Sensitization of Human Carcinoma of Nasopharynx Cells to Doxorubicin and Induction of Apoptosis by *Sargassum baccularia* Lipophilic Fraction

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Abstract

The pharmaceutical properties of marine bioactive compounds derived from variants of the *Sargassum* species have long been recognized as important features for medicinal use. However, the molecular mechanisms of their anticancer activities are unclear. This study aimed to investigate the apoptosis-modulating activities of *Sargassum baccularia* lipophilic fraction (SBL fraction) in human carcinoma of nasopharynx cells (KB cells). *Sargassum baccularia* was extracted with 95 % ethanol and then fractionated through solvent-solvent partitioning with chloroform: methanol: water. It was found that SBL fraction and doxorubicin (Dox) alone inhibited proliferation in KB cells with IC_{50} of 85.04 ± 5.28 and 2.0 ± 0.1 µg/ml, respectively. The combined SBL fraction (10 - 50 µg/ml) and Dox (1 µg/ml) treatment produced greater cytotoxicity associated with the increasing of chromatin condensation, DNA fragmentation, and hypo-diploid cells (DNA < 2n), compared with each SBL fraction and Dox alone. Apoptosis induced by SBL fraction was associated with caspase-3 activation that was attenuated in a caspase-3 inhibitor. Thus, SBL fraction acts as potent sensitizer for doxorubicin-induced apoptosis, and it can be potentially developed into a promising adjuvant therapy for nasopharynx carcinoma.

Keywords: *Sargassum baccularia*, lipophilic fraction, apoptosis, carcinoma of nasopharynx cells

Introduction

Brown seaweeds, a rich source of bioactive natural compounds, are widely distributed in warm and temperate waters, especially in those of the east coast of the Gulf of Thailand [1]. *Sargassum* (Phaeophyceae) is the largest brown seaweed, and has been consumed not only as food but also as alternative medicine since ancient times in many countries. The medicinal uses of *Sargassum* in Traditional Chinese Medicine include treatment of thyroid related disease, arteriosclerosis, high blood pressure, angina pectoris, and acute esophagitis. A wide range of pharmacological properties of *Sargassum* extracted or isolated pure compounds have been studied, *in vivo* and *in vitro* using scientific-based methodologies, which suggested that *Sargassum* has anti-inflammatory, anti-cancer, anti-microbial, anti-viral, liver protective, and antioxidant activity. *Sargassum* is therefore has valuable health benefits due to its vast biodiversity and safety [2].

In recent years, many biomolecules from lipophilic extracts of *Sargassum*, such as meroterpenoids (vitamin E), phytosterols, polyphenols, polyunsaturated fatty acids, and fucoxanthin (carotenoid) have been identified [3-5]. Meroditerpenoids from the southern Australian marine brown alga *Sargassum fallax* show anticancer activity against the P388 murine leukaemia cell line, with a concentration of these

compounds required to reduce the cell viability to 50 % (IC_{50}) at a value of 17 µM [6]. The meroditerpenoids (chromene) isolated from a chloroform fraction of *Sargassum siliquastrum* showed significant cytotoxicity against human leukemia HL-60 cells by mechanisms involving the induction of apoptosis [3]. The methanolic extraction of *Sargassum thunbergii* contains polyunsaturated fatty acids, such as arachidonic acid, arachidic acid, palmitic acid, elaidic acid, linoleic acid, stearic acid, and cis-5, 8, 11, 14, 17-eicosanoic acid, that have potent antioxidant activities related to oxidative stress [4]. The sargaquinoic and sargachromenol have been identified in *Sargassum micracanthum* showed *in vitro* anti-oxidant activities; therefore, they could be useful in pharmaceutical industries [7].

