JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant and Antiproliferative Activities of Spirulina and Chlorella Water Extracts

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Liver fibrosis is a chronic liver disease that will further develop to cirrhosis if severe damage continues to form. A potential treatment for liver fibrosis is to inhibit activated hepatic stellate cell (HSC) proliferation and, subsequently, to induce HSC apoptosis. It has been reported that antioxidants are able to inhibit the proliferation of HSCs. In this study, the aqueous extract of spirulina was chosen as the source of antioxidant to investigate the inhibitory effect on the proliferation of HSC. The growth inhibitory effects of aqueous spirulina and chlorella extract on human liver cancer cells, HepG2, were also studied and compared in pairs. Results indicated that the total phenol content of spirulina was almost five times greater than that of chlorella (6.86 ± 0.58 vs 1.44 ± 0.04 mg tannic acid equivalent/g of algae powder, respectively). The antioxidant activity of spirulina determined by the $ABTS^{\bullet+}$ method was higher than chlorella (EC_{50}: 72.44 ± 0.24 μmol of trolox equivalent/g of spirulina extract vs 56.09 ± 1.99 μmol of trolox equivalent/g of chlorella extract). Results of $DPPH^{\bullet}$ assay also showed a similar trend as the $ABTS^{\bullet+}$ assay (EC_{50}: 19.39 ± 0.65 μmol of ascorbic acid equivalent/g of spirulina extract vs 14.04 ± 1.06 μmol of ascorbic acid equivalent/g of chlorella extract). The aqueous extracts of these two algae both showed antiproliferative effects on HSC and HepG2, but spirulina was a stronger inhibitor than chlorella. Annexin-V staining showed that aqueous extract of spirulina induced apoptosis of HSC after 12 h of treatment. In addition, the aqueous extract of spirulina triggered a cell cycle arrest of HSC at the G2/M phase.

KEYWORDS: Spirulina; chlorella; antioxidant; activated hepatic stellate cells; liver fibrosis; apoptosis; phenolics

INTRODUCTION

The blue−green microalgae spirulina, an important source of nutrients in the traditional diet of natives of Africa and Mexico, have been found to be a rich natural source of protein (*1*), carotenoids (*2*), ω-3 and ω-6 polyunsaturated fatty acids, provitamins, and other nutrients such as vitamin A (*3*), vitamin E (*4*), and selenium (*5*). These algae can be extensively grown for alimentary use (dietary supplements) or industrial use (blue colorant, emulsifiers, and thickening and gelling agents). They have been gaining increasing attention relative to the role of antioxidants in improving health and preventing disease (*6*). Experimental studies have demonstrated the antioxidant activity of spirulina in decreasing lead-induced lipid peroxidation and brain lead deposition (*7*). Furthermore, their efficacy in anti-inflammation (*8*), inhibition of zymosan-induced arthritis (*9*), reversing age-induced increases in proinflammatory cytokines, and decreases in cerebellar β-adrenergic function (*10*), as well as inhibition of tumor incidence (*11*, *12*) and prevention of chronic diffusion of liver disease (*13*), have also been reported.

Hepatic stellate cells (HSCs) play a key role in the pathogenesis of hepatic fibrosis (*14*). HSCs are activated after liver damage of any etiology to form myofibroblast-like cells and become the primary source of excessive extracellular matrix (ECM) (*15*, *16*). It was suggested by Murphy (*17*) and Saile (*18*) that a possible approach for the recovery from liver fibrosis is to induce the apoptosis of activated HSCs. Oxidative stress has been implicated in hepatic fibrosis (*19*, *20*). It is reported that oxidants or their byproducts act directly upon HSCs to stimulate collagen synthesis (*21*, *22*). Studies have indicated that oxidative stress may enhance the activation of HSC, whereas antioxidants may suppress this process (*23*, *24*). Antioxidants such as the natural phenolic compounds resveratrol and quercetin could significantly suppress the proliferation of HSC (*24*). Many of the chemical components of spirulina such as phenolic compounds exhibit antioxidant properties (*25*). It would therefore be of interest to investigate the antioxidant activity of spirulina and its effect on the suppression of the proliferation of activated HSC. With the progression of liver fibrosis to cirrhosis, the risk of hepatocellular carcinoma increases substantially. Accordingly, spirulina's effects on the human liver cancer cell line HepG2 were investigated.

