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Induction of Apoptosis by Three Marine Algae through Generation of Reactive Oxygen Species in Human Leukemic Cell Lines

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In this study, we examined the antitumor effect of marine algae extracts on human hepatoma and leukemia cells. Ethyl acetate extracts from *Colpomenia sinuosa* (*Cs*-EA), *Halimeda discoidae* (*Hd*-EA), and *Galaxaura oblongata* (*Go*-EA) directly inhibited the growth of human hepatoma HuH-7 cells and leukemia U937 and HL-60 cells in a time- and dose-dependent manner. Specifically, these algae extracts induced apoptosis of U937 and HL-60 cells as evaluated by detection of hypodiploid cells using flow cytometry and observation of condensed and fragmented nuclei in algae extract-treated cells. Intracellular reactive oxygen species (ROS), especially hydrogen peroxide and superoxide anion, were increased about 2−3-fold in U937 cells treated with *Cs*-EA for 3−5 h. Interestingly, antioxidant *N*-acetylcysteine effectively blocked *Cs*-EA-, *Hd*-EA-, and *Go*-EA-induced apoptosis, suggesting that ROS is a key mediator in the apoptotic signaling pathway. In conclusion, our results show that algae extracts induce apoptosis in human leukemia cells through generation of ROS.

KEYWORDS: ***Colpomenia sinuosa*; *Halimeda discoidea*; *Galaxaura oblongata*; apoptosis; reactive oxygen species**

INTRODUCTION

Scientists have been trying to find antitumor agents in natural products to develop novel therapeutic agents for cancer, a leading cause of death worldwide. Compounds or extracts from Chinese herbal medicines (*1*−*8*), foods (*9*, *10*), tea (*11*), or honeybee propolis (*12*, *13*) have been demonstrated to have cytotoxic effect or apoptosis-inducing activity in tumor cell lines. In addition, some reports have demonstrated the suppression of tumor growth by seaweed or algae extracts (*14*−*17*). For example, wakame and mekabu have been shown to reduce the growth of breast tumors in rats (*14*, *16*). However, there are relatively few studies demonstrating possible antitumor agents found in algae, a rich resource found throughout the world.

In general, most antitumor agents act by inducing apoptosis in tumor cells. Apoptosis is initiated by activation of either the tumor necrosis factor (TNF) receptor superfamily at the cell membrane or other intrinsic signaling pathways such as DNA damage in the nucleus (*18*, *19*). Caspase family members figure prominently in the control of all known apoptotic signaling pathways. Mitochondria also play pivotal roles in activating the caspase cascade in most such pathways, for example, the intrinsic signaling pathway. During the early stages of apoptosis, oligomerization of members of the Bcl-2 superfamily, including proapoptotic Bax and Bak, control the permeability of the outer mitochondrial membrane, with rapid loss of the transmembrane potential. Cytochrome *c* and apoptosis-inducing factor are then released, followed by activation of caspase 9 and other execution caspases, thus turning on the irreversible apoptotic signaling pathway.

Generation of reactive oxygen species (ROS) also plays a role in many of these pathways, such as TNF-α-, ceramide-, cisplatin-, etoposide-, and UV-induced apoptosis (*20*−*28*). ROS are primarily derived from normal cellular respiration in mitochondria. Increased ROS generation also plays a role in many signaling cascades, such as activation of protein tyrosine kinase or transcription (*29*). The antioxidant drugs *N*-acetylcysteine (NAC) and pyrrolidenedithiocarbamate (PDTC) or overexpression of the antioxidant enzyme manganese superoxide

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dismutase (MnSOD) protects cells from apoptosis initiated by TNF-α, camptothecin, adriamycin, inostamycin, vinblastine, staurosporine, etoposide, cisplatin, or UV light (*21*, *23*−*28*), indicating the close association between apoptosis and ROS. In this study, we assessed the antiproliferative effect of three marine algae, *Colpomenia sinuosa*, *Halimeda discoidea*, and *Galaxaura oblongata*, on three different tumor cell lines.

MATERIALS AND METHODS

Cell Cultures. The human myeloid leukemia cell lines U937 and HL-60 (*8*) were grown in RPMI 1640 medium (Gibco), and the human hepatoma cell line HuH-7 (*30*) was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Hyclone), 2 mM L-glutamine, and 1% nonessential amino acids (Atlanta Biologicals) at 37 °C in a humidified atmosphere containing 5% CO_2.