Fucoxanthin is a carotenoid in the chloroplasts of *Sargassum*, and has become of great interest for its unique structure, called allenic carotenoid fucoxanthin. Recent pharmacological research indicates that fucoxanthin shows anti-oxidant, anti-inflammatory, anti-cancer, anti-obese, anti-diabetic, anti-angiogenic, anti-malarial activities, as well as a protective effect on the liver [5]. Based on *in vitro* researches, fucoxanthin induces apoptosis and enhances anti-proliferative effect on human leukemia HL-60 cells [8], adult T-cell leukemia (ATL) [9], human colon cancer cells [10,11], human prostate cancer cells [12,13], human gastric adenocarcinoma MGC-803 cells [14] and osteoblast-like cell line MC3T3-E1 [15]. In animal experiments, Japanese kelp (Kombu) extract significantly inhibited the formation of aberrant crypt foci, a pre-neoplastic marker for colon cancer, induced by azoxymethane challenged mouse colon [16]. The apoptosis-inducing effect of fucoxanthin has been demonstrated to be mediated through the ROS-mediated bcl-xl pathway [8], caspase-3 activation and DNA fragmentation [13], cell cycle arrest at the G0/G1 phase [17-19] and the JAK/STAT signal pathway [14]. Thus, fucoxanthin is supposed to have multiple mechanisms underlying its potential anti-cancer activity.

Cancer is a particularly complex disease, and much research has demonstrated the link between cyclooxgenase-2 overexpression and cancer cell proliferation. Cyclooxgenase-2 is inflammatory signaling and the cyclooxgenase-2 inhibitor has established potent anti-cancer activity [20]. The dichloromethane and ethanol extraction of the *Sargassum fulvellum* and *Sargassum thunbergii* have shown potent anti-inflammatory and anti-edema activities, without any acute toxicity [21]. Thus, the anti-inflammatory molecules from *Sargassum* that can be used to prevent cancer are attractive. A large number of studies suggest that *Sargassum* has a multitude of potential applications in human health benefits. Although approximately 80 of the 400 known *Sargassum* species have been analyzed [2], the bioactive compounds of *Sargassum baccularia* (*S. baccularia*), collected from the east coast of the Gulf of Thailand, have not been studied. The search for bioactive natural compounds sensitizing cancer cells to apoptosis induction by chemotherapeutic agents is a novel strategy of anticancer therapy. The objectives of this study were to evaluate the apoptogenic and apoptosis-sensitizing activities of the lipophilic fraction of *S. baccularia* against human carcinoma of nasopharynx cells, (KB cells).

Materials and methods

Total lipid extraction
Fresh *S. baccularia* was collected from the east coast of the Gulf of Thailand, Trat province, Thailand, in December, 2010. The seaweed sample was identified by Assoc. Prof. Anong Chirapart, Department of Fishery Biology, Faculty of Fisheries, Kasetsart University. The voucher specimen (ABRC0036) was deposited in the algal Bioresources Research Center of the same university. It was washed with tap water to remove salt, small shells, and sand on the surface, and then stored at −20 °C until use. Moisture content of the algal samples was determined by an oven-drying method as per AOAC (1995) and used for expressing fucoxanthin, tocopherol and polyphenol content on a dry weight (dwt) basis. The frozen *S. baccularia* was thawed and extracted at room temperature (25 °C) twice, with 95 % ethanol at a ratio of 1: 10 (w/v) for 24 h under dark conditions. The supernatants were filtered and concentrated using a rotary evaporator to obtain the crude extract, which was fractionated through solvent-solvent partitioning with chloroform: methanol: water at a ratio of 10: 5: 3 (v/v/v). The lower layer was collected and the solvent removed under vacuum at 30 °C using a rotary flash evaporator (Eyela N1000; Tokyo Rikakikai Ltd., Tokyo, Japan) to obtain *S. baccularia* lipophilic fraction (SBL

fraction), and then stored at −20 °C until use. For preparation of stock solution, the powder samples were solubilized in 70 % EtOH at 25 °C before analysis of apoptotic properties.

Fucoxanthin analysis

All the HPLC analyses were carried out using a Hitachi L-7000 HPLC system (Hitachi, Tokyo, Japan) equipped with a pump (L-7100), an auto sampler (L-7200), a photodiode array (PDA) spectrophotometric detector (Hitachi L-7455), and online analysis software (Hitachi HPLC system-5-manager; Model D-7000). Fucoxanthin content in SBL fraction was determined by reversed-phase HPLC (RP-HPLC) with methanol-acetonitrile (7: 3 v∕v) as the mobile phase at a flow rate of 1.0 mL min^{-1}. All RP-HPLC analyses were carried out at 28 °C using an RP column (Develosil-ODS, UG-5, particle size, 250 × 4.6 mm i.d.; Nomura Chem. Co., Seto, Aichi, Japan) protected with a guard column (10 × 4.0 mm i.d.) having the same stationary phase. Fucoxanthin was detected at 450 nm and its content was estimated by the standard calibration curve using purified fucoxanthin (purity > 98 %). Purified fucaxanthin was isolated from the brown seaweed, Wakame (*Undaria pinnatifida*), as described previously [22].