Chlorella, another microalgae, has also been reported to show antioxidant activity (*26*) in exhibiting attenuating effects on oxidative stress and suppressing inflammatory mediator activa-

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10.1021/jf0479517 CCC: $30.25
Published on Web 04/13/2005

tion in peritoneal macrophages and liver (*27*). However, there is little information about the effect of spirulina and chlorella on HSC and HepG2. It would therefore be useful to compare the antioxidant activity of spirulina and chlorella and to study their antiproliferative effects on HSC and HepG2 cells.

The characteristics of natural substances in inhibiting carcinogenesis have become an important issue for cancer research. Marine bioresources are one of the bioactive substances that demonstrate potential in anticarcinogenesis (*34*, *35*) and other biofunctions such as the bioactivity of algae aqueous extracts against murine immunocytes (*36*). Natural antioxidants have been suggested to demonstrate antifibrosis effects in liver and lung (*37*−*39*). However, there has been limited information about the effects of algae aqueous extracts' antioxidant capacity on HSCs and liver tumor cells. In this study, aqueous extracts of spirulina and chlorella were therefore investigated as chemoprevention agents for their antioxidant activity and their effects on HSCs and human liver tumor cell line HepG2.

The present work has been carried out to study the total phenolic content, the antioxidant activity, and the antiproliferative activities of aqueous extracts of spirulina (*Spirulina maxima*) and chlorella (*Chlorella vulgaris*). The induction of apoptosis and triggered cell cycle arrest of spirulina aqueous extract on HSCs were determined to delineate the underlying mechanism.

MATERIALS AND METHODS

Materials. Folin−Ciocalte reagent, catechin, gallic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for bioassay, and all other chemicals and organic solvents were purchased from Sigma Chemical Co. (St. Louis, MO). Whatman no. 1 filter paper was bought from Fisher Scientific (Fair Lawn, NY). Chorella (*C. vulgaris*) and spirulina (*S. maxima*) powder were purchased locally (Puli, Nantou, Taiwan). Fetal bovine serum (FBS) was purchased from Gibco Life Technologies (Grand Island, NY).

Sample Preparation. Only a product sample from spirulina and chlorella was used. The tested samples were prepared as follows. Spirulina and chlorella powders were extracted with Millipore water (20 mg/mL) by stirring for 30 min. Extracts were then filtered through Whatman no. 1 filter paper in a Buchner funnel under vacuum. The filtrate was lyophilized until use.

Determination of Total Phenolic Compounds. The total phenolic contents of samples were determined using a modified Folin−Ciocalteu method (*28*) as described previously (*29*). In short, an aliquot of 2.5 mL of 7% sodium carbonate solution was added into the test tubes, which contained a 1 mL aliquot of a known dilution of the extract combining with 0.5 mL of Folin−Ciocalteu's reagent. Color developed for 40 min, and absorbance was measured at 760 nm using the Beckman UV−vis spectrophotometer (Fullerton, CA). The measurement was compared to a standard curve of prepared tannic acid solution and expressed as tannic acid equivalent in milligrams.

DPPH• Radical Scavenging Activity. The radical scavenging activity was assayed by the DPPH• method as described previously (*30*). A 0.5 mL aliquot of 80% ethanol dissolved extract was mixed with 0.25 mL of ethanolic 0.5 mM DPPH• solution and 0.5 mL of 100 mM acetate buffer (pH 5.5). The decrease in absorbance of DPPH• at 517 nm was measured by a Beckman UV−vis spectrophotometer. All tests were performed in triplicate. The antioxidant activity of the test samples was expressed as the median effective concentration for radical scavenging activity (EC_{50}): tested algae extract (mg) required for a 50% decrease in absorbance of DPPH• radicals and expressed relative to ascorbic acid in terms of ascorbic acid equivalent. The inhibition (%) of DPPH• absorbance was calculated based on the formula that $(A_{control} - A_{test}) \times 100/A_{control}$. $A_{control}$ is the absorbance of the control (DPPH• solution without the test sample), and A_{test} is the absorbance of the test sample.

ABTS•+ Assay. ABTS•+ used in the assay was generated from ABTS salt, in which 2.45 mM potassium persulfate ($K_2S_2O_8$) was reacted with 7 mM ABTS salt in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 16 h at room temperature in the dark (*31*). The resultant ABTS•+ radical cation was diluted with 0.01 M PBS, pH 7.4, to give an absorbance of around 0.70 at 734 nm. The standard and sample spirulina and chlorella extracts were diluted 100× with the ABTS•+ solution to a total volume of 1 mL and allowed to react for 3 min. Control (without a standard or sample) was used as blank. Trolox, the water soluble α-tocopherol (vitamin E) analogue, served as a standard, and the results of the assay were expressed relative to trolox in terms of trolox equivalents.