Preparation of Algae Extracts. The brown algae *C. sinuosa*, green algae *H. discoidea*, and red algae *G. oblongata* were collected as fresh specimens from the southern coast of Taiwan. The algae were air-dried, ground into powder, and then extracted with ethyl acetate at a 1:10 (w/v) ratio for 24 h, and the solvent was removed under reduced pressure to dryness. The ethyl acetate extracts from *C. sinuosa*, *H. discoidae*, and *G. oblongata* are referred to as *Cs*-EA, *Hd*-EA, and *Go*-EA. The extract powder was dissolved and diluted with dimethyl sulfoxide (DMSO), and then the cells were incubated with 0.25% DMSO containing different concentrations of extracts for 23 h (apoptosis assay of leukemia cells) or 24 h (MTT assay of HuH-7).

Cytotoxicity. U937 or HL-60 cells at 1×10^5/mL were incubated with various concentrations of algae extracts for 5 days. Viable cells were then counted by trypan blue dye exclusion. To assess cell growth kinetics, 1×10^5/mL U937 cells were treated and counted on days 1, 2, and 3. Each experiment was performed in triplicate, and the data from each are expressed as the mean ± SEM.

Inhibition of HuH-7 Cell Growth. HuH-7 cells at 1×10^5/mL were seeded in 96-well culture plates for 24 h before treatment. After incubation of HuH-7 cells with algae extracts for 3 days, the viability of the cells was measured by a tetrazolium (MTT) assay (*31*). In brief, 1 mg/mL MTT (Sigma) was added to the culture medium, and the cells were incubated at 37 °C for 3 h. Afterward, the culture medium was removed, and DMSO was added to each well to dissolve the MTT dye in the viable cells. The absorbance was measured at 540 nm using a Power Wavex Microelisa reader (Bio-tek). OD values from untreated control cells were designated 100% as a standard.

Hypodiploid Cells. Human leukemia cells were treated with various concentrations of algae extracts for 22 h, after which the cells were collected and resuspended in propidium iodide (PI) hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100, and 20 μg/mL PI) (Sigma) as described by Nicoletti et al. They noted that cells dying by necrosis have no detectable oligonucleosomal degradation of nuclear DNA. Therefore, cells with hypodiploid DNA must be dying by apoptosis (*32*). The DNA content of the cells was measured by using a FACScalibur flow cytometer (Becton Dickinson), and the percentage of sub-G1 cells was calculated by using CellQuest software. For some experiments, cells were preincubated with 6 mM *N*-acetylcysteine (NAC, Sigma) for 1.5 h and then treated with algae extracts for 22 h. The assay for sub-G1 cells was then performed in a similar manner.

Apoptotic Cellular Morphology. After 14 h of treatment with 75 μg/mL *Cs*-EA, U937 cells were washed twice with PBS and fixed with 3.7% formaldehyde for 10 min. They were washed again, stained with 4′,6-diamidino-2-phenyindole (DAPI; 5 μg/mL; Sigma) for 5 min, and washed one more time. A confocal laser scanning microscope (Zeiss) was used to count cells containing apoptotic bodies.

Detection of Intracellular ROS. A 2′,7′-dichlorofluorescein diacetate probe (DCFH-DA, Molecular Probes) was used to detect cytosolic hydrogen peroxide (H_2O_2), and dihydroethidium (DHE, Sigma) was used to detect cytosolic superoxide anion ($O_2^{\bullet -}$). First, U937 cells were preincubated with 2 μM DCFH-DA-containing Hanks' balanced salt solution (HBSS) or 1 μM DHE-containing culture medium and incubated at 37 °C for 30 min. The cells were then washed, resuspended in culture medium, and treated with DMSO, 200 μM H_2O_2 (as a positive control), or 75 μg/mL *Cs*-EA for 2−5 h. The intracellular levels of H_2O_2 and $O_2^{\bullet -}$ were finally measured by flow cytometry and mean fluorescence intensity.

NAC is a potent antioxidant. It is a glutathione precursor which accelerates the conversion of H_2O_2 into H_2O, thus removing excess ROS. To further examine the association between apoptosis and ROS, we used NAC to attempt to block ROS generation in U937 cells treated with the algae extracts.

