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Antiproliferative Effects of Carotenoids Extracted from *Chlorella ellipsoidea* and *Chlorella vulgaris* on Human Colon Cancer Cells

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The antiproliferative activity of carotenoids separated from marine *Chlorella ellipsoidea* and freshwater *Chlorella vulgaris* has been evaluated. HPLC analysis revealed that the main carotenoid from *C. ellipsoidea* was composed of violaxanthin with two minor xanthophylls, antheraxanthin and zeaxanthin, whereas the carotenoid from *C. vulgaris* was almost completely composed of lutein. In an MTT assay, both semipurified extracts of *C. ellipsoidea* and *C. vulgaris* inhibited HCT116 cell growth in a dose-dependent manner, yielding IC_{50} values of 40.73 ± 3.71 and 40.31 ± 4.43 μg/mL, respectively. In addition, treatment with both chlorella extracts enhanced the fluorescence intensity of the early apoptotic cell population in HCT116 cells. *C. ellipsoidea* extract produced an apoptosis-inducing effect almost 2.5 times stronger than that of the *C. vulgaris* extract. These results indicate that bioactive xanthophylls of *C. ellipsoidea* might be useful functional ingredients in the prevention of human cancers.

KEYWORDS: ***Chlorella ellipsoidea*; *Chlorella vulgaris*; carotenoid; antiproliferative effects; apoptosis**

INTRODUCTION

Carotenoids are a family of pigmented compounds that are synthesized by plants and microorganisms but not animals. They consist of long, aliphatic, conjugated double-bonded systems, which serve as light-absorbing chromophores, and are usually composed of eight isoprene units with the molecular formula $C_{40}H_{56}$ (*1, 2*). Many carotenoids contribute to light harvesting, maintain the structure and function of photosynthetic complexes, quench chlorophyll triplet states, and dissipate excess energy (*1, 3, 4*). Alternatively, they can act as potent free radical quenchers, singlet oxygen scavengers, and lipid antioxidants, thereby acting as photoprotectants under conditions of excess light (*5*−*7*). Some carotenoids such as β-carotene and lycopene may lessen the risk of cardiovascular diseases and certain cancers, whereas lutein and zeaxanthin may lessen the risk of eye disorders (*8*−*11*). These beneficial effects may be due to their antioxidant properties. Recent studies have also shown that carotenoids may act through other mechanisms such as gap junction communication, cell growth regulation, and modulation of gene expression (*5*). For structure identification, many researchers have undertaken carotenoid analysis involving high-performance liquid chromatography (HPLC) coupled to mass detectors using electrospray or atmospheric pressure chemical ionization (*12*−*16*).

Microalgae are a major natural source for a vast array of novel compounds: nutrients, including proteins, vitamins, minerals, and fatty acids, as well as carotenoid pigments, such as xanthophylls and carotenes. Recently, microagal biotechnology has advanced considerably. It is now possible to produce some carotenoids commercially through aquaculture. These include β-carotene from *Dunaliella*, astaxanthin from *Haematococcus*, and lutein from *Chlorophycean* strains (*16*−*22*).

Chlorellas are also a good commercial source of many carotenoids, such as lutein, zeaxanthin, β-carotene, and astaxanthin. Green algae have frequently been used as a model organism for research into genetics and the molecular biology of photosynthesis. Recent developments in genetic modification and metabolic engineering technology have enabled the extensive growth of chlorella for industrial use (*19, 20, 23*). One marine strain, *Chlorella ellipsoidea*, is widely used in Japan and Korea as feed for rotifers and brine shrimp, which then are fed to fish larvae. This alga in particular contains large amounts of lipids and eicosapentaenoic acid, compared to other lake chlorella species (*24, 25*). Several studies have reported on carotenoids from chlorella (*14, 16, 18, 19, 23, 26*), but no research has been published about carotenoids from seawater *Chlorella ellipsoidea* and their function. We analyzed carotenoids separated from marine *C. ellipsoidea* and from freshwater *Chlorella vulgaris*. We also evaluated the antiproliferative activity of their carotenoid extracts by measuring cytotoxicity and apoptosis-inducing activity.

