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Molecular Mechanisms of Apoptosis Induced by *Scytosiphon gracilis* Kogame in HL-60 Cells

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Abstract: Many seaweed species are used as traditional medicine in different parts of the world. However, little data on the anti-cancer effect of seaweed has been published. In this study, we investigated the ability of ethyl acetate extracts of *Scytosiphon gracilis* Kogame (SGE) to induce apoptosis in cultured human promyelocytic leukemia HL-60 cells. Treatment of HL-60 cells with various concentrations of SGE extracts (12.5-100 μg mL^{-1}) exhibiting IC_{50} values of 15.68 μg mL^{-1} resulted in sequences of events marked by apoptosis, such as loss of cell viability, morphology change and internucleosomal DNA fragmentation. The SGE-induced apoptotic cell death was associated with caspase-3 and -9 activation and poly ADP-ribose polymerase degradation in HL-60 cells. This increase in SGE-induced apoptosis was also associated with a reduction in the levels of Bcl-xL, a potent cell-death inhibitor and an increase in levels of the Bax protein, which heterodimerizes with and thereby, inhibits Bcl-2. Present findings suggested that *S. gracilis* exerts antiproliferative action and growth inhibition on human promyelocytic leukemia HL-60 cells through a mitochondria-dependent apoptotic pathway. Therefore, *S. gracilis* may have anticancer properties valuable for application in food and drug products.

Key words: Apoptosis, Bax, Bcl-xL, caspase, poly ADP-ribose polymerase, *Scytosiphon gracilis*

INTRODUCTION

Despite modern advancements in diagnosis, prevention and therapy, cancer is still the single largest cause of death for humans worldwide. There are several natural resources, including seaweeds, which are considered to possess significant anticancer activity (Kong *et al*., 2009; Palozza *et al*., 2009; Ren *et al*., 2009). However, the offered proof is inadequate and their use as therapeutic agents cannot be accepted yet. There is a need for comprehensive, systematic and multidisciplinary evaluation of these natural resources before their introduction in modern clinical practice (Mishra *et al*., 2008). Another problem concerns resistance to anticancer drugs and side effects were discovered. Therefore, it is necessary for pharmaceutical and alternative medicinal industries to study and develop new and safe drugs (Yi *et al*., 2003; Kraft, 2009). At one time, the cytotoxic actions of chemotherapeutic drugs were attributed solely to their ability to induce genotoxic damage. However, there is recent substantial evidence that many cancer chemotherapeutic agents induce a cell death process known as programmed cell death or apoptosis. Although, the mechanisms by which chemotherapeutic agents can eliminate tumours via apoptotic pathways have been controversial, eliminating tumours by inducting apoptosis has now been recognized as a novel strategy in the development of anticancer drugs (Ward *et al*., 2008; Tan *et al*., 2009; Yang *et al*., 2009; Sun and Peng, 2009). These agents often induce tumour cells to undergo apoptosis with limited damage to surrounding normal cells. Therefore, it appears that exploiting the apoptotic potential of cancer cells might lead to new therapies that could be less toxic to normal cells because of their physiologically controlled survival pathway (Hsu *et al*., 2004; Padma *et al*., 2007). To study the ability of chemotherapeutic agents to induce apoptosis, it is essential to accurately define this mode of cell death (Oh *et al*., 2001). Generally, the following traditional criteria are required: (1) typical morphological changes, including chromatin condensation and nuclear fragmentation, must be observed under light microscopy and (2) a DNA ladder must be demonstrated by agarose gel electrophoresis. Several new potentially useful assays to detect apoptosis have recently been developed on the basis of biochemical events associated with apoptosis,

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such as DNA fragmentation by flow cytometry and caspase activation (Otsuki *et al*., 2003; Kurokawa and Kornbluth, 2009).

The brown alga *Scytosiphon gracilis* Kogame was initially described in Japan by Kogame (1998) and it was subsequently reported only in Korea (Aguilar-Rosas *et al*., 2006). Owing to these novelties, their pharmacological properties and bioactive constituents were left uncharacterised. In the present study, we investigated the cytotoxic and apoptogenic effects of ethyl acetate extract of *S. gracilis* (SGE) in HL-60 cell lines. To our knowledge, this is the first report on SGE-induced apoptosis in these cells.