Table 1. Effect of Algae Extracts on Growth of U937 and HL-60 Cells

treatment (μg/mL)		inhibition rate[a] (%) U937	HL-60
Cs-EA	25	16.4 ± 3.2	39.2 ± 8.7
	50	37.5 ± 4.6	60.8 ± 9.1
	100	75.7 ± 9.1	99.8 ± 0.2
	200	96.1 ± 3.1	98.3 ± 1.6
Hd-EA	25	31.8 ± 9.1	15.4 ± 4.8
	50	58.6 ± 3.3	36.1 ± 8.4
	100	55.4 ± 6.7	61.8 ± 9.5
	200	87.3 ± 7.7	98.1 ± 1.9
Go-EA	25	6.3 ± 6.5	32.3 ± 4.1
	50	24.9 ± 5.4	46.9 ± 8.3
	100	41.3 ± 9.2	96.7 ± 1.2
	200	95.9 ± 4.1	97.0 ± 2.9

[a] U937 or HL-60 cells were incubated with various concentrations of *Cs*-EA, *Hd*-EA, or *Go*-EA for 5 days. The number of viable cells was counted in a trypan blue dye exclusion test. Data are reported as the percent of inhibition compared to untreated controls and are recorded as the mean ± SD from three separate experiments.

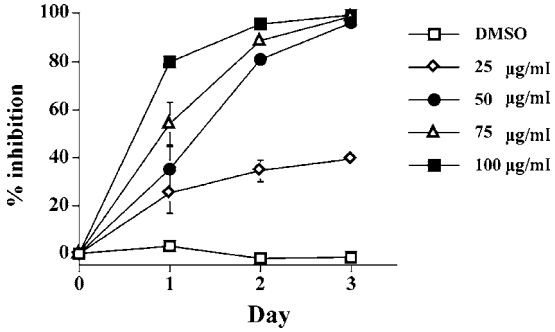

Figure 1. Growth kinetics of U937 cells treated with *Cs*-EA. U937 cells were incubated at 1×10^5/mL in the presence of DMSO or 25, 50, 75, or 100 μg/mL *Cs*-EA for 1, 2, or 3 days, and then the numbers of cells were counted. Data are reported as the percent of inhibition as compared to untreated control cells.

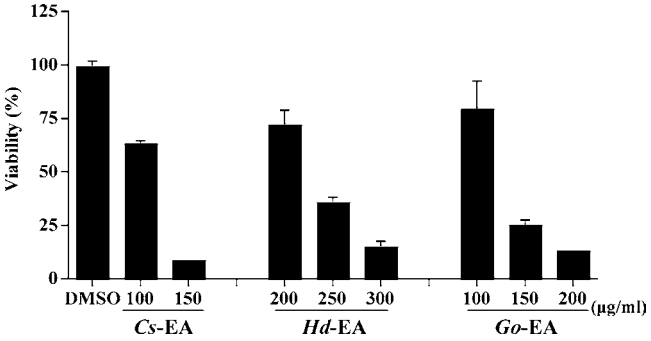

Figure 2. Cytotoxic effect of algae extracts on human hepatoma HuH-7 cells. Human hepatoma HuH-7 cells were seeded at 1×10^5/mL and cultured for 24 h, followed by treatment with various concentrations of *Cs*-EA, *Hd*-EA, or *Go*-EA for 3 days. Cell viability was measured by an MTT assay and compared with that of untreated cells.