Polyphenol analysis

Total polyphenols in the SBL fraction was measured by the Folin-Ciocalteu method; 0.1 ml of the sample solution in methanol was mixed with 0.75 ml of 10 % Folin-Ciocalteu reagent and incubated at 37 °C. After 5 min, 0.75 ml of 6 % sodium carbonate solution was added and allowed to stand for 30 min at 37 °C in the dark; the color developed was measured at 750 nm. The total phenolic of algal extract was then calculated on the basis of a phloroglucinol standard curve and expressed as mg of the phloroglucinol equivalent (PGE)/g extract [23].

Tocopherol analysis

HPLC was also used for the analysis of α-, γ-, and δ-tocopherol. The column used was Develosil 30-3 silica column (250 × 4.6 mm i.d.). The mobile phase was hexane-2-propanol (99.2: 0.8, v/v). The flow rate was 0.1 mL/min. The fluorescence detector was set at Ex. 298 nm and Em. 325 nm. The quantitative analysis was done with the calibration curve made using standard *dl*-α-tocopherol. *dl*-γ-tocopherol, and *dl*-δ-tocopherol. All tocopherol was obtained from Kanto Chemical Co. Inc., Tokyo, Japan.

Fatty acid composition analysis

Fatty acid (FA) analysis was accomplished by injecting FA methyl esters (FAMEs) into a gas chromatography (GC) system (Shimadzu GC-14B; Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame-ionization detector (FID) and a capillary column (Omegawax-320; 30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA, USA). The carrier gas was helium, at a flow rate of 50 kPa. The detector, injector, and column temperatures were 260, 250, and 200 °C, respectively. FAMEs from algal extract were prepared to an aliquot of extract (∼ 9 - 12 mg). Briefly, 1 mL n-hexane and 0.2 mL 2N NaOH in methanol were added, gently vortexed, and incubated at 50 °C for 30 min. Post-incubation, 0.2 mL 2N HCl in methanol solution was added, and gently mixed to recover the upper n-hexane layer containing FAMEs. The FA content was expressed as a weight percentage of total FAs in the algal samples [22].

Cell viability assay

The KB cells were obtained from the National Cancer Institute, (Bangkok, Thailand). The percentage of living cells was determined by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromides (MTT) assay (Sigma Chemical Co., USA). This assay is based on the conversion of tetrazolium salt into a blue formazan product by mitochondrial dehydrogenase of viable cells. KB cells were seeded at a density of 5×10^4 cells/well in a 96-well for 24 h to allow surface attachment of the cells. After 24 h of seeding, KB cells were treated with EtOH (0.5 %), SBL fraction (0 - 250 μg/ml), Doxorubicin (Pfizer, Australia) (Dox) (0 - 10 μg/ml) and SBL fraction + Dox for 48 h. The MTT solution (20 μl of 5 g/L) was added to each well for 4 h at 37 °C and 5 % CO_2. The MTT fluid was

removed, and 100 μl dimethyl sulfoxide (DMSO) was added per well. The blue formazan product was then measured at 570 nm with a microplate reader (Cecil Bioquest 2000 Series). The 50 % inhibition of cell proliferation was determined from the dose-response curves [24,25]. Experiments were performed in triplicate. The results were expressed as a percentage of control, and the relative cell viability (%) was calculated by using the following equation;

$$\text{Percentage of cell viability} = \frac{\text{Absorbance at 570 nm of treated cells}}{\text{Absorbance at 570 nm of control cells}} \times 100$$

Toxicity assay with *Artemia salina*

The SBL fraction was tested for toxicity with one-day old brine shrimp, *Artemia salina*, at 25 °C for 72 h in a 24-well plate. Sterilized sea water was used in preparing each SBL concentration. Four fold dilutions (400, 100, 25, 6.25, 1.56, and 0.39 μg/ml) were made to each well in triplicate. The control experiment had no SBL fraction. Then, 10 brine shrimps were added to each well by micropipette. The dead brine shrimp larvae were counted at 12 h interval up to 72 h, and the data for the tree parallels were averaged.