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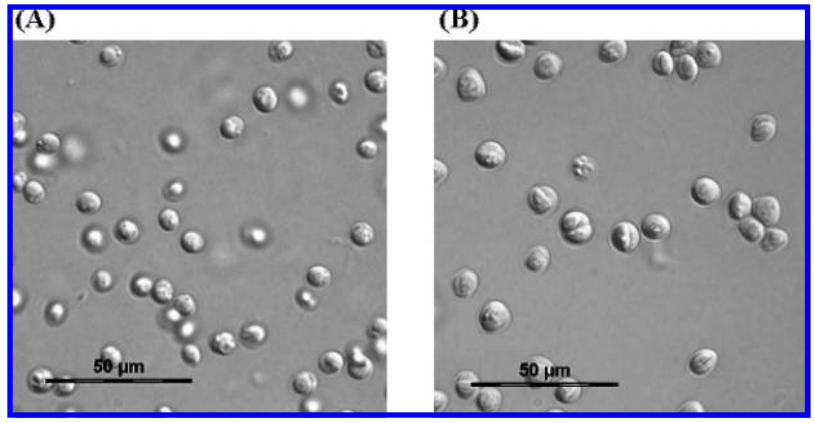

Figure 1. Microscopic images of two chlorella strains, *Chlorella ellipsoidea* (**A**) and *Chlorella vulgaris* (**B**). The cells were observed by a differential interference contrast microscope (×1500).

MATERIALS AND METHODS

Materials. Cultured solutions of *C. vulgaris* (KMCC C-024) and *C. ellipsoidea* (KMCC C-020) were purchased from the Korea Marine Microalgae Culture Center (Busan, Korea). The shape of each strain was identified using a differential interference contrast microscope (TE2000U, Nikon, Japan). Cultured solutions were washed twice in distilled water and harvested using a centrifuge. Harvested chlorella cells were freeze-dried in a vacuum freezer-dryer (Ilshin Laboratory, Korea) and refrigerated for subsequent chemical analysis. All HPLC solvents were purchased from Fisher Scientific (Springfield, NJ) and were of analytical grade. Standard lutein (98.2%), zeaxanthin (95.8%), neoxanthin (94%), and violaxanthin (98.3%) were purchased from Chromadex Inc. (Santa Ana, CA). Butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich (St. Louis, MO). The cell culture medium, Dulbecco's modified Eagle's medium (DMEM), was obtained from Gibco BRL (Gaithersburg, MD), as were the samples of fetal bovine serum (FBS), phosphate buffer solution (PBS), penicillin, streptomycin, and trypsin/EDTA. All other chemicals and solvents were of reagent grade.

Sample Preparation. A 1 g sample of freeze-dried chlorella powder was extracted in a 100 mL ethanol solution (100%) containing 0.1% (w/v) BHT at room temperature in the dark. After 3 h of extraction under continuous shaking, the mixture was filtered through a Whatman no. 1 filter paper (Whatman, Maidstone, U.K.). Still at room temperature and in the dark, 120 μL of 100% KOH was added to 5 mL of the contents for saponification. After saponification for 1 h, 3 mL of hexane was added to partition carotenoids; this mixture was shaken for 1 min and then diluted with 3 mL of water. The mixture was allowed to stand in an amber separatory funnel until the two phases had clearly separated. The upper layer containing carotenoids was collected, and the residue was repeatedly extracted until it contained no trace of carotenoids. The extract from the upper layer was then collected and evaporated to dryness using nitrogen purging. For HPLC analysis, the residue was redissolved in 1 mL of methanol, and fat-soluble impurities were extracted with hexane. Samples were shielded from strong light and kept on ice during all procedures. The authentic standards were dissolved in their respective solvents with the addition of 0.1% (w/v) BHT and dried under a stream of nitrogen gas prior to storage at −20 °C to prevent isomerization. These standards were redissolved in the appropriate solvent prior to use. All dilutions were conducted in methanol.

HPLC Conditions and Peak Identification. HPLC analysis was carried out in a Jasco HPLC 2000 Plus Series System equipped with a PU-2089 Plus quaternary gradient pump, an AS-2055 Plus Intelligent autosampler, a CO-2065 Plus column oven, a MD-2015 Plus Intelligent diode array detector, and a LC Net II/ADC, controlled by EZChrom Elite software version 3.16. Each 20 μL sample was injected into a YMC carotenoid column (3 μm particle size, 250 mm × 4.6 mm, Waters, Milford, MA) and then eluted in isocratic mode with an acetonitrile/methanol (75:25) mobile phase at flow rate of 1.0 mL/min by modification of the HPLC method of Gouveia (*16*). The column was equilibrated for 30 min at the starting conditions before each injection. The various carotenoids were identified by comparing the retention times and absorption spectra of unknown peaks with reference standards. Carotenoids containing epoxy were identified by comparing peak absorption characteristics before and after the addition of 10 μL of methanolic HCl (0.1 N) to the sample extract (*22*).

HPLC-ESI-MS for Identification. Extracted carotenoids were identified using a LC-MS (HP-1100MSD, Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization interface in the positive mode according to the method of Stepnowski (*15*). Separation was performed in a SunFire C18 column (150 mm × 4.6 mm × 3.5 μm, Waters, Milford, MA) using the isocratic solvent system, acetonitrile/methanol (0.1 M ammonium formate)/dichloromethane (71:22:7, by volume). The flow rate was set at 0.5 mL/min, and the injection volume was 20 μL. ESI-MS operating parameters were optimized using a direct infusion of a lutein standard solution in the mobile phase: positive ion mode, 13 L/min dry gas flow (N_2), 30 psi nebulizer pressure, 350 °C drying gas temperature, and 4500 V capillary voltage. Mass spectra were acquired over the *m*/*z* 400−700 scan range using a 0.1 unit step size.

Cell Culture. HCT-116 human colon cancer cells were obtained from the American Type Culture Collection (Rockville, MD). These cells were maintained as monolayer cultures at 37 °C in a humidified 95% air and 5% CO_2 atmosphere in T-75 flasks containing DMEM with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. For all experiments, cells were harvested by trypsinization and then seeded and grown in fresh media in a humidified 95% air and 5% CO_2 atmosphere. Cells were counted using a hemocytometer, and the number of viable cells was determined using trypan blue dye exclusion.

Cell Viability. For the cell viability test and flow cytometric analysis, all extracts were evaporated to dryness, and the stock solutions of these extracts were prepared in DMSO. The final concentration of DMSO was 0.5% and was tested as negative control. The cytotoxicity of chlorella extracts was evaluated using the Cell Counting Kit (CCK-8) produced by Dojindo Laboratories (Tokyo, Japan). In brief, 5×10^3 cells per well were plated into 96-well plates and incubated at 37 °C for 24 h. The medium was replaced with a fresh supply, and then cells were treated with various concentrations of chlorella extracts and incubated at 37 °C for an additional 24 h. Next, 10 μL of the CCK-8 solution was added to each well, and incubation was conducted for another 1 h. Absorbance values were measured at 450 nm using a PowerWave XS microplate reader (Bio-Tek Instruments, Winooski, VT).

Flow Cytometric Analysis. Any phosphatidylserine (PS) appearing on the extracellular side of a membrane was analyzed using Annexin V-FITC and propidium iodide (PI) staining (Annexin-V-FLUOS staining kit, Roche, Basle, Switzerland) followed by a flow cytometric analysis of cells. Briefly, exponentially growing cells were seeded in 6-well plates. Various concentrations of chlorella extracts were added to the cells 24 h after seeding. After the treatment, cells were trypsinized, counted, washed twice in ice-cold PBS, and resuspended in a binding

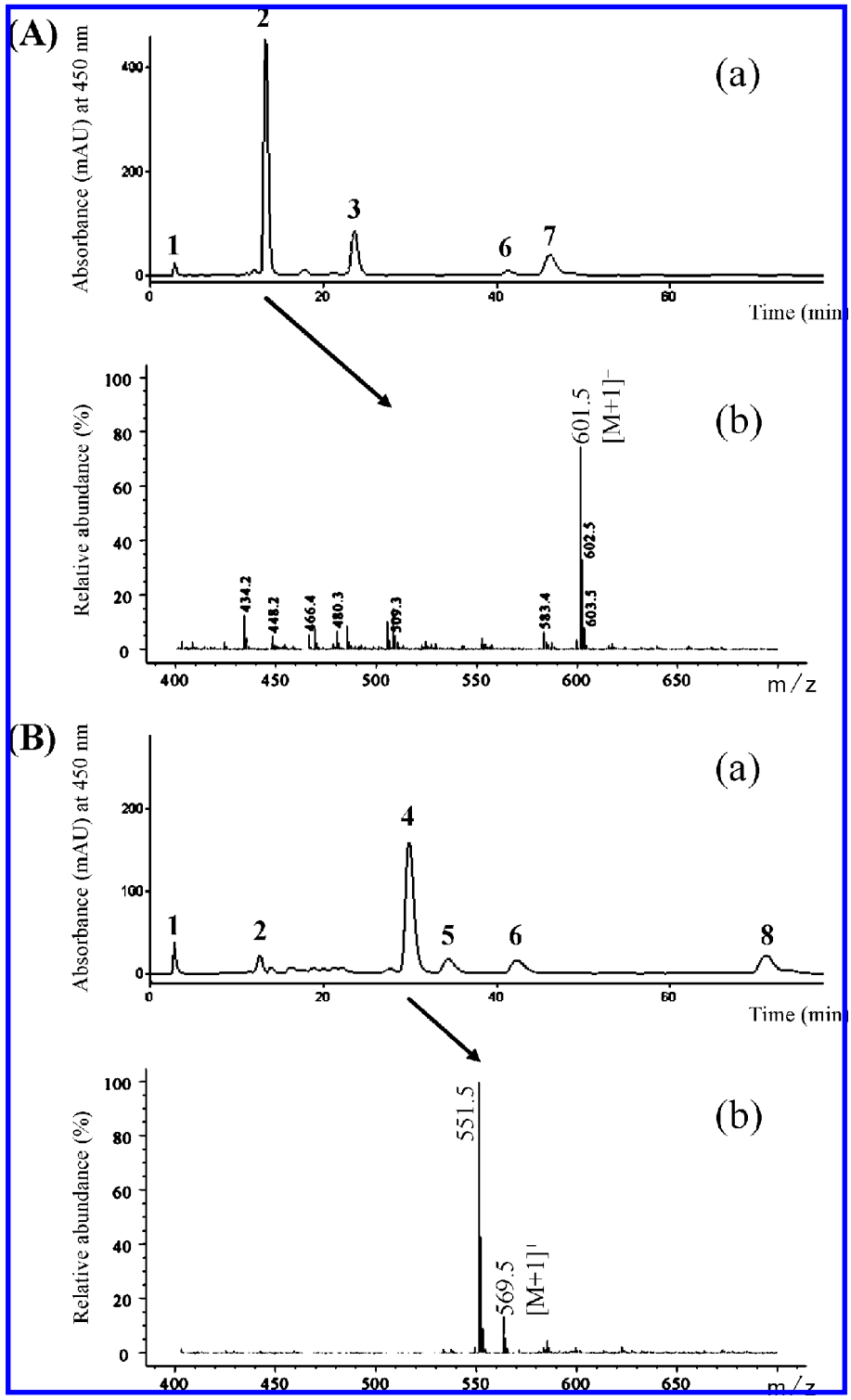

Figure 2. HPLC profiles (**a**) of total carotenoids and mass spectrum (**b**) of the main carotenoid extracted from *Chlorella ellipsoidea* (**A**) and *Chlorella vulgaris* (**B**). HPLC conditions are described in the text. **Table 1** shows the identities of other numbered peaks by LC-DAD and LC-ESI-MS.

buffer. Annexin V-FITC (2 μL) and propidium iodide (2 μL) were added to 100 μL of each cell suspension and incubated for 30 min at room temperature under dim lighting conditions. Next, binding buffer (400 μL) was added to the samples; these mixtures were maintained under ice-cold conditions until they were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). A total of 20000 cells were analyzed per sample.

RESULTS AND DISCUSSION

Analysis of Major Carotenoids in Chlorellas. Two strains of chlorella were selected for the production of bioactive carotenoids: the marine *C. ellipsoidea* and the freshwater *C. vulgaris*. Both strains exhibited typical microalgae appearance: diameter smaller than 10 μm and globular cell shapes under differential interference contrast microscope (**Figure 1**).

Carotenoid pigments were extracted from the two chlorella species and identified using HPLC. The various pigment ingredients were separated on the C30 column under a range of mobile phase conditions. Methylene chloride is generally used as a cosolvent to separate nonpolar carotenoids such as α-carotene and β-carotene, because nonpolar compounds are rela-

Table 1. Peak Identification of Carotenoids Isolated from Two *Chlorella* Strains and Their Spectral Characteristics following DAD

peak	t_R (min)	λ_{max}[a] (nm)	hypsochromic shift (nm)	epoxide test	%III/II[b]	ESI-MS (positive, *m*/*z*)	tentative identification
1	3.35						degradation product of chlorophyll
2	13.42	417, 441, 470	381, 404, 428	diepoxide	86.6	601.5	violaxanthin
3	24.14	424, 447, 475	402, 428, 452	monoepoxide	51.6	585.6	antheraxanthin
4	30.70	426, 446, 476	426, 446, 476	non-epoxide	55.6	569.5	lutein
5	34.88	423, 440, 470	423, 440, 470	non-epoxide	51.4	569.5	*cis*-lutein
6	42.28	470, 662	470, 662	non-epoxide	0	ND[c]	chlorophyll *b*
7	46.61	427, 453, 479	427, 453, 479	non-epoxide	18	569.5	zeaxanthin
8	71.44	426, 448, 478	426, 448, 478	non-epoxide	47	569.5	*cis*-lutein

[a] A mobile phase of methanol/acetonitrile (25:75, v/v) was used. [b] Ratio of the height of the longest wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between two peaks as baseline, multiplied by 100, in mobile phase. [c] Not determined.

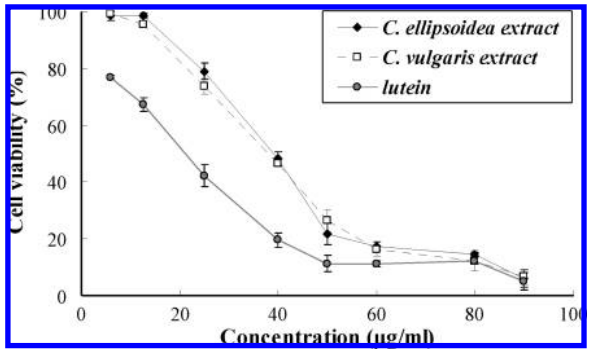

Figure 3. Effects of semipurified chlorella extracts and lutein on viability of HCT116 cells. The cells were treated with CEE (◆), CVE (□), and lutein (●) in designated concentrations for 24 h. Cell viability was measured with the MTT assay and is presented as a percentage of the control value. Data are expressed as mean ± SD of triplicate cultures.

tively sensitive to a hydrophobic solvent (*14, 27*). However, in our preliminary research, all constituents from *C. ellipsoidea* and *C. vulgaris* were completely separated using two solvents: acetonitrile and methanol (data not shown). In addition, it is preferable to limit the use of hydrophobic solvents when testing *C. ellipsoidea*, because most *C. ellipsoidea* pigments are polar xanthophylls, which can be damaged by hydrophobic solvents (*28*). Therefore, we used acetonitrile and methanol as binary mobile phases in all analyses, with the exception of the LC-MS analysis, which had to be conducted at a low flow rate.

Figure 2 presents a typical chromatogram of the major carotenoids in two chlorella strains. Unknown peaks were identified on the basis of their retention behavior and visible absorption spectra in comparison to available authentic standards (**Table 1**). When a standard was not commercially attainable, peaks were tentatively identified according to previously published results (*28*) and HPLC-ESI-MS. Peaks 2, 4, and 7 were identified as violaxanthin, lutein, and zeaxanthin, respectively, by comparing their retention times and spectra with corresponding standards. Under acidic conditions, violaxanthin converts to auroxanthin because two 5,8-epoxides form from their 5,6-epoxy groups, and it causes a hypsochromic shift, which is a change of spectral band position in the absorption spectrum of a molecule to a shorter wavelength (*12, 14*). After the epoxide test, we confirmed peak 2 as violaxanthin because we observed a hypsochromic shift of approximately 40 nm, which resulted from the two epoxy groups. Peak 3 was identified as antheraxanthin due to its distinguishing molecular weight (584.9), and peak 6 was identified as chlorophyll *b* by comparison of retention time and spectra with published data (*13*). Peaks 5 and 8 were identified tentatively as *cis*-lutein because of their concordance with *trans*-lutein in visible absorption spectra and LC-MS data. Peak 1 was identified as a degradation product of chlorophyll, as its spectrum was similar to that of chlorophyll. Interestingly, the major mass spectrum of peak 4 and lutein standard was not *m*/*z* 569.5, but *m*/*z* 551.5. This result was in agreement with another study (*29*), and the loss of the mass spectrum might be affected by the formation of water molecule.

We observed clear differences in two chromatograms between *C. ellipsoidea* (**Figure 2A**) and *C. vulgaris* (**Figure 2B**). The main carotenoid from marine *C. ellipsoidea* was violaxanthin with two minor xanthophylls, antheraxanthin and zeaxanthin, whereas the carotenoid from *C. vulgaris* was almost completely composed of lutein. A few studies have reported that this species of green algae chlorella frequently produces lutein as the major xanthophyll (*16, 23, 26*). However, only one study has reported results similar to our findings; it concluded that violaxanthin was the only xanthophyll in a seawater eustigmatophyte identified previously as *Chlorella minutissima* (*30*). Recently, some researchers have demonstrated that *Nannochloropsis* sp., known as a marine chlorella, is not a member of the class chlorophyceae but, belongs, instead, to eustigmatophyceae (*31, 32*). Other researchers have reported that members of the class eustigmatophyceae contain violaxanthin as their major carotenoid (60%), together with vaucheriaxanthin (free or ester) and some other minor components including zeaxanthin and antheraxanthin (neither lutein nor chlorophyll *b*) (*33, 34*). In addition, random amplified polymorphic DNA identification of chlorella species revealed a significantly high *F* value (a coefficient of genetic similarity) between *C. ellipsoidea* and *Nannochloropsis* sp. (*35*). On the basis of these results, we can hypothesize that *C. ellipsoidea* has a close evolutionary relationship with the class eustigmatophyceae.

Effects of Chlorella Extracts on the Viability of HCT116 Cells. We evaluated the cytotoxicity of chlorella extracts using an MTT assay. To determine the functions of the xanthophylls from the extracts, we refined the crude ethanol extracts of *C. ellipsoidea* and *C. vulgaris* using saponification and fractionation of hexane. The hexane phase was washed several times with deionized water to remove water-soluble impurities. Because fat-soluble impurities were not liquefied in this condition, the dried residue was dissolved in 100% methanol. For the cell treatment, the methanol solution was re-evaporated to dryness and a final stock solution was prepared in DMSO. These semipurified extracts of *C. ellipsoidea* (CEE) and *C. vulgaris* (CVE) both inhibited the growth of HCT116 cells in a dose-dependent manner, as shown in **Figure 3**. The antitumorigenic activities of CEE and CVE were similar, with IC_{50} values of 40.73 ± 3.71 and 40.31 ± 4.43 μg/mL, respectively, whereas pure lutein yielded an IC_{50} of 21.02 ± 0.85 μg/mL. The nearly 2-fold difference between the growth inhibition ability of CVE and pure lutein probably resulted from the low lutein purity of CVE (50%). A previous study reported that spirulina extracts markedly inhibited growth in human liver cancer cells, whereas

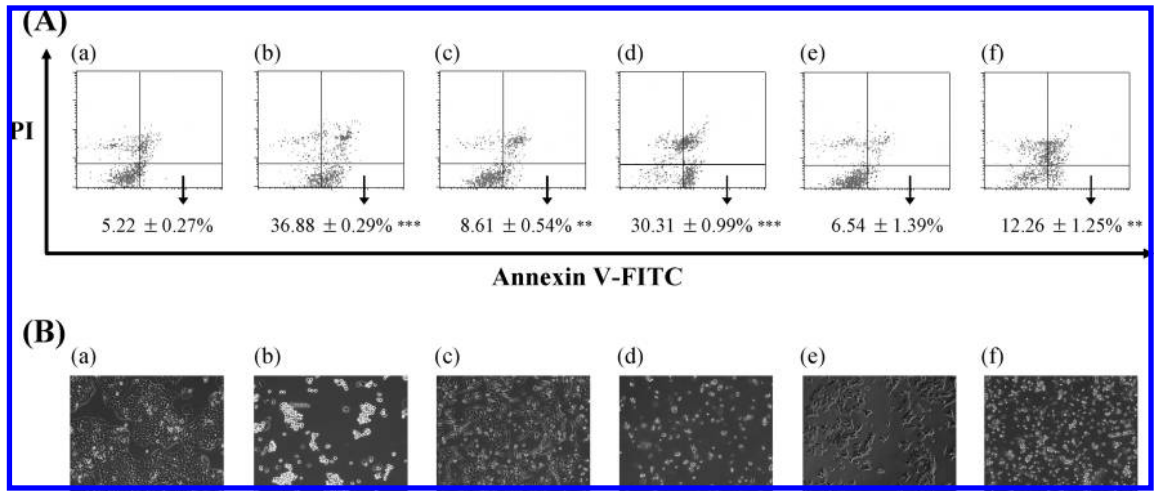

Figure 4. Effects of semipurified chlorella extracts on apoptosis in HCT116 cells. PS externalization by chlorella extracts was determined by annexin-V/PI assay with flow cytometry (**A**). The progression of apoptotic cells and respective phase were analyzed with a microscope (×200) (**B**). Cells were treated with the media containing 0.5% DMSO as a negative control (**a**), 100 nM of paclitaxel as a positive control (**b**), 40 μg/mL of CEE (**c**), 80 μg/mL of CEE (**d**), 40 μg/mL of CVE (**e**), and 80 μg/mL of CVE (**f**). The values marked with an asterisk are significantly different from control (***, $p < 0.001$; **, $p < 0.01$) using Student's t test, with $n = 3$.

chlorella extracts produced only a slightly inhibitory effect on the growth of the same cell lines (*36*). These inconsistent results may have originated from variations in extraction solvent; the previous study involved extracting phenolic compounds using water, whereas, in contrast, we extracted carotenoids using organic solvents.

Chlorella Extracts Induced Apoptosis on HCT116 Cells. To determine whether the antiproliferative activity of CEE and CVE was related to apoptosis, we used an annexin V−flouoresein and PI double staining assay to investigate the translocation of phosphatidylserine from the cytoplasm to the extracellular side (**Figure 4A**). The flow cytometry analysis indicated that treating HCT116 cells with CEE and CVE enhanced the intensity of fluorescence in the early apoptotic cell population (annexin V−fluoresein positive and PI negative, inverse phosphatidylserines without loss of membrane integrity). At 24 h post-treatment with CEE (40 and 80 μg/mL), early apoptotic cells increased 1.6- and 5.8-fold, respectively, over the control containing 0.5% DMSO. At 24 h post-treatment with CVE (40 and 80 μg/mL), we also observed phosphatidylserine externalization of HCT116 (1.3- and 2.3-fold, respectively) over the control. Our data revealed that CEE had an apoptosis-inducing effect almost 2.5 times stronger than that of CVE, especially at a concentration of 80 μg/mL; this result was comparable to the effects of 100 nM paclitaxel, which is known to have a strong apoptosis-inducing effect on human colon cancer cells (*37*). We also observed that after treatment with a range of CEE and CVE concentrations over 24 h, HCT116 cells exhibited drastic morphological changes such as membrane blebbing and cell shrinkage (**Figure 4B**). After 24 h of incubation, apoptotic cells began to lift from the surface monolayer, forming assorted sizes of spherical shapes. Apoptotic cells were more obviously apparent after treatment with CEE and CVE in concentrations of 80 μg/mL.

Considerable evidence supports the theory that some carotenoids, such as β-carotene and lycopene, may interfere with cancer-related molecular pathways and change the expression of many proteins involved in apoptosis (*38*−*41*). In addition, recent research has shown that dietary lutein can inhibit growth in mouse mammary tumors by regulating angiogenesis and apoptosis (*42*); this finding is of particular relevance because the primary pigment of *C. vulgaris* in our study was lutein. Research in the field of biological functions has tended to underestimate violaxanthin, which is the major carotenoid of *C. ellipsoidea*, because of its facile degradation. However, recent studies have demonstrated that neoxanthin, an epoxycarotenoid like violaxanthin, can vigorously induce apoptosis in human colon and prostate cancer cell lines (*43, 44*). Our findings produced convincing evidence that CEE causes antiproliferative activity in HCT116 cells. This result might be due to structural similarity; both neoxanthin and violaxanthin contain the epoxy group at the 5,6-position, although an allenic group in the polyene chain exists only in neoxanthin. A recent study found that zeaxanthin, one of the minor carotenoids in *C. ellipsoidea*, strongly induced apoptosis in neuroblastoma cells (*45*). Further study on each isolated carotenoid will be required to determine if the apoptosis-inducing activity of CEE was caused by violaxanthin or zeaxanthin.

In conclusion, we analyzed the bioactive carotenoids from marine *C. ellipsoidea* and *C. vulgaris*. We found that the extract of *C. ellipsoidea* exerted strong antiproliferative effects, including induction of apoptosis in in vitro cellular models. These findings suggest that bioactive xanthophylls of *C. ellipsoidea* could be potential therapeutic agents in the prevention of human cancers. Further research to isolate active xanthophylls and to verify molecular mechanisms will be required to clarify fully the value of these effective constituents to cancer therapy.

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