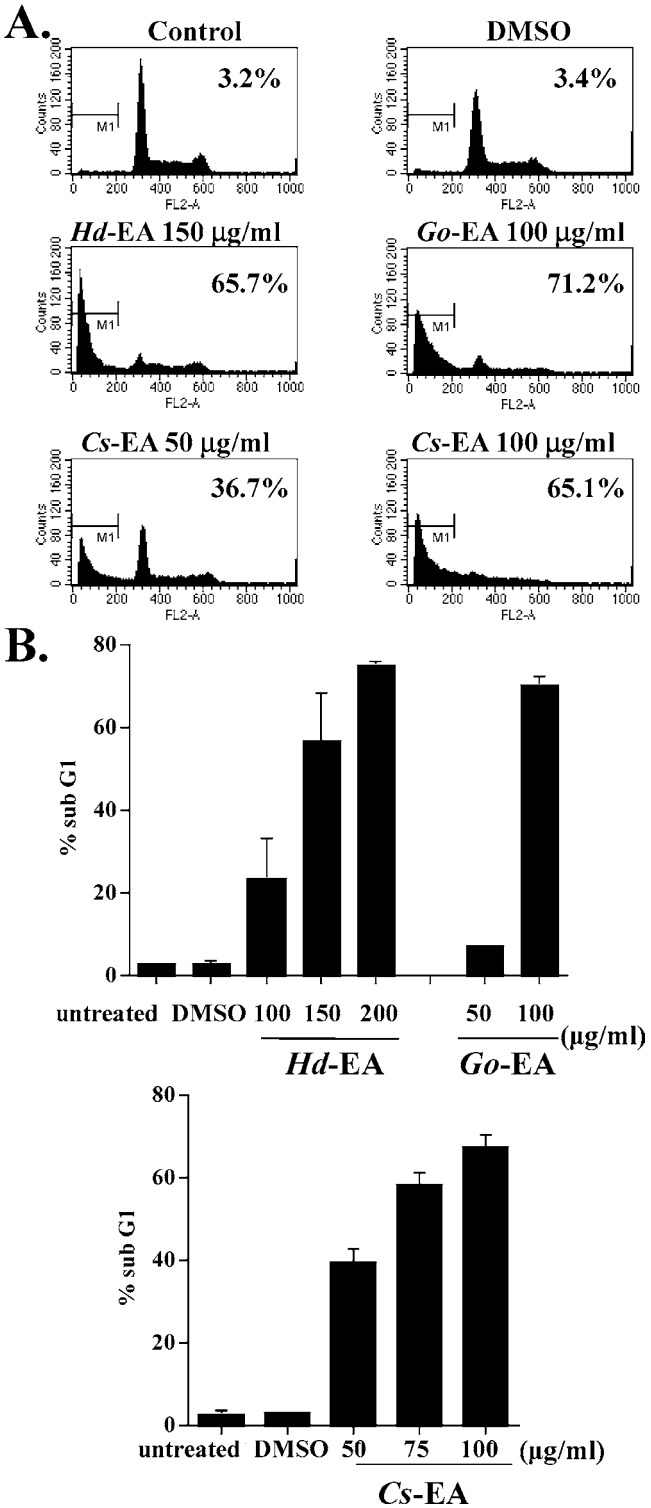

Figure 3. Algae extracts induce apoptosis in U937 human leukemia cells. (**A**) U937 cells were incubated with various concentrations (μg/mL) of *Cs*-EA, *Hd*-EA, or *Go*-EA as indicated in each graph. Twenty-two hours later, the cells were harvested, stained with PI, and assessed using flow cytometry. (**B**) Percentage of sub-G1 cells, which was analyzed by CellQuest software.