HepG2 Cell Culture. The human hepatoma cell line HepG2/C3A (CCRC 60177) was obtained from the Food Industry Research and Development Institute (FIRDI, Hsin-Chu, Taiwan) and was cultured in a minimal essential medium (MEM) containing 10% FBS. At confluence, HepG2 cells were subcultured by trypsinization with 0.25% sterile trypsin at 1:6 split ratios approximately every 5 or 6 days. HepG2 cells were cultured in the respective tissue culture plates and incubated at 37 °C in humidified air containing 5% CO_2. For the MTT assay, HepG2 cells were seeded in clear 96 well microplates and cultured for 3 days. Cell viability was checked by adding 0.4% Trypan Blue. The number of Trypan Blue-positive dead cells and Trypan Blue-negative live cells was counted on a hemocytometer under a microscope.

HSC Isolation and Culture. Stellate cells were prepared from normal rat liver as described in Kawada et al. (*24*). Briefly, the liver was perfused with a Ca^{2+}/Mg^{2+}-free Gey's balanced salt solution for 10 min at 37 °C (10 mL/min) followed by Pronase and collagenase digestion for 40 min at 37 °C. The digested liver was excised, cut into small pieces, and incubated with gentle stirring in Gey's balanced salt solution containing 0.08% Pronase E, 0.04% collagenase, and 20 μg/mL DNase for 30 min at pH 7.3. The resulting suspension was filtered through a 150 μm steel mesh and centrifuge (1400*g* at 4 °C for 20 min) on an 8.2% Nycodenz (Sigma) cushion, which produced a stellate cell-enriched fraction in the upper whitish layer. Those cells were washed by centrifugation (400*g* at 4 °C for 10 min), cultured in DMEM supplemented with 10% FBS and antibiotics (70 mg/L penicillin and 100 mg/L streptomycin), and used for the experiments. Cell purity was around 95%, as assessed by a typical starlike configuration.

Determination of Inhibition of HepG2 Cell Proliferation. The MTT assay was used to measure the ability of extracts to inhibit HepG2 human liver cancer cell proliferation (*32*). The cell cultures were exposed to various concentrations of the extracts during a 72 h growth period. Cell proliferation was measured by the ability of viable cells to reduce MTT to formazan based on the ability of living cells to utilize thiazolyl blue and convert it into purple formazan, which absorbs light at 570 nm and could be analyzed spectrophotometrically. Measurement was performed in triplicate. The absorbance was measured using the Beckman UV−vis spectrophotometer. The effective median dose (EC_{50}) was determined and expressed as milligrams of extracted sample ± SD.

Flow Cytometric Quantification of Apoptosis of Isolated HSCs. Annexin-V-fluorescein isothiocyanate (FITC) (Pharmingen, Becton Dickinson, Franklin Lake, NJ) was known to have high affinity to phosphatidylserine, which was used to stain cells and to detect early apoptotic changes. Measurement of annexin-V binding was performed simultaneously with a dye exclusion test using propidium iodide (PI) (Sigma Chemical Co.) to discriminate between apoptosis and necrosis. Briefly, cells treated with extracts and untreated ones were trypsinized from six well dishes and then stained with annexin-V and PI. After the addition of 5 μL of annexin-V-FITC and 10 μL of PI to the mixture, followed by incubation for 15 min in the dark, cells were analyzed on a flow cytometer (FACS, Beckton Dickinson, CA). Only fluoresceine-positive cells without PI staining were regarded as apoptotic cells.

DNA Content Assay by Flow Cytometry. Cultured cells with and without exposure to algae extracts were analyzed by flow cytometry at 24 h after the beginning of the treatment. Floating and trypsinized adherent cells were collected and adjusted to 1×10^6 cells, washed with PBS, and mixed in cold 70% ethanol for 4 h at 37 °C. Then, the cells were washed with PBS again, incubated in phosphate citrate buffer

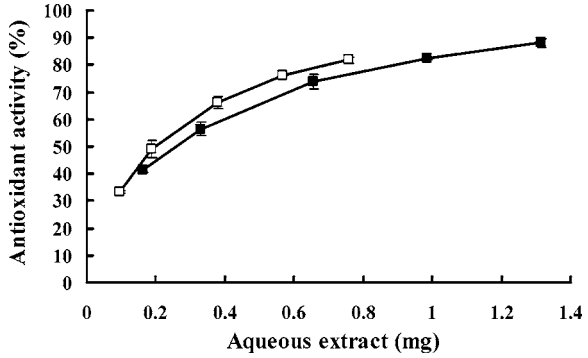

Figure 1. Antioxidant activity of aqueous extracts of spirulina (□) and chlorella (■) determined by ABTS method. Values are means ± SD (n = 3); error bars represent ± 1 SD.

for 30 min, and treated with RNase A for 30 min at room temperature. Finally, the cells were stained with PI (*33*). After 1 h of incubation, cells were analyzed on a flow cytometer (FACS Calibur).