Nuclear staining with DAPI

The KB cells at a density of 5×10^4 cells/well were grown on coverslips attached with 6-well plate. After treatments, the cells were washed with phosphate-buffered saline (PBS), incubated with RNase A (10 μg/ml), and fixed with 2.5 % glutaraldehyde for 10 min at room temperature. The fixed cells were washed twice with PBS and stained with 4, 6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA) 5 μg/ml for 10 min in dark conditions. After removing unbound dye, cells were mounted on a glass slide with mounting solution (PBS: glycerol), and the edges were sealed with nail polish. The nuclear morphology of the cells was then visualized through a fluorescence microscope (Olympus BX51) at 100 × magnification. Typical apoptotic nuclei could be identified by the condensation of chromatin and segmentation of the nucleus. Approximately 500 different nuclei were counted in random microscopic fields [24,25]. Data were expressed as a percentage of apoptotic nuclei. At least three separate experiments were executed.

Agarose gel electrophoresis for DNA fragmentation assay

The GF-1 Tissue DNA Extraction Kit (Vivantis, Poland) was used according to the manufacturer's instructions. After treatments, the cells were washed with PBS and then lysed with digestion buffer containing proteinase K (400 μg/ml) for 10 min at 60 °C, and subsequently with RNase A (10 μg/ml) for 10 min at 37 °C. Genomic DNA was extracted with ice-cold absolute ethanol. The concentration of DNA and RNA should be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. High-purity genomic DNA is eluted in low salt buffers and has an absorbance 260/280 ratio between 1.7 and 1.9, making it ready to use. Equal amounts of DNA samples were mixed with the loading dye containing SYBER Gold (Invitrogen, USA), and then loaded onto pre-solidified 1 % agarose. The fragmentation of DNA was electrophoresed at 125 V for 30 min in TBE buffer [24,25]. Gels were photographed under a transilluminator (Clare Chemical Research).

Propidium iodide staining for measurement of sub-G1 phase

After treatments, cells were harvested and washed once with cold PBS, fixed in 4 % paraformaldehyde (cold) for 15 min, and stored at 4 °C. Prior to analysis, the cells were washed twice again with PBS, suspended in 1 ml of a cold propidium iodide (PI) solution containing RNase A (10 μg/ml), PI (50 μg/ml), EDTA disodium (0.1 mM), Triton X-100 (0.1 %) (v/v), and further incubated on ice in the dark until analysis. Apoptotic cells with hypo-diploid DNA content were detected using a flow cytometer (FACS Calibur, Becton Dickinson, USA). Data were analyzed by using CellQuest software (BD Biosciences, USA) which was used to determine the cellular DNA content based on the presence of PI-labeled cells [14].

Caspase-3 activity assay

After treatments, caspase-3 activity in cell lysates was measured using a colorimetric assay kit (Clontech, Texas, USA) based on the manufacturer's protocol. Briefly, cell lysates from 4×10^6 cells in each group were prepared using the lysis buffer (50 µl) and then incubated on ice for 30 min. After centrifugation at 14,000 g, 4 °C for 5 min, the supernatant (50 µl) was collected and mixed with 2× reaction buffer (50 µl) and caspase-3 substrate (DEVD-pNA) (5 µl). The samples were incubated at 37 °C in the dark for 4 h and the results were read on a spectrophotometer at a wavelength of 405 nm, which represented the intra-cellular activity of caspase-3 [26]. Data were expressed as a fold increase on the control level.

Data processing and statistical analysis

Data were expressed as a mean ± standard error of the mean (SEM) from 3 or 4 independent experiments and analyzed with the software Microcal TM Origin 6. Statistical comparisons were performed using Student's t-test. A *P*-value < 0.05 was considered to be statistically significant.

Results and discussion

Chemical composition

Marine brown seaweeds are rich in pharmacological properties, and *Sargassum* spp. extracts, or isolated pure components, are promising sources of novel chemotherapeutics [2,5]. In recent studies, many biomolecules from lipophilic extracts such as meroterpenoids (vitamin E), phytosterols, polyphenols, polyunsaturated fatty acids and fucoxanthin (carotenoid) have been identified from *Sargassum spp*. [3-5]. In this study, the yield of SBL fraction was 4.71 ± 0.10 % dry weight (% dwt) and the chemical composition of the fractions are illustrated in **Table 1**. There were also essential fatty acids, such as linoleic acid or LA (C18:2ω6, 5.94 % weight (wt)), γ-linolenic acid or GLA (C18:3ω6, 0.59 % wt), alfa-linolenic acid or ALA (C18:3ω3, 6.18 % wt), dihomo-γ-linolenic acid or DGLA (C20:3ω6, 1.15 % wt), arachidonic acid or AA (C20:4ω6, 10.46 % wt) and eicosapentaenoic acid or EPA (C20:5ω3, 3.56 % wt).

Table 1 Yield and chemical composition of *Sargassum baccularia* extract.

Yield and chemical composition	Content
Yield of SBL extract (% dwt)	4.71 ± 0.10
Fucoxanthin (mg/g extract)	43.90 ± 3.90
Total phenolic (mg PGE/g extract)	87.23 ± 7.56
α-tocopherol (mg/g extract)	1.70 ± 0.46
γ-tocopherol (µg/g extract)	241.24 ± 7.02
δ-tocopherol (µg/g extract)	50.71 ± 5.15

Inhibition of cell viability

After 48 h treatment, the SBL fraction and Dox treatment inhibited KB cells in a dose-dependent manner with IC_{50} values of 85.04 ± 5.28 and 2.0 ± 0.1 µg/ml, respectively (**Figure 1a**). However, the combination of SBL fraction (10, 25, 50 µg/ml) and Dox (1 µg/ml) significantly inhibited the cell viability as compared to treating cells with each agent alone ($p < 0.05$). The SBL fraction acts as a sensitizer for a Dox-induced cytotoxic effect. The SBL fraction (50 µg/ml) plus doxorubicin (1 µg/ml) combination showed the best sensitization effect, causing an approximately 94 % decrease in the number of cell viability (**Figure 1b**). The morphological changes after treatment included cell volume shrinkage,

cytoplasm condensation, and membrane blebbing, compared with the controls, which showed cuboid and polygonal shapes.

This is the first study to report on the anti-proliferative activities of lipophilic extracts from *S. baccularia* using human carcinoma of nasopharynx cells as a model. Recently, we reported that the crude extract of 4 out of 15 brown seaweeds along the east coast of the Gulf of Thailand (*Sargassum oligocystum*, *Sargassum swartzii*, *Sargassum binderi* and *Turbinaria conoides*) showed dose-dependent cytotoxic activity (IC_{50} < 50 μg/ml). *Turbinaria conoides* was most toxic on cervical, oropharyngeal, breast and glioma cancer cell lines, with an IC_{50} of 19, 18, 22 and 25 μg/ml, respectively [27]. Interestingly, the toxicity assay on brine shrimp, *Artemia salina*, revealed that the SBL fraction was not toxic to this animal. None of the brine *shrimp nauplii* died in each well throughout the experiment (data not shown). Consistent with the result of the toxicity assay, *Sargassum* should be regarded as non-toxic. After intra-peritoneal injection of 500 mg/kg of body weight, the hexane, methanol and butanol extracts of *Sargassum wightii* showed no acute toxicity in mice [28].
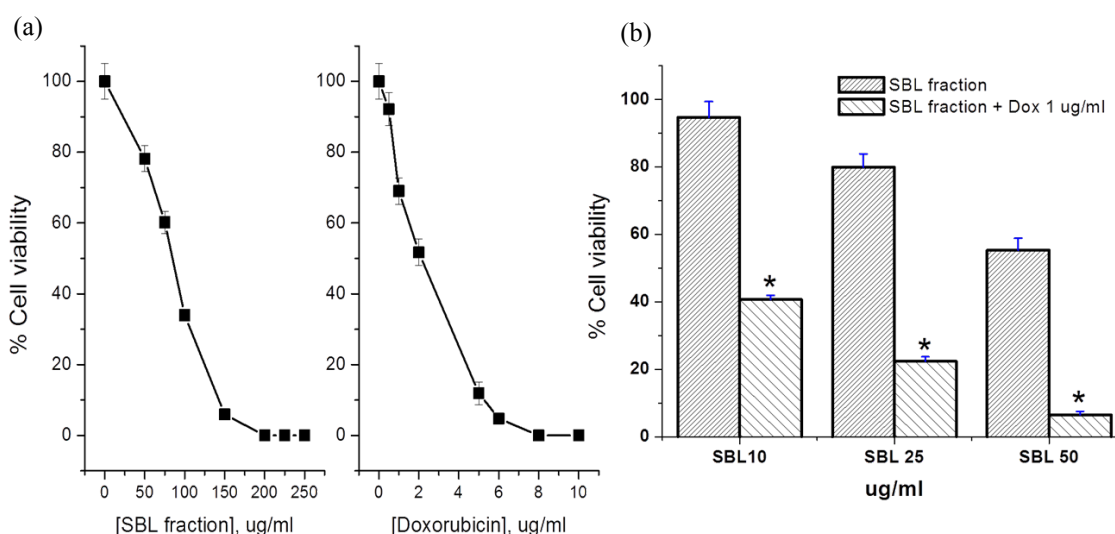

Figure 1 (a) Inhibition of cell viability by SBL fraction and Dox on KB cells. (b) Inhibition of cell viability by SBL fraction plus Dox on viability of KB cells. *p < 0.05 vs. SBL fraction alone. Data were expressed as mean ± SEM of n = 3.

Induction of apoptosis

In order to determine whether the growth inhibitory effect of SBL fraction could be related to apoptotic cell death, further experiments using fluorescent microscopy, agarose gel electrophoresis, and flow cytometer analyzes were carried out. The nuclear morphological features were visualized by staining with the cell-permeable DNA dye DAPI (**Figure 2a**). In the control group (0.5 % EtOH), the nuclei were round, with homogeneously staining, and the quantitative estimation of normal cells was 100 %. The apoptotic nuclei showed chromatin condensation and fragmentation of nuclear chromatin into irregular size. When the KB cells were treated separately with SBL fraction (50 μg/ml) and Dox (1 μg/ml), the apoptotic nuclei were 33.33 ± 2.15 and 42.39 ± 4.93 %, respectively. In the combined treatment group, the sensitization effect was significantly evident in the increase of apoptotic nuclei to 78.37 ± 5.37 %.

In addition, agarose gel electrophoresis indicated that treatment with SBL fraction and Dox alone induced some fragmented DNA, whereas control cells showed no evidence of DNA fragmentation. The

combined SBL fraction and Dox treatment showed progressive accumulation of nucleosomal fragmentation, and the intensity of smear-like DNA degradation was more projecting compared to individual treatment (**Figure 2b**). The key mechanism of apoptosis is endonuclease activation, leading to internucleosomal DNA breaks that consist of laddered DNA fragmentation. In contrast, the key mechanism of necrosis is random nucleosomal DNA breaks that consist of smeared fragments of multiple sizes after gel electrophoresis [29]. In this study, both apoptosis and necrosis were considered, because there were no phagocytic cells to destroy apoptotic cells in the culture system. The initial alterations of cellular apoptosis induced by these agents may be activated *in vitro*, and the apoptotic cells eventually reached a late stage similar to necrosis at 48 h of incubation.

The magnitude of apoptosis was determined by analyzing the percentage of sub-G1 DNA that was in the KB cells treated with SBL fraction, Dox, and SBL fraction + Dox, using a flow cytometer. As shown in **Figure 3a**, the KB cells individually treated with SBL fraction or Dox resulted in a significant accumulation of cells, with sub-G1 DNA content at 16.29 ± 1.83 % and 30.26 ± 4.28 %, respectively, as compared to the control cells (2.71 ± 0.2 %). On the other hand, the sensitization effect was also evident in the combined treatment of SBL fraction and Dox that resulted in an obvious increase in sub-G1 phase to 35.92 ± 3.47 %. So, there is a good correlation between the accumulation of cells in the sub-G1 phase and the inhibition of cell viability after exposure to SBL fraction. In addition, caspase-3 was used to be a specific marker for the apoptotic pathway, and the activities of caspase-3 were detected by colorimetric analysis. The KB cells treated with SBL fraction and Dox significantly increased the relative activity of caspase-3, by 2.0 ± 0.01 and 3.0 ± 0.15 folds, respectively, when compared with the control cells. The z-DEVD-fmk (caspase-3 inhibitor) significantly inhibited the SBL fraction induced caspase-3 activation (**Figure 3b**) by 1.3 ± 0.06 folds. These results indicate that SBL fraction induces apoptotic death in KB cells, at least in part through a caspase-3-dependent pathway.

Induction of apoptosis in cancer cells is a very important mechanism, because one of the features of cancer cells is their ability to evade apoptosis. To understand the mechanism of how SBL fraction produces cytotoxic effects in KB cells, the measurement of selected markers was utilized. This compound was found to trigger the activation of caspases-3, which is the hallmark of apoptosis that in turn causes DNA fragmentation and cell death. Recent studies have reported that *Turbinaria conoides*, (J. Agardh) Kutzing [24], and *Sargassum binderi*, Sonder [25], from the east coast of the Gulf of Thailand, induced human cervical cancer cell death via morphological changes typical of apoptosis, including membrane blebbing, chromatin condensation, and nuclear and DNA fragmentation. Several investigations have been focused on the apoptosis mechanism of the fucoxanthin derived from different *Sargassum* species. Generally, the apoptosis-inducing effect of marine carotenoid fucoxanthin may vary, depending on the carotenoid structure [30] and the cell line employed [31]. For example, fucoxanthin obtained from the brown seaweed, *Undaria pinnatifida*, induced apoptosis on human leukemia HL-60 cells, while B-carotene did not show an apoptosis-inducing effect [30]. Further studies are needed to assess the specificity of the molecular mechanisms.

Nasopharynx carcinoma is the most common cancer originating in the nasopharynx, and less curable, because of its position near the cranial nerves and brain. The KB cell line was derived from human carcinoma of the nasopharynx, and has been reported to contain human papillomavirus18 (HPV-18) sequences [32]. The KB cell line has also been used by the National Cancer Institute for anticancer assays for screening herbal extracts. In this study, the SBL fraction from *Sargassum baccularia* sensitizes KB cells to Dox-induced apoptosis. Doxorubicin is a potent anticancer drug that caused the DNA double strand to break, and the cells be injured mainly by apoptosis. However, doxorubicin can damage normal cells and vital organs, resulting in serious and long-term side effects [33]. SBL fraction may be used in combination with chemotherapy in order to have milder side effects, because the marine brown seaweeds offer not only nutritional properties but also a wide range of health benefits. Recent studies have shown that fucoidan, a major sulphated polysaccharide of the brown seaweed, enhances etoposide induced caspase-3 dependent cell death pathways in human leukemia MT-4 cells [34]. The synergistic effect of the combined treatment of dietary carotenoids (halocynthiaxanthin) and tumor necrosis factor–related apoptosis, inducing ligand (TRAIL), significantly induced apoptosis in colon cancer DLD-1 cells, whereas each agent alone only slightly induced apoptosis [35]. The combined treatment with SBL

fraction and conventional antitumor agents, such as doxorubicin, is a promising strategy against cancer, but an optimizing combination therapy needs to be established for further investigation.
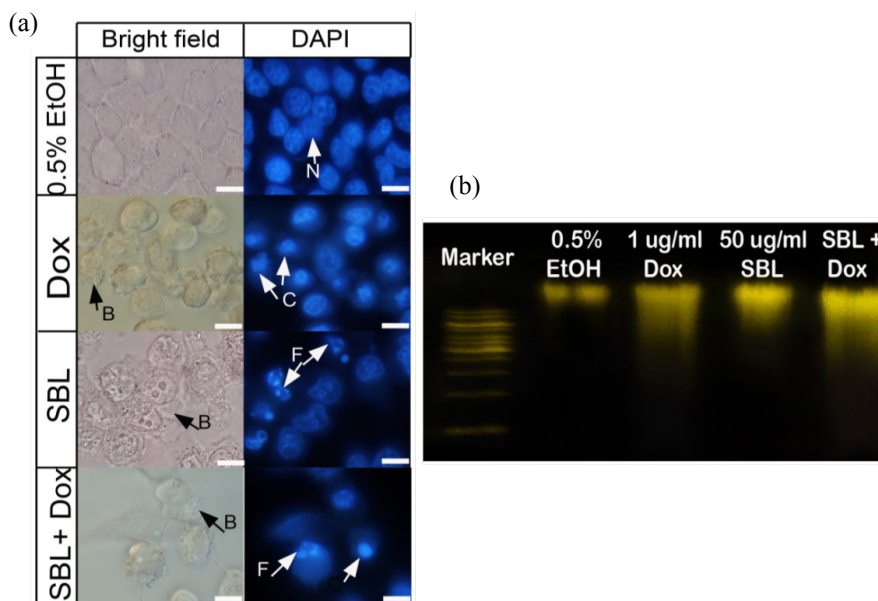