MATERIALS AND METHODS

Preparation of *S. gracilis* extracts: *Scytosiphon gracilis* was collected in Jeju Island on May 2006 and was then identified by Dr. Wook Jae Lee. Previously, sample specimens were deposited at the herbarium of the Jeju Biodiversity Research Institute (JBRI), Jeju, Korea. The materials for extraction were cleaned, dried at room temperature for 2 weeks and then ground into a fine powder. The dried algae (100 g) were extracted with 80% ethanol (EtOH; 2 L) at room temperature for 24 h and then evaporated in a vacuum. The evaporated EtOH extract was then suspended in water (4 L) and partitioned with ethyl acetate (EtOAc; 4 L); this partitioning was repeated 3 times.

Cell culture: The HL-60 (human promyelocytic leukemia cell line), HT-29 (human colon carcinoma cell line) and A549 (human lung cancer cell line) cells were grown in RPMI-1640 medium and B16F10 (mouse melanoma cell line) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). Cultures were maintained at 37°C in a 5% CO_2 incubator.

MTT assay: Cytotoxicity of *S. gracilis* against tumour cells was determined by using a colorimetric MTT assay, as described previously (Kim *et al*., 2009). Suspension cells (HL-60 cells) were seeded (5×10^4 cells mL^{-1}) together with various concentrations of SGE and incubated up to 72 h before MTT treatment. Attached cells (HT-29, B10F16 and A549 cells) were seeded in a 96-well plate at a concentration of 2×10^4 cells mL^{-1}. Sixteen hours after seeding, the cells were treated with SGE and then incubated for an additional 72 h at 37°C. The MTT stock solution (50 μL; 2 mg mL^{-1} in PBS) was then added to each well for a total reaction volume of 250 μL. After incubating for 4 h, the plate was centrifuged at 2,000 rpm for 10 min and the supernatant was aspirated. The formazan crystals in each well were dissolved in dimethyl sulphoxide (DMSO). The amount of purple formazan was determined by measuring the absorbance at 540 nm.

Nuclear staining with Hoechst 33342: The nuclear morphology of cells was studied by using cell-permeable DNA dye Hoechst 33342. Cells with homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard *et al*., 1995). The HL-60 cells were placed in a 24-well plate at a concentration of 4×10^5 cells mL^{-1}. The cells were treated with various concentrations of the SGE and further incubated for 24 h. Next, Hoechst 33342, a DNA specific fluorescent dye, was added to the culture medium at a final concentration of 10 μg mL^{-1} and the plate was incubated for another 10 min at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera (Media Cybernetics, Silver Spring, MD) to examine the degree of nuclear condensation.

Determination of DNA fragmentation: The characteristic ladder pattern of DNA breakage was analysed by agarose gel electrophoresis. The HL-60 cells were placed in a 6-well plate at a concentration of 4×10^5 cells mL^{-1}. The cells were treated with various concentrations of SGE and were further incubated for 24 h. The DNA was isolated using a Promega Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and electrophoretically analysed on 1.2% agarose gel containing 0.1 μg mL^{-1} ethidium bromide.

Cell cycle analysis: Cell cycle analysis was performed to determine the proportion of apoptotic sub-G_1 hypodiploid cells (Nicoletti *et al*., 1991). The HL-60 cells were placed in a 6-well plate at a concentration of 4.0×10^5 cells mL^{-1}. The cells were treated with various concentrations of SGE. After 24 h, the cells were harvested at the indicated time and fixed in 1 mL of 70% ethanol for 30 min at 4°C. The cells were washed twice with SGE and incubated in the dark in 1 mL of PBS containing 100 μg PI and 100 μg RNase A for 30 min at 37°C. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Kim *et al*., 2009).

Western blot analysis: Western blot analysis was performed as described by Kim *et al*. (2009). Cells

(2×10^5 cells mL^{-1}) were treated with various concentrations of SGE for 24 h and were then harvested. The cell lysates were prepared with lysis buffer [50 mmol L^{-1} Tris-HCl (pH 7.4), 150 mmol L^{-1} NaCl, 1% Triton X-100, 0.1% Sodium Dodecyl Sulphate (SDS) and 1 mmol L^{-1} EDTA]. Cell lysates were then washed by centrifugation and protein concentrations were determined using a BCA™ protein assay kit. The lysates containing 30 μg of protein were subjected to electrophoresis on 10 or 12% sodium dodecyl sulfate-polyacrylamide gel and the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibody against Bax, Bcl-xL, cleaved caspase-3, -9, Poly ADP-Ribose Polymerase (PARP) and β-actin in TTBS (25 mmol L^{-1} Tris-HCl, 137 mmol L^{-1} NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% non-fat dry milk for 1 h. Membranes were washed with TTBS and incubated with secondary antibodies. Signals were developed using an ECL Western blotting detection kit and were exposed to x-ray films.

Analysis of total phlorotannin: To determine the total phlorotannin content in the seaweed extracts, the adjusted method with Folin-Ciocalteu reagent (Merck) was used (Ham *et al*., 2010). We added 550 μL of distilled water/Folin-Ciocalteu solution (10:1, v/v) to 50 μL of diluted extract (1 mg mL^{-1} of ethanol). After 3 min, 200 μL of 2 M sodium carbonate (Na_2CO_3) and 300 μL of distilled water were added. After 1 h standing at laboratory temperature, absorbance was measured at 725 nm. The total phlorotannin content was calculated as a phloroglucinol equivalent from the calibration curve of phloroglucinol standard solutions (concentration range, 0-1.0 mg mL^{-1}). All measurements were conducted in triplicate.

Statistical analysis: The data are expressed as Mean±SD from at least 3 independent experiments. Statistical analysis was performed using Student's t-test, with $p<0.05$ as a criterion of significance.

RESULTS

Cytotoxic activities of *S. gracilis* against cancer cell lines: In this study, we tested the cytotoxic effects of SGE on HL-60, HT-29, B16 and A549 cells. These cells were subjected to 72 h of exposure at either a 100 μg mL^{-1} concentration (Fig. 1a). As a control group, some cells were incubated with the same concentration of DMSO. The percentage inhibition of SGE (100 μg mL^{-1}), on HL-60, HT-29, B16 and A549 cell lines was 88.5, 70.2, 55.3 and 5.7%, respectively. Of the 4 cancer cell lines, SGE displayed the strongest cytotoxic effects on human HL-60. We compared the cytotoxic effects of seaweed extracts on cancer cells with the effects on normal cells (human keratinocytes, HaCaT). The viability of human keratinocytes (HaCaT) that were exposed to concentrations up to 50 μg mL^{-1} for periods of 72 h was barely affected. These results suggest that SGE has irreversible cytotoxic effects on HL-60 cells, but not on normal cells. To determine the concentration dependent effects of SGE on the HL-60 cells, an MTT conversion assay was performed. Cells were exposed to various concentrations of SGE (13, 25, 50 and 100 μg mL^{-1}) for 72 h. Cells treated with 0.1% DMSO were used as a control. As shown in Fig. 1b, SGE exerted a cytotoxic effect on HL-60 cells in a dose-dependent manner. The IC_{50} value of SGE, calculated from the graph, was 15.68 μg mL^{-1}.

Induction of apoptotic DNA fragmentation by *S. gracilis*: Agarose gel electrophoresis of chromosomal DNA-treated

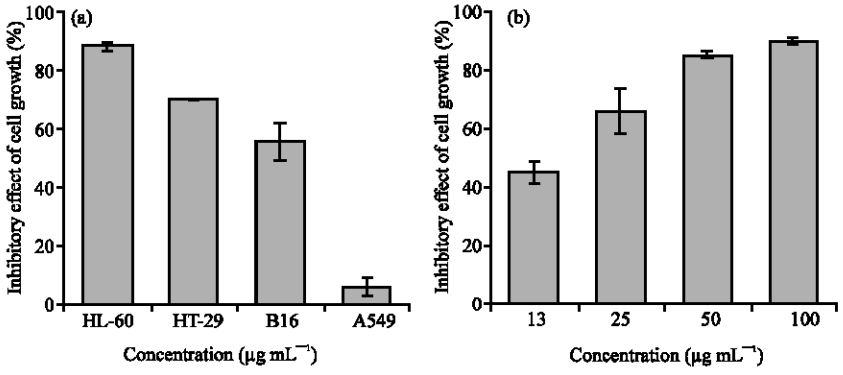

Fig. 1: Inhibitory effect of SGE against growth of tumour cells. (a) HL-60, HT-29, B16 and A549 cells were incubated with SGE extract (100 μg mL^{-1}) for 72 h and (b) HL-60 cells were incubated with various concentrations of SGE extract for 72 h and the cell viability was examined by an MTT assay

SGE (at 13, 25, 50 μg mL^{-1}) showed a ladder-like pattern of DNA fragments consisting of multiples of approximately 180-200 base pairs. The apoptosis-inducing activity of SGE was dose-dependent (Fig. 2).

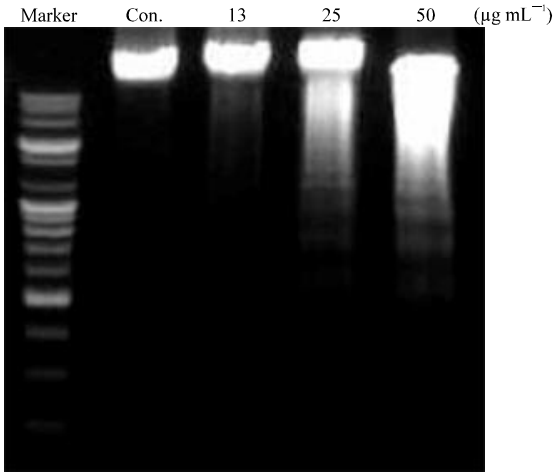

Fig. 2: Induction of apoptosis by SGE extract in HL-60 cells. HL-60 cells were incubated with various concentrations of SGE extract for 24 h. DNA was isolated and subjected to 1.2% agarose gel electrophoresis after staining with ethidium bromide

Observation of *S. gracilis*-induced morphological changes in HL-60 cells under a fluorescent microscope: To test whether, the decrease in cell viability observed after treatment with SGE was due to apoptosis, the HL-60 cells were stained with Hoechst 33342 dye. This dye stains the condensed chromatin of apoptotic cells more brightly than it stains the chromatin of normal cells. As shown in Fig. 3a-d, Hoechst staining, which correlates with the presence of cells with a typical apoptotic nuclear morphology (nuclear shrinkage, DNA condensation and fragmentation), was present in the SGE-treated cells, but not in the untreated controls.

Effects of *S. gracilis* on the population of hypodiploid cells among the HL-60 cells: In flow cytometry histograms, apoptotic cells produce DNA fluorescence in the subdiploid regions. Therefore, we used flow cytometric analysis to assess the progression of the cell cycle and to discriminate between cells that were experiencing apoptosis and those that were undergoing necrosis as a result of the lethal effects of SGE. In HL-60 cells that were treated with SGE, there was an appearance of a hypodiploid peak, which we attributed to the presence of apoptosing cells and/or apoptotic bodies. Quantitation of the apoptotic cells showed a dose-dependent response to exposure to SGE; this result is in accordance with the finding of morphological changes. To determine the

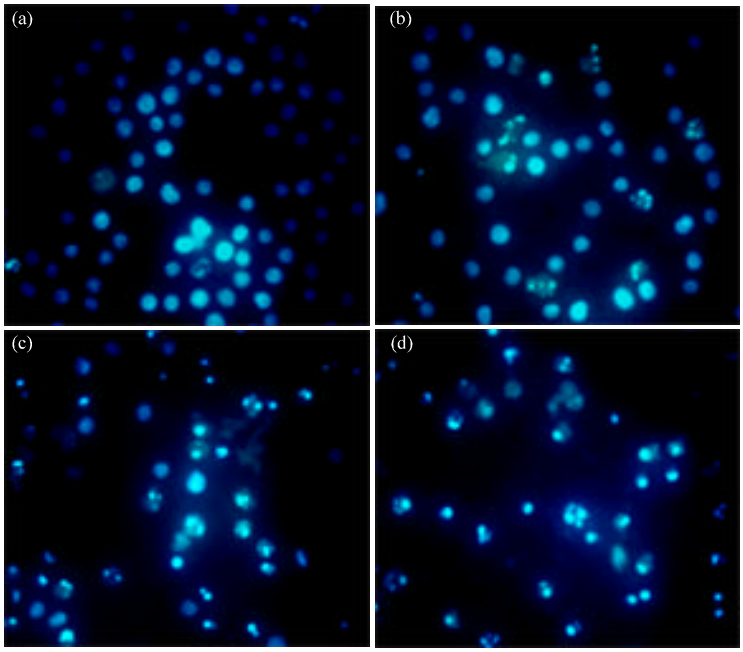

Fig. 3: Induction of apoptosis by SGE extract in HL-60 cells. HL-60 cells were incubated with various concentrations of SGE extract for 24 h. Cells were stained with Hoechst 33342 and the stained nuclei were observed under a fluorescent microscope. (a) Con., (b) 13 μg mL^{-1}, (c) 25 μg mL^{-1} and (d) 50 μg mL^{-1}

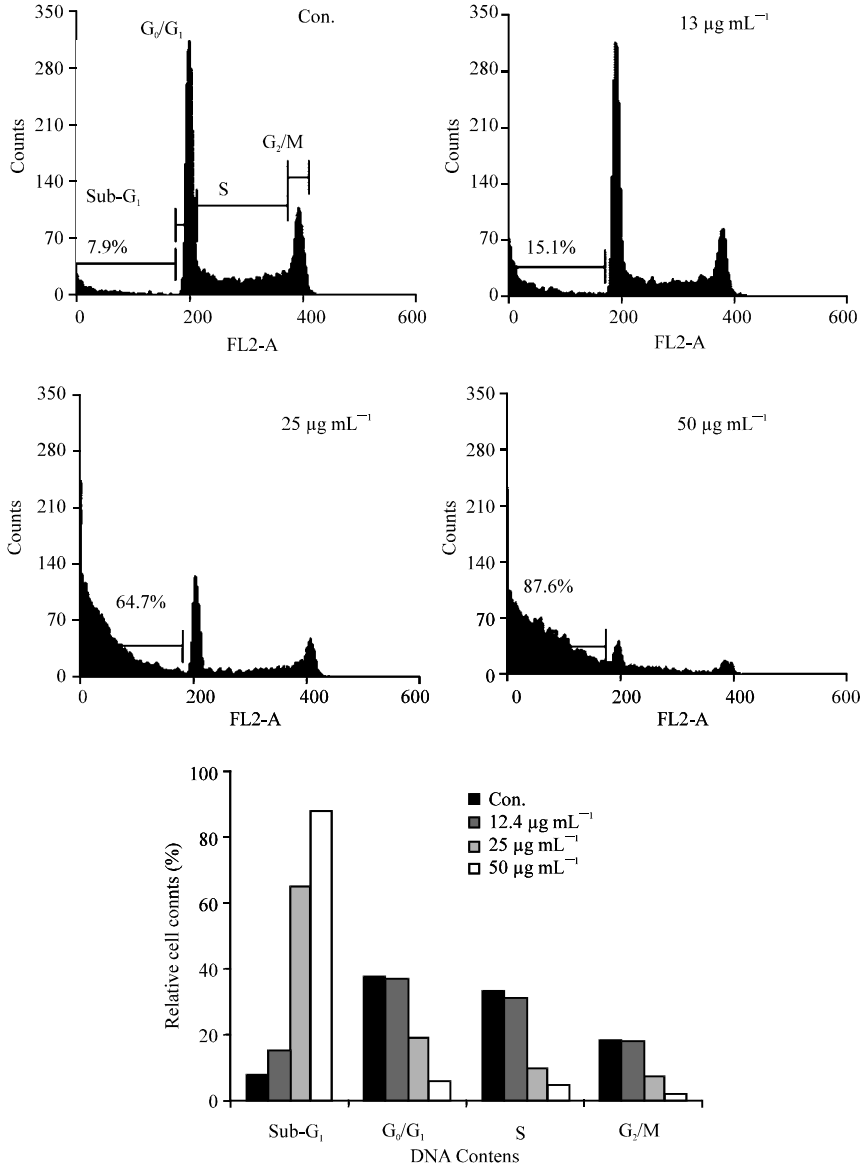

Fig. 4: Induction of apoptosis by SGE extract in HL-60 cells. HL-60 cells were incubated with various concentrations of SGE extract for 24 h. To quantify the degree of apoptosis induced by SGE extract, the cells were evaluated for sub-G1 DNA content represent the fractions undergoing apoptotic DNA degradation use a flow cytometer

percentage of cells in the sub-G_1, G_0/G_1, S and G_2/M phases for both the control cells and the cells treated with SGE, quantitative analyses of the histograms for 24 h are shown. These data clearly indicate that SGE causes an increase in the percentage of cells at the sub-G_1 phase and a significant decrease in the percentage of cells at the G_0/G_1 phases (Fig. 4). Present results suggested that cell cycle-induced apoptosis is a mechanism by which SGE exerts antiproliferative effects.

Effect of *S. gracilis* on caspase-3 and -9 activities and on poly ADP-ribose polymerase cleavage: Many reports indicate that treatment of cells with a variety of chemotherapeutic agents is accompanied by increased cytosolic translocation of cytochrome c, activation of caspase-3 and degradation of PARP. We investigated the role of caspase-3 and -9 in the cellular response to SGE. Immunoblotting analysis revealed that SGE induced proteolytic cleavage of pro-caspase-3 and pro-caspase-9

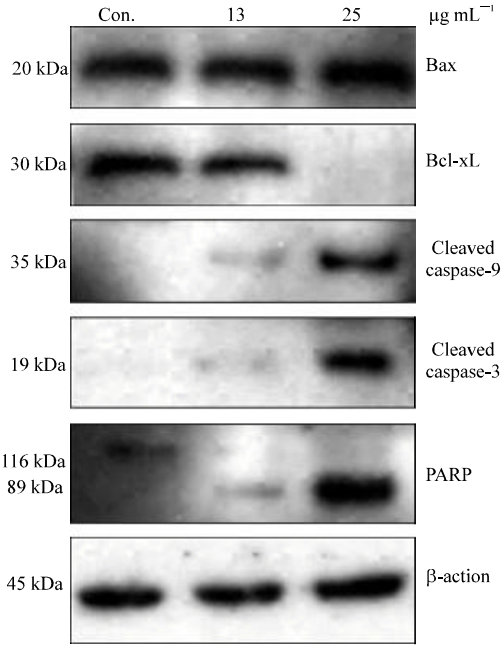

Fig. 5: Expression levels of apoptosis-related proteins by SGE extract treatment in HL-60 cells. HL-60 cells were incubated with various concentrations of SGE extract for 24 h. Cell lysates (30 μg) were immunoblotted with the indicated antibodies (Bax, BclxL, capsase-3, -9, PARP and β-actin) for Western blotting

in the active form in treated HL-60 cells. Since, PARP-specific proteolytic cleavage by caspase-3 is considered to be a biochemical characteristic of apoptosis, a Western blotting experiment was conducted using the antibody against PARP. The PARP is a nuclear enzyme involved in DNA repair and it has been demonstrated that the 116-kDa PARP protein is cleaved into an 89 kDa fragment. As shown in Fig. 5, PARP was cleaved into an 89 kDa fragment after the addition of SGE.

Effect of *S. gracilis* on Bcl-xL and Bax protein: Recently, it has been shown that the Bcl-2 family plays an important regulatory role in apoptosis, either as an activator (Bax) or as an inhibitor (Bcl-xL). Bcl-xL and Bax protein levels were studied in cultured HL-60 cells to examine the involvement of Bcl-xL and Bax in SGE-mediated apoptosis. Western blot analysis of Bcl-xL and Bax exposed to SGE was resolved on 10% SDS-polyacrylamide gel electrophoresis. Incubation of HL-60 cells with SGE reduced Bcl-xL, a potent cell-death inhibitor and increased Bax protein levels (Fig. 5). The increase in SGE-induced apoptosis was associated with the reduction in Bcl-xL and an increase in Bax. These results indicate that SGE induces dysregulation of Bcl-xL and Bax in HL-60 cells.

DISCUSSION

Apoptosis plays an essential role in controlling cell numbers in many developmental and physiological settings and in chemotherapy-induced tumour cell killing. The hindrance of this process leads to the development of many severe diseases and disorders, including tumours. Selective induction of the apoptosis process may become the fundamental strategy in developing anti-cancer drugs. Most available chemotherapy drugs break down tumour cells by inducing apoptosis (Bremer *et al*., 2006; Yang *et al*., 2009; Sun and Peng, 2009). In this study, we demonstrated that SGE markedly inhibits HL-60 cell growth and efficiently induces apoptosis. More specifically, our results shows that SGE inhibits the growth of human promyelocytic leukemia cells (Fig. 1). SGE treatment of HL-60 cells induced apoptotic characteristics, such as DNA fragmentation and chromatin condensation (Fig. 2). These changes result from the proteolytic cleavage of various intracellular polypeptides, which is most often caused by a family of cysteine-dependent proteases called caspases. As a major downstream effector of apoptosis, the caspase-3 mediated proteolytic cleavage of PARP and other substrates. This process is a critical step leading to DNA fragmentation and chromatin condensation. We observed these major apoptotic events in the SGE-treated HL-60 cells in the current study. Successive SGE treatment increased the proteolytic cleavage of PARP in HL-60 cells. Moreover, SGE can modulate the process of apoptosis in tumour cells through caspase activation, a decrease of Bcl-xL expression and an increase in Bax expression.

The Bcl-2 protein family is a critical regulator of apoptotic pathways. Many types of cancer, including leukemia, are associated with the overproduction of Bcl-2 protein. The antiapoptotic Bcl-2 and Bcl-xL proteins reside on the outer membranes of mitochondria and can inhibit apoptosis in the presence of various apoptotic stimuli and promote cell survival (Kang and Reynolds, 2009). Bax is a proapoptotic member of the Bcl-2 family that resides in the cytosol and translocates to the mitochondria upon the induction of apoptosis. It was reported that changes in the ratio of proapoptotic and antiapoptotic members of the Bcl-2 family, rather then the absolute expression level of any single Bcl-2 member protein, could determine apoptotic sensitivity (Damial, 2008; Ghiotto *et al*., 2010). In the present study, the data showed that the decreased level of antiapoptotic Bcl-2 protein and increased level of proapoptotic Bax protein may play a key role in SGE-induced apoptosis of HL-60 cells. In conclusion, this study clearly demonstrates that SGE strongly inhibits cell proliferation and induces apoptosis in HL-60 cells. The SGE induced apoptosis through the activation of

caspases-9 and -3, degradation of PARP and up-regulation of the proapoptotic Bax/Bcl-2 ratio. Because apoptosis was regarded as a new target in the discovery of anticancer drugs, these results confirm the potential of *S. gracilis* as an agent of chemotherapeutic and cytostatic activity in human promyelocytic leukemia cells. Many of the anti-cancer drugs that induce apoptosis are oxidants or stimulators of cellular oxidative metabolism, while many inhibitors of apoptosis show antioxidant activity. Indeed, Reactive Oxygen Species (ROS) can play a pivotal role in apoptosis by regulating the activity of certain enzymes involved in the cell-death pathway (Yang *et al*., 2008). However, SGE did not induce the ROS generation.

Taken together, we suggested that SGE may possess anticancer properties and, therefore, may be potentially valuable for application in food and drug products. Phlorotannin are aromatic secondary plant metabolites, widespread in the seaweeds that are associated with the anti-inflammatory and anticancer properties of various foods. Therefore, the total phlorotannin content of SGE was assessed in this study. Though, the SGE showed high phlorotannin content (61.5 μg mg^{-1}), further investigation of its activity including *in vivo* and purification of bioactive compounds is now in progress in order to elaborate this nascent possibility

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