RESULTS AND DISCUSSION

Total Phenolic Compounds and Antioxidant Activity. Our results showed that spirulina extracted with water had almost five times more total phenolic compounds than did chlorella (6.86 ± 0.58 vs 1.44 ± 0.04 mg tannic acid equivalent/g of algae powder). It has been suggested that the antioxidant activity had a positive relationship with the total phenolic content (*29*). As expected, with the increase of the extracts, the antioxidant activity increased in both algae groups (**Figure 1**). The values of EC_{50} determined by $ABTS^{\bullet+}$ method of spirulina and chlorella extract were 72.44 ± 0.24 and 56.09 ± 1.99 μmol of trolox equivalent/g of aqueous extract, respectively. Similarly, the values of EC_{50} measured by $DPPH^{\bullet}$ method of spirulina and chlorella aqueous extracts were 19.39 ± 0.65 and 14.04 ± 1.06 μmol of ascorbic acid equivalent/g of aqueous extract, respectively.

Spirulina extract was a stronger antioxidant than was chlorella, which was probably because of the higher content of phenolic compounds (23.87 vs 15.25 mg tannic acid equivalent/g of algae aqueous extract). Moreover, per gram of the algae powder, the antioxidant capacity of spirulina was better ($ABTS^{\bullet+}$ assay: 19.74 ± 0.64 vs 4.60 ± 0.19 μmol of trolox equivalent/g of algae powder).

Miranda et al. (*25*, *26*) studied the antioxidant activity of chlorella methanolic extracts. Those results were higher than those for aqueous extract reported here. This could be due to differences in the solubility of phenolic compounds in different extraction solvent systems and constituent variance among algae species. There was little information on the spirulina aqueous extract's total phenolic content, but its methanolic extract was reported to have antioxidant activity in vitro determined on a brain homogenate (*25*). Evidence indicated that antioxidants inhibit HSC proliferation (*40*) or hepatocellular carcinoma (*41*). Because it was observed in our study that spirulina showed a higher antioxidant activity than chlorella, it was, therefore, valuable to compare their antiproliferative effects on HSC and HepG2.

Antiproliferative Effect on HSCs and HepG2. HSCs and HepG2 cells were cultured in 10% FBS containing medium with or without aqueous extracts of spirulina and chlorella for 72 h. Cell proliferation was evaluated by the MTT assay. Under those experimental conditions, a dose-dependent decrease in cell proliferation was observed with treatment of spirulina aqueous extract on both sets of cells (**Figure 2**). The antiproliferative

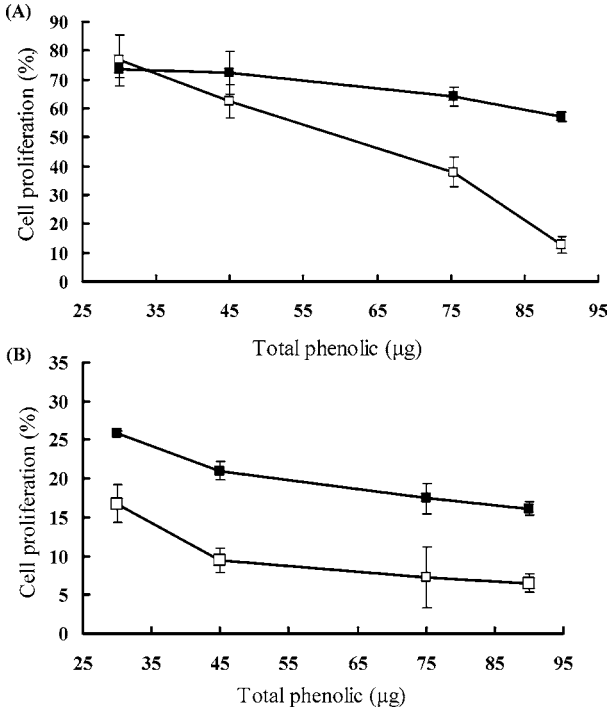

Figure 2. Antiproliferative effect of aqueous extracts of spirulina (□) and chlorella (■) on HepG2 cells (**A**) and HSCs (**B**). Values are means ± SD (n = 3); error bars represent ± 1 SD.

effect of spirulina aqueous extract on HepG2 and HSCs was stronger than that of chlorella (**Figure 2**). Results indicated that HSCs were more sensitive to these two algae aqueous extracts than were HepG2 cells. The value of EC_{50} of spirulina on HepG2 was at a total phenolic concentration of 60.22 μg, whereas the value of EC_{50} for chlorella was not able to be obtained from the curve (**Figure 2A**).

Antioxidants regulate stellate cells, which is not necessarily related to their antioxidative action. Natural phenolics regulate the activity of receptor tyrosine kinase and the expression of cell cycle protein cyclin D1, thereby modulating functions of stellate cells (*24*). On the other hand, sulfhydryl antioxidants exert their reducing activity to regulate stellate cells (*23*). Accumulated evidence indicates that natural phenolic compounds such as resveratrol and quercetin are potent inhibitors for the growth of stellate cells by perturbing the signal transduction pathway and cell cycle protein expression (*24*). Additionally, it was reported that quercetin was capable of inducing selective growth inhibition and apoptosis in hepatic tumor cells but not in normal cells (*42*). It was likely that the algae extracts in this study might constitute specific compounds that are potential anticancer or antifibrosis reagents. Further study on identification of these agents is under way. Spirulina aqueous extracts showed significant antiproliferative effect on HSC. The mechanism behind this effect is further discussed below.

Spirulina Aqueous Extract Induced Apoptosis on HSCs. A 150 μg amount of spirulina aqueous extract (2 mL of 75 μg/mL) treated HSCs were stained with FITC-conjugated annexin-V. Cells were stained with PI at the same time. Flow cytometry analysis was used to quantify apoptotic cells after excluding PI-positive necrosis cells. It was indicated by flow cytometry analysis that the fluorescence intensity of the stained cell population increased with the progression of time. Apoptotic cells stained with annexin-V increased to 25% after 48 h of culture (**Figure 3A**). The respective phase contract images were

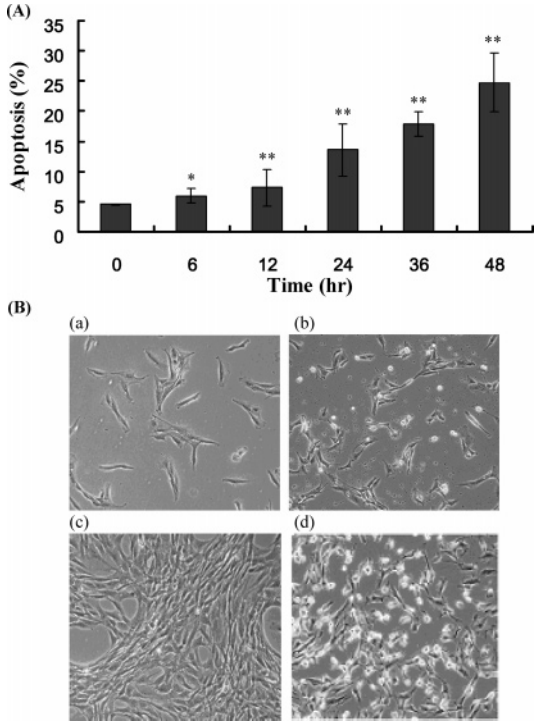

Figure 3. Induction of HSC apoptosis by spirulina aqueous extract determined by annexin-V/PI assay with flow cytometry (**A**). The progression of apoptotic cells with time (**B**) and respective phase contrast microscopic images of control after 12 (a) and 48 (c) h of incubation. The corresponding treated groups were at 12 (b) and 48 (d) h. Values are means ± SD ($n = 3$); error bars represent ± 1 SD. *$p < 0.05$; **$p < 0.01$.

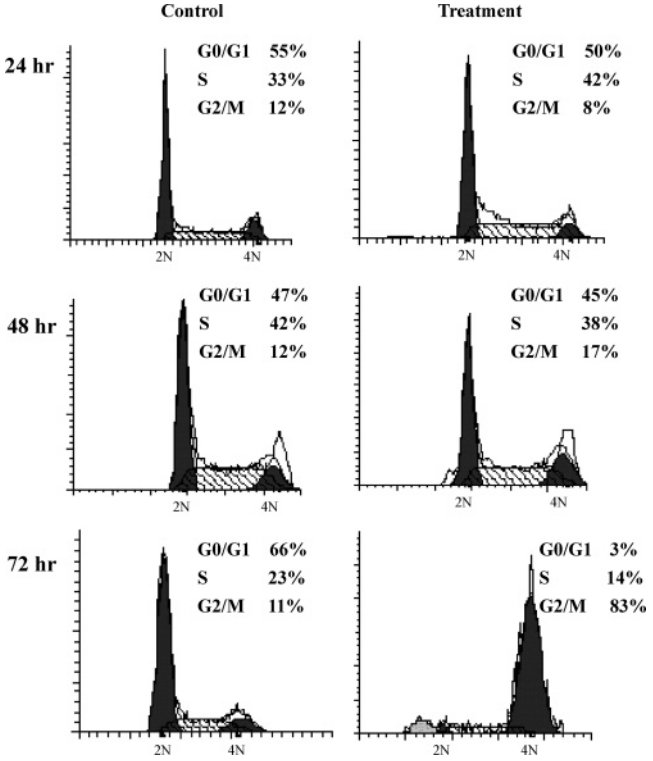

Figure 4. Cell cycle analysis of HSC by PI staining after the treatment of spirulina aqueous extract.

shown in **Figure 3B**. Apoptotic cells were rounded up and lifted from the surface monolayer (**Figure 3B**,**b**) after 12 h of incubation. The algae extract-treated cells showed pronounced morphological changes such as cell shrinkage and formation of membrane blebs. More significant apoptotic cells were observed after 48 h of incubation (**Figure 3B**,**d**).

The resolution of hepatic fibrosis is associated with remodeling of the excess liver matrix and may result in restitution of near-normal liver architecture (*43*−*45*). An essential element of the recovery process of liver fibrosis may be the apoptosis of activated HSCs (*43*). Apoptosis is a physiological process critical to the removal of damaged, senescent, or unwanted cells. The literature has suggested that spirulina demonstrates an important role in the amelioration of liver disease (*46*). However, little attention has been focused on the induction of apoptosis by spirulina on HSCs. In the current study, we found that the spirulina aqueous extract was able to induce apoptosis of HSC, which could explain in part the beneficial (therapeutic) effects of spirulina on liver disease.

Spirulina Induced Cell Cycle Arrest at the G2/M Stage. The antiproliferative effect observed in spirulina extract-treated HSCs was further verified by flow cytometric analysis of DNA content. Loss of DNA is a typical feature of apoptotic cells. Exploration of the intrinsic mechanism of the inhibitory effect of the extract on cell growth and cell cycle arrest was performed with 100 μg of spirulina (2 mL of 50 μg/mL) extract or without by FACS analysis after 24, 48, and 72 h of culture. The DNA content histograms obtained after PI staining of permeabilized cells are shown in **Figure 4**. The FACS analysis of control cells showed prominent G1, followed by S and G2/M phases. In the treated group, a typical subdiploid peak was observed after 72 h of culture, which implied the presence of cells with fragmented DNA. It was necessary to distinguish the source of cell death as from either necrotic or apoptotic cells. Other methods such as TUNEL or annexin-V assay were required for the reconfirmation of the types of cell death. In agreement with the annexin-V assay (**Figure 3**), the aqueous extract of spirulina induced the apoptosis of HSC. From the results of flow cytometric analysis (**Figure 4**), it was observed that there was a significantly increased percentage at the G2/M phase, along with a dramatically decreased cell population of the G1 phase. The value of G2/M phase increased from 8 to 83% in treated cells after 72 h of culture, whereas the value of G2/M phase in control cells was around 12%. These results suggested that the aqueous extract of spirulina could inhibit cell proliferation by inducing the G2/M arrest in HSC through regulating the G2 checkpoint. Additionally, induced apoptosis by aqueous extract of spirulina was observed with the increased hypodiploid apoptotic cells.

Liver fibrosis requires the maintenance of activated HSCs, which overproduce ECM. Potential strategies to terminate activated HSC for prevention and treatment of liver fibrosis are to inhibit cell proliferation and to induce apoptosis. Aqueous extracts of blue−green algae spirulina and chlorella demonstrated antiproliferative effects on HSC and HepG2, suggesting that these microalgae possess the potential for amelioration of liver fibrosis and probably liver tumors. The results of this study are in agreement with clinical and laboratory studies (*13*) that suggest that administration with spirulina would be a valid treatment in chronic diffuse liver conditions due to its hepatoprotective properties. Spirulina aqