software, version 6.0 for windows; GraphPad Software, La Jolla, CA, USA).

## Results

*FT-IR spectroscopic analysis of L. papillosa extract*. The simple yet efficient extraction method yielded considerable levels of SPs, which are originally found in appreciable levels in the red alga *L. papillosa*. Comparing the FT-IR spectrum of the ESC with the standard FT-IR spectra of the most common carrageenans (κ-, ι- and λ-carrageenans) revealed that the obtained ESC FT-IR spectrum is similar to the standard FT-IR spectrum of ι-Carrageenan (Fig. 1) (22,23). Notably, the spectrum obtained in the current study possessed two characteristic absorption bands at 847 and 802 $cm^{-1}$ which are associated with ι-carrageenan. The distinctive band at 802 $cm^{-1}$ is associated with the sulfate group linked to the anhydrogalactose ring, whereas the clear 847 $cm^{-1}$ band corresponds to the sulfated group bonded to the galactose ring. The broad band at ~1250 $cm^{-1}$ is readily assigned to the S=O stretching vibration of sulfate groups (24,25). Furthermore, the spectrum showed no marked peaks around 823 or 835 $cm^{-1}$ which are usually observed in the other SPs, confirming the lack of other sulfate ester substitutions.

*ESC inhibits the proliferation of MDA-MB-231 cells in a time- and concentration-dependent manner*. Cells treated with series of increasing concentrations of ESC showed a reduction in cell viability in a time- and concentration-dependent manner (Fig. 2). Therefore, to elucidate the pathway that cells

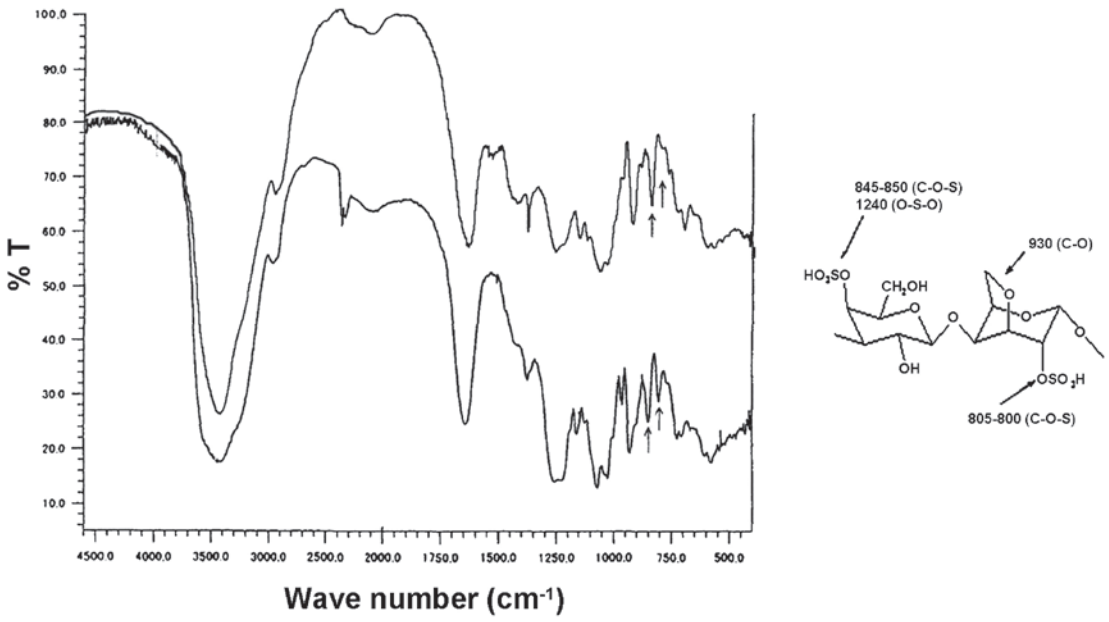


Figure 1. Molecular structure of ι-carrageenan and its characteristic infrared (IR) bands ($cm^{-1}$). The Fourier transform (FT)-IR spectrum of the extracted polysaccharide in a potassium bromide (KBr) disc (upper line) and the ι-Carrageenan reference-standard (lower line). The IR spectra were obtained using a Nicolet 6700 FT-IR spectrometer, with the samples dispersed in KBr disks. Arrows indicate the characteristic IR bands of iota-Carrageenan.

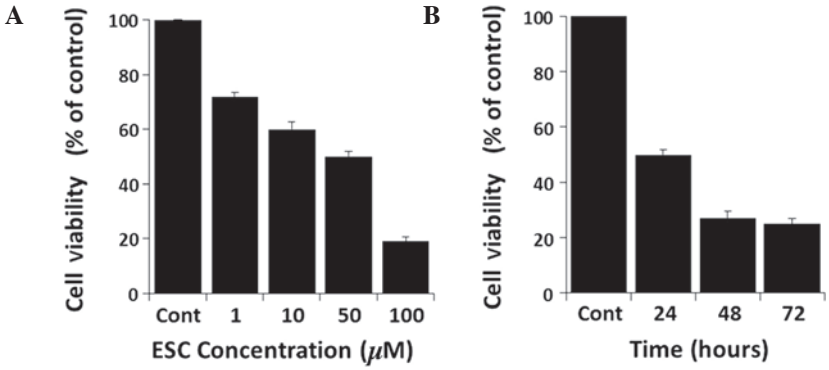


Figure 2. Extracted sulfated carrageenan (ESC)-induced cell injury and affected cell proliferation in a time- and concentration-dependent manner. (A) Cells were treated with five concentrations of ESC (1, 10, 50 and 100 $\mu$M) over 24 h. (B) Cells were treated with 50 $\mu$M ESC for 24, 48 and 72 h. Cell viability was monitored using XTT assay. The percentage of cell viability was expressed as a ratio from the total viable cells of control (Cont), with each data point representing the mean ± the standard error of the mean of three independent experiments.

were following when exposed to ESC, it was important to determine the half maximal inhibitory concentration ($IC_{50}$). ESC inhibited the proliferation of MDA-MB-231 cells in a concentration-dependent manner, with an $IC_{50}$ value of ~50 $\mu$M (Fig. 2A). A time-course study at a concentration of 50 $\mu$M revealed that ESC markedly reduced the cell viability in a time-dependent manner, as shown in Fig. 2B.

*ESC induces morphological changes in MDA-MB-231 cells.* To visually confirm the aforementioned biochemical results, bioimaging of ESC-treated cells was performed. Fig. 3A shows that cell proliferation was notably reduced by increasing concentrations of ESC. Control cells showed healthy growing patterns compared with those of the cells treated with 5 $\mu$M of ESC, which had a rounded, shrunken appearance reflecting the classical signs of programmed cell death. The signs of biological injury in the compound-treated cells were clearer at higher concentrations (20 and 50 $\mu$M). Higher concentrations of ESC led to a reduction in cell proliferation, evident by a greater number of shrunken rounded cells compared with those observed at lower concentrations. In addition, the morphological changes of ESC-treated cells were observed via DAPI staining. Apoptotic bodies, one of the morphological signs of apoptosis, were present in the 50 $\mu$M ESC-treated cells stained with DAPI (Fig. 3B). Furthermore, nuclear condensation, which leads to the breakdown of nuclear DNA strands into multiple oligonucleosomal-sized fragments, was observed. ESC-treated cells showed an increase in the levels of DNA fragmentation following 12, 16 and 24 h of ESC treatment, confirming activation of apoptosis (Fig. 4A).

*ESC alters apoptotic gene activity and caspase activation in MDA-MB-231 cells.* The relative expression levels of genes involved in apoptosis were analyzed, including those of caspase-8, caspase-3, caspase-9, p53, Bcl-2 and Bax.

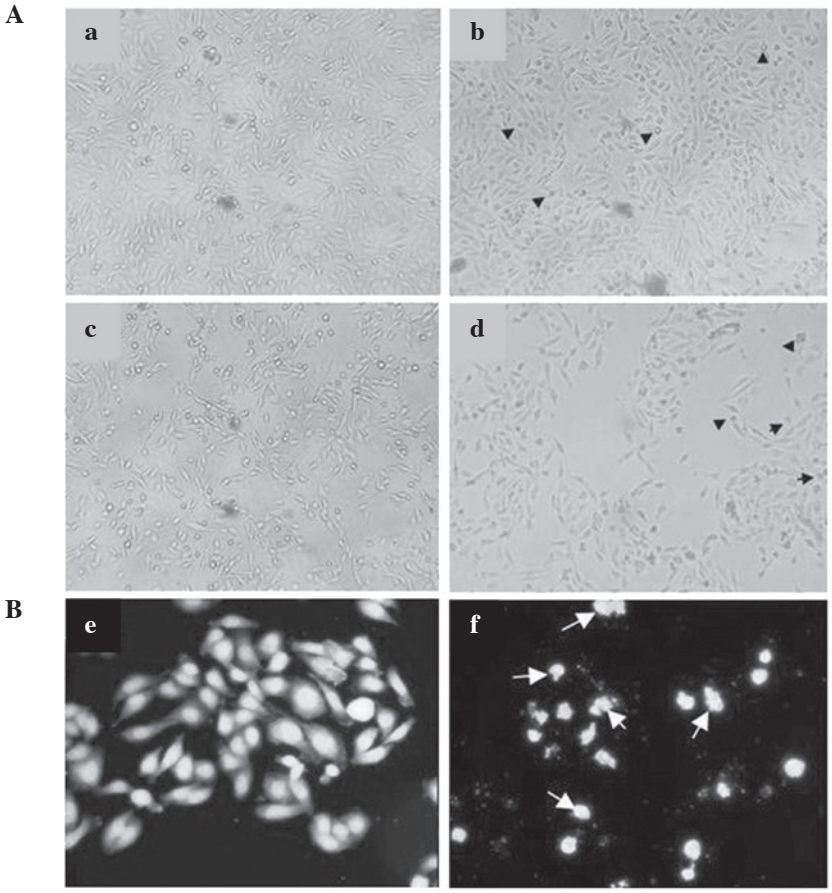


Figure 3. (A) Cell bioimaging investigation of ESC-treated cells. Cells were exposed to either (a) vehicle control DMSO or ESC (b) 5 (c) 10 or (d) 50 $\mu$M for 48 h. Following treatment, the formaldehyde-fixed cells were viewed under a microscope. Arrows show apoptotic cells as a result of ESC treatment. (B) The nuclear morphology and DNA condensation of the ESC-treated cells was evaluated using DAPI staining. Cells were exposed to either (e) vehicle control DMSO or (f) 50 $\mu$M ESC for 24 h. Cells were DAPI-stained and viewed under a fluorescence microscope. Arrows show nuclear condensation in apoptotic cells as a result of ESC treatment. (A-D, magnification, x100; E and F, magnification, x400).

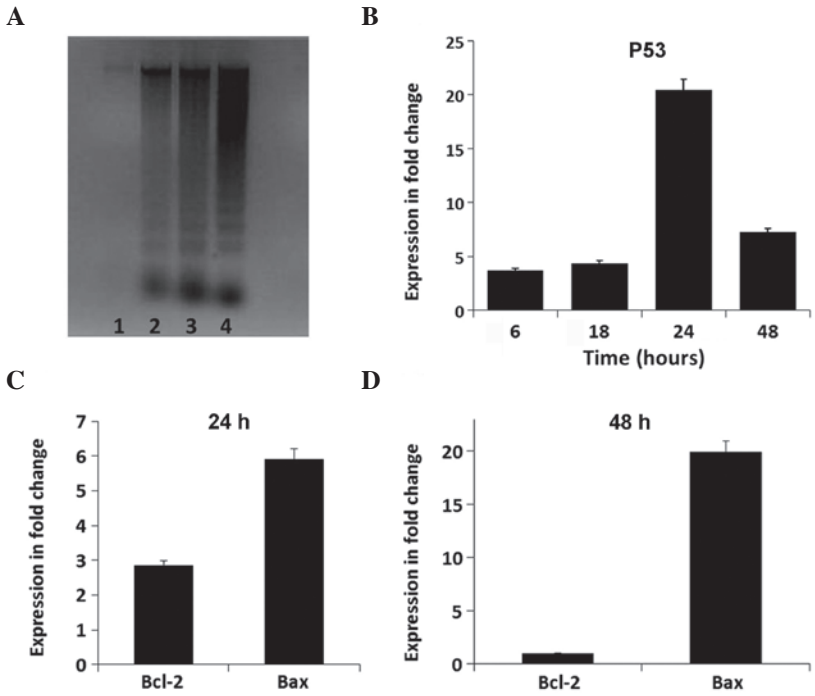


Figure 4. Induction of apoptosis via DNA fragmentation and regulation of p53, Bcl-2 and Bax. (A) DNA fragmentation assay: Cells were treated with 50 $\mu$M extracted sulfated carrageenan (ESC) (lanes 2, 3 and 4) for 12, 16 or 24 h, respectively. The non-treated control cells are in lane 1. (B) Comparison of p53 gene expression levels in cells as a relative fold change (ratio of target to reference gene) after 6, 18, 24 and 48 h of exposure to 50 $\mu$M of ESC. The ratio of Bax:Bcl-2 gene expression levels as a relative fold change in treated cells after (C) 24 and (D) 48 h of exposure to 50 $\mu$M ESC.

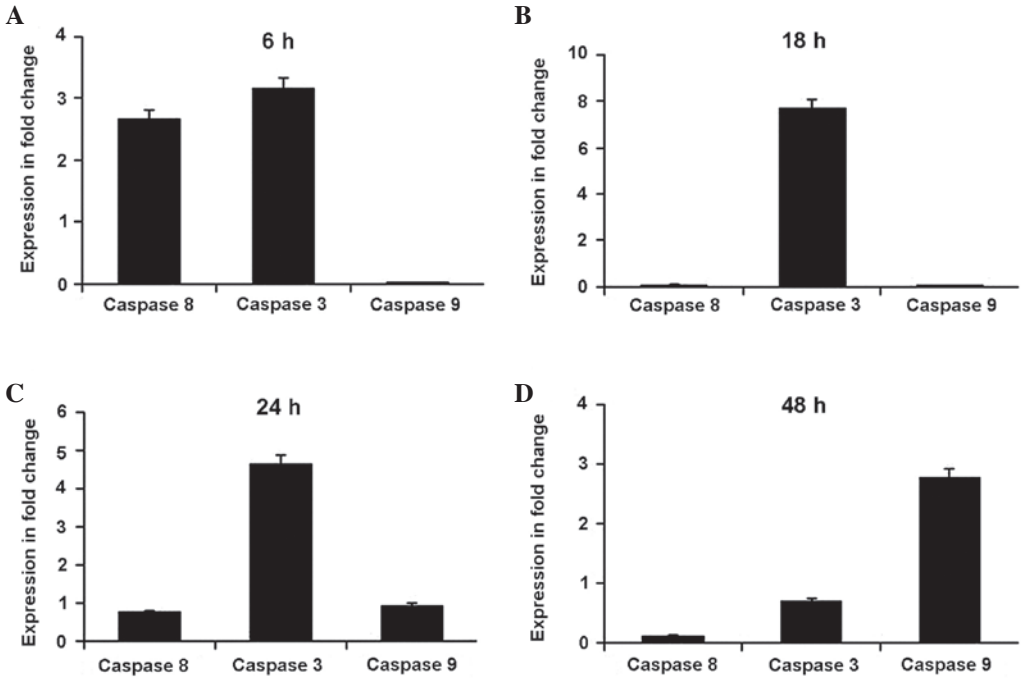


Figure 5. Expression of caspase genes after various periods of exposure to extracted sulfated carrageenan (ESC). Comparison of change in expression of caspase genes as a relative fold change (ratio of target to reference gene) in MDA-MB-231 cells after (A) 6, (B) 18, (C) 24 and (D) 48 h of exposure to 50 $\mu$M ESC.