Figure 2 (a) Correlation of surface morphologies with nuclear features. KB cells treated with SBL fraction (50 µg/ml), Dox (1 µg/ml) and SBL fraction (50 µg/ml) + Dox (1 µg/ml). B: membrane blebbing, C: chromatin condensation, F: nuclear fragmentation and N: normal nuclei. Scale bar is 10 µM. (b) Fragmented DNA was extracted and electrophoresed on agarose gel containing SYBER Gold.
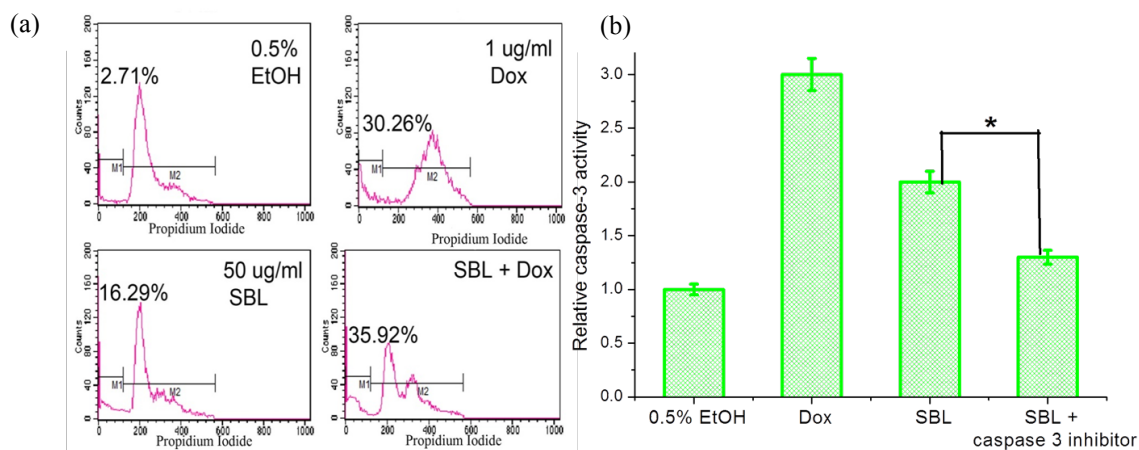

Figure 3 (a) Histograms of flow cytometric analysis of KB cells stained with PI. M1 was the percentage of cells in sub-G1 phase. (b) Relative caspases-3 activities were measured by spectrophotometry. The control group (0.5% EtOH) was set as 1, and the values of other groups were standardized against it. Data were expressed as a mean ± SEM of n = 3 samples, *$p < 0.05$ vs. SBL fraction alone.

Conclusions

The present study demonstrated that the lipophilic fraction of the brown seaweed *S. baccularia* has moderate cytotoxic activity, and the combination of SBL fraction and Dox enhances more apoptosis than treating with each agent alone in KB cells. Apoptosis is characterized by cytoplasmic shrinkage, nuclear condensation, and activation of caspase-3. The chemical compositions of *S. baccularia*, such as fucoxanthin, phenolic compounds, tocopherols, and fatty acids, have been also reported. The synergistic cytotoxic effect of SBL fraction against nasopharyngeal cancer cells may provide a rationale for future investigations, which are needed to assess the details of the molecular mechanisms against different types of cancer cells or animal models.

Acknowledgements

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