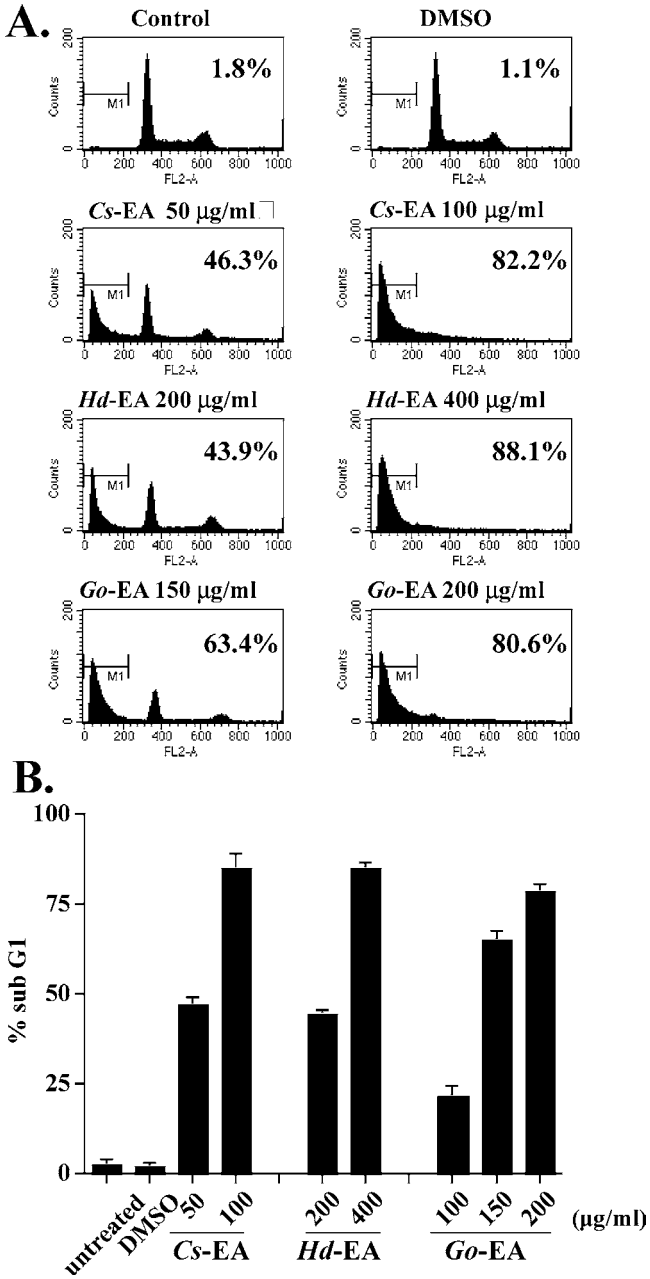

Figure 4. Algae extracts induce apoptosis in HL-60 human leukemia cells. (**A**) HL-60 cells were treated with the extracts, and the number of apoptotic cells was determined by the method described in the caption of **Figure 3**. (**B**) Percentage of sub-G1 cells, which was analyzed by CellQuest software.

RESULTS

Growth Inhibitory of *Cs*-EA, *Hd*-EA, and *Go*-EA on Human Leukemia Cells. For preliminary screening of the cytotoxic effect of marine algae extracts, human U937 and HL-60 leukemia cells were mixed with *Cs*-EA, *Hd*-EA, and *Go*-EA for 5 days. As shown in **Table 1**, all three extracts inhibited the growth of both U937 and HL-60 cells in a dose-dependent manner. *Cs*-EA also had dose- and time-dependent growth inhibition on U937 cells (**Figure 1**).

To further confirm the cytotoxic effect of the algae extracts, HuH-7 cells were incubated with various concentrations of *Cs*-EA, *Hd*-EA, and *Go*-EA for 3 days, and then the viability of the cells was assessed with an MTT assay. Consistent with the previous data, the algae extracts all decreased the number of viable cells (**Figure 2**). The 50% effective concentrations (EC_{50}) for *Cs*-EA, *Hd*-EA, and *Go*-EA against HuH-7 cells were 112.38, 230.53, and 123.54 μg/mL, respectively.

Induction of Apoptosis in U937 and HL-60 Cells. Since all three algae extracts had cytotoxic activity in the cell lines tested, we examined whether the extracts induced apoptosis in the cells. After the leukemia cells were incubated with different

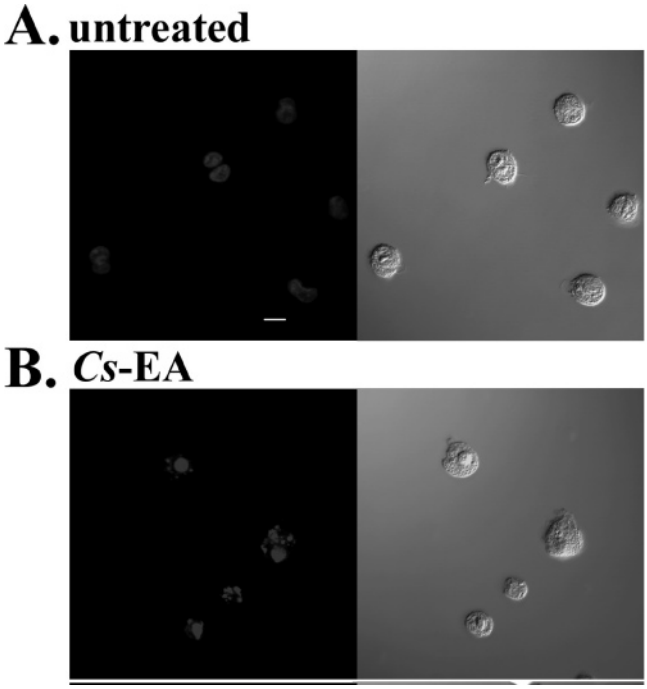

Figure 5. Morphology of cells undergoing *Cs*-EA-induced apoptosis. U937 cells, either untreated (**A**) or treated for 14 h with 75 μg/mL *Cs*-EA (**B**), were stained with DAPI and examined with confocal microscopy. The left panel shows a fluorescent view of the nuclei, and the right panel shows a bright view of the cells. The bar represents 10 μm.

concentrations of *Cs*-EA, *Hd*-EA, and *Go*-EA for 22 h, they were harvested and stained with PI, and hypodiploid or sub-G1 DNA containing cells were counted by flow cytometry. As shown in **Figures 3** and **4**, all three algae extracts induced apoptosis in U937 and HL-60 cells in a dose-dependent manner. The EC_{50} values for induction of apoptosis in U937 cells by *Cs*-EA, *Hd*-EA, and *Go*-EA were 63.45, 139.37, and 83.79 μg/mL, respectively. The comparable EC_{50} doses in HL-60 cells were 53.35, 226.35, and 132.73 μg/mL. *Cs*-EA was thus more effective (that is, required a lower dose) in inducing apoptosis in U937 and HL-60 cells.

We examined the nuclear morphology of U937 cells treated with *Cs*-EA. Compared to intact nuclei from control cells, *Cs*-EA treatment resulted in at least 70% of the cells containing condensed and fragmented nuclei, which are typical of apoptosis (**Figure 5**).

Increased Intracellular ROS by *Cs*-EA in Human Leukemia Cells. After *Cs*-EA treatment, we assessed the intracellular H_2O_2 and $O_2^{\bullet -}$ levels by DCFH-DA- and DHE-labeling and flow cytometry. Compared to those of untreated and DMSO-treated control cells, intracellular H_2O_2 levels were enhanced about 3-fold in cells treated with *Cs*-EA for 3−5 h (**Figure 6**). The amount of H_2O_2 in *Cs*-EA-treated cells was similar to or even higher than the amount of H_2O_2 in positive control cells directly treated with 200 μM H_2O_2. The intracellular $O_2^{\bullet -}$ level was also increased about 2-fold in *Cs*-EA-treated cells compared to untreated and DMSO-treated cells (**Figure 7**). Thus, *Cs*-EA increased ROS levels in human leukemia cells. NAC pretreatment (6 mM for 1.5 h) partially blocked the H_2O_2 and $O_2^{\bullet -}$ generation induced by Cs-EA in U937 cells.

Suppression of Algae Extract-Induced Apoptosis by NAC. We assessed the ability of NAC to block apoptosis. As shown in **Figure 8**, NAC pretreatment effectively blocked apoptosis induced by *Cs*-EA (**A**, **B**) and *Hd*-EA and *Go*-EA (**C**) in U937 cells. H_2O_2 (200 μM), a positive control, induced 43.4 ± 6.1% sub-G1 population of U937 cells. This suggests that the excess ROS generation is a key mediator in the apoptotic signaling pathways stimulated by these algae extracts.

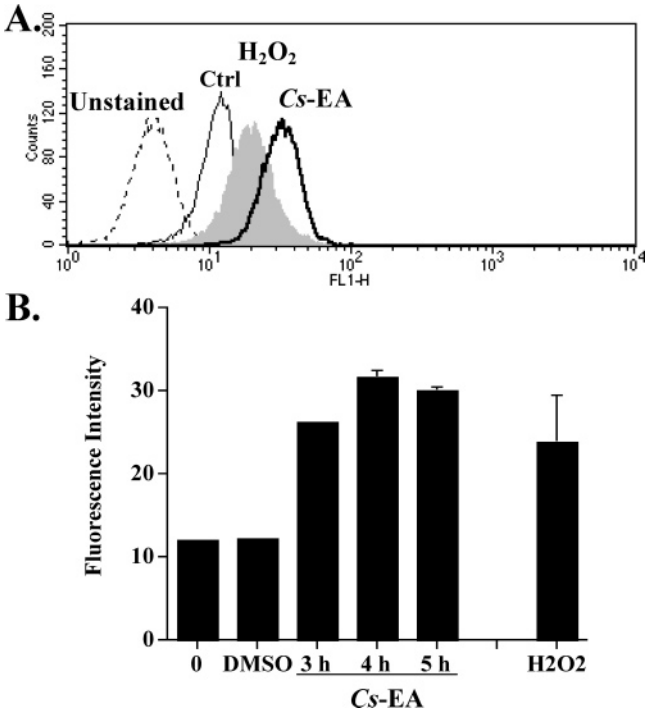

Figure 6. Intracellular H_2O_2 production in *Cs*-EA-treated cells. (**A**) U937 cells were first incubated with DCFH-DA for 30 min and then treated with 200 μM H_2O_2 (as a positive control, shaded curve) or 75 μg/mL *Cs*-EA (bold curve) for 4 h. Intracellular H_2O_2 was measured by flow cytometry, and fluorescence intensity was calculated by CellQuest software (Becton Dickinson). The light curve represents untreated control cells (ctrl), and the dashed curve represents unstained control cells (unstained). (**B**) U937 cells, either untreated (0) or treated with DMSO or 200 μM H_2O_2 for 5 h or with 75 μg/mL *Cs*-EA for 3–5 h, were assayed for intracellular H_2O_2 levels. Data from three separate experiments are expressed as the mean ± SEM.

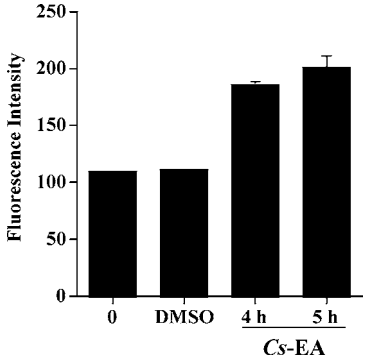

Figure 7. Intracellular $O_2^{\bullet -}$ generation in *Cs*-EA-treated cells. U937 cells were first incubated with DHE, and then were either untreated (0) or treated with DMSO or 75 μg/mL *Cs*-EA for 4 or 5 h. Intracellular $O_2^{\bullet -}$ levels were determined by flow cytometry, and fluorescence intensity was calculated by CellQuest software. Data from three separate experiment are expressed as the mean ± SEM.

DISCUSSION

In this study, we demonstrated the cytotoxic effects of ethyl acetate extracts of three algae, *Cs*-EA, *Hd*-EA, and *Go*-EA, against human hepatoma HuH-7 cells and human leukemia U937 and HL-60 cells (**Table 1**, **Figures 1** and **2**). *Cs*-EA, *Hd*-