Figs. 4 and 5 summarize the gene expression changes. In the initial 6 h, ESC increased the expression levels of caspase-8 and caspase-3 by several fold compared with those of the untreated cells (Fig. 5A). Subsequently, ESC maintained high levels (a several fold increase) of caspase-3 expression for 18 and 24 h as a result of the early expression of caspase-8, compared with those of the untreated cells (Fig. 5B and C). The expression levels of caspase-9 only began to increase relative to the control cells after 24 h, indicating the possibility of indirect activation by ESC. The expression levels were markedly increased by several fold after 48 h (Fig. 5D). In parallel, the expression levels of the oncogenic p53 gene were increased by 3.72-fold at 6 h and increased to 20.45-fold relative to the controls at 24 h (Fig. 4B). In the same time period, the apoptotic Bax:Bcl-2 expression ratio in 50 $\mu$M ESC-treated cells was increased in a time-dependent manner. Comparably, the expression levels of Bcl-2 were reduced from2.85-fold at 24 h to 0.97-fold at 48 h. Conversely, Bax expression levels were significantly increased in ESC-treated cells to5.8-fold at 24 h and to 19.95-fold at 48 h compared with those in the controls, indicating that the ESC-treatment induced apoptosis by increasing the Bax:Bcl-2 ratio (Fig. 4C and D).

## Discussion

Marine alga metabolites have been recognized as a source of diverse and novel pharmacological molecules and compounds. Carrageenans, complex SPs, are considered to be major constituent compounds in a large group of edible red algae (8,26). In the present study, FT-IR analysis revealed that the SPs extracted from *L. papillosa* have the characteristic spectra of ι-carrageenan carbohydrate. The IR spectroscopic analysis of an algal extract from *Eucheuma serra* highlighted similar unique characteristics of ι-carrageenan (23). In the last decade, several studies have indicated that a number of red algal SPs demonstrate anti-proliferative, pro-apoptotic, DNA-damaging, anti-angiogenic, growth-inhibiting, cell cycle-arresting and anti-metastatic functions (27,28). Therefore, algal polysaccharides have become compounds of great interest due to their anti-cancerous activity (6,7). The anti-cancer mechanisms of SPs have been hypothesized to involve inhibition of the proliferation of tumor cells via the induction of apoptosis, which has been demonstrated in a number of tumor models, including those of melanoma, nasopharyngeal and gastric carcinomas and breast cancer (11,29). The current study revealed that ESC exerts a cytotoxic inhibitory effect on MDA-MB-231 cells in a time- and concentration-dependent manner. Subsequently, the inhibition of the ESC-treated cells led to apoptosis. Similar studies have shown that the sulfated oligosaccharide PI-88 demonstrates effective anti-tumor activity in a pancreatic islet mouse melanoma via apoptosis (30). These results, along with the results of the present study, demonstrate that ESC has anti-proliferative properties that lead to apoptosis.

Induction of apoptosis via cytotoxic drug treatment has been shown to be a significant method of triggering cell death in a number of types of cancer (31). Therefore, an understanding of the events of apoptosis and its signaling pathway may allow for the development of novel agents for cancer treatment (32). Notably, the results of the current study demonstrated that ESC effectively induces the extrinsic pathway of apoptosis via regulation of the key molecule caspase-8 in MDA-MB-231 cells. In addition, ESC-treated cells exhibit features that characterize apoptosis induced by the main executors caspase-3 and caspase-9 (33), including nuclear condensation, DNA fragmentation and cell shrinkage (34-36). The Bax and Bcl-2 proteins are also known to regulate apoptosis promoted by different stimuli (37,38). p53 is a direct transcriptional activator of the Bax gene (39), which interacts with Bcl-2 to enhance outer

mitochondrial membrane permeabilization. The increased expression levels of p53 induce an increase in the Bax:Bcl-2 ratio, resulting in the release of cytochrome *c*, the activation of caspase and ultimately apoptosis (40,41). The biological activity of ESC algal extracts observed in the present study indicates a potential mechanism for the induction of cell apoptosis. These results concur with those of several previous studies on apoptosis induction via SP treatment (27,28).

In conclusion, the results of the current study demonstrate that ESCs from the red alga *L. papillosa* inhibit cell growth and induce apoptosis in MDA-MB-231 cells via the recruitment of caspase-3, caspase-8 and caspase-9, the re-modulation of the Bax:Bcl-2 ratio, and DNA damage. ESC may serve as a potential therapeutic agent and could be a promising target molecule in cancer prevention. Further studies are required to evaluate the potential anti-proliferative and anti-cancer activities of this extract *in vivo*.

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