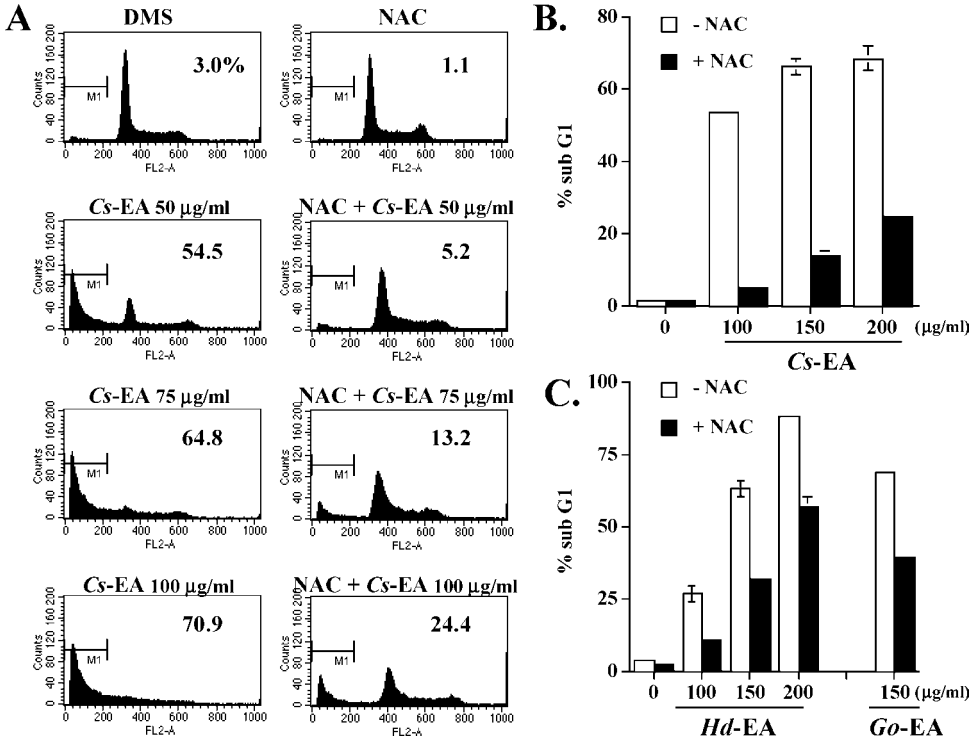

Figure 8. Inhibition of algae extract-induced apoptosis by antioxidant NAC. U937 cells were preincubated with 6 mM NAC (right panels of (**A**) and solid columns of (**B**) and (**C**)) for 1.5 h and then treated with various concentrations of *Cs*-EA, *Hd*-EA, or *Go*-EA, as indicated in each graph. The percentage of sub-G1 cells was detected by flow cytometry as described in the caption of **Figure 3**. For the positive control (right panels of (**A**)), viable U937 cells were counted by trypan blue dye exclusion after the cells were incubated with H_2O_2 or H_2O_2 plus 6 mM NAC for 23 h.

EA, and *Go*-EA were shown to induce apoptosis in the leukemia cells (**Figures 3**−**5**). Sterols isolated from *Galaxaura marginata*, similar to *G. oblongata*, have been shown to be cytotoxic in several cancer cell lines (*33*, *34*). An algal lectin from *G. marginata* exhibited antibacterial activity (*35*). In addition, fatty acids, such as palmitic acid, have been isolated from *C. sinuosa* (*36*), and another report has demonstrated antitumor activity of palmitic acid from marine algae (*15*). Our results are consistent with these findings in that *Cs*-EA and *Go*-EA are cytotoxic for HuH-7 cells and induce apoptosis in U937 and HL-60 cells. As a result of these findings, we are working on further isolation of potential chemotherapeutic agents from these algae extracts.

It is well-known that ROS generation plays an important role in many signaling pathways, including apoptosis, kinase activation, transcriptional regulation, and cell adhesion (*29*). We found that intracellular H_2O_2 and $O_2^{\bullet -}$ levels increased dramatically after U937 cells were treated with *Cs*-EA (**Figures 6** and **7**). This effect was much greater than that caused by direct treatment of control cells with 200 μM H_2O_2. In addition, intracellular generation of H_2O_2 and $O_2^{\bullet -}$ occurred after 3−5 h of incubation with *Cs*-EA (**Figures 6** and **7**) but not after only 2 h (data not shown). This is similar to the timing of ROS generation by the anticancer drugs camptothecin, adriamycin, inostamycin, and vinblastine (*28*).

Interestingly, we demontrated that the antioxidant NAC effectively inhibited *Cs*-EA-, *Hd*-EA-, and *Go*-EA-induced apoptosis in U937 cells (**Figure 8**). The generation of ROS by treatment of these algae extracts may correlate with the mitochondrial death pathway in the leukemic cells we used. In our previous investigation, we found that caffeic acid phenethyl ester also induced apoptosis of HL-60 cells via interference of mitochondrial transmembrane potential, and this effect involves ROS (*13*).

Our data indicate that these algae extracts may work via apoptotic signaling pathways in ways similar to those of other agents known to cause DNA damage, including effective anticancer drugs. There may be a potential for developing new algae-derived chemotherapeutic agents.

ABBREVIATIONS USED

ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; $O_2^{\bullet -}$, superoxide anion; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PI, propidium iodide; NAC, *N*-acetylcysteine; DAPI, 4′,6-diamidino-2-phenyindole; DCFH-DA, 2′,7′-dichlorofluorescein diacetate probe; DHE, dihydroethidium; HBSS, Hank's balanced salt solution; TNF-α, tumor necrosis factor-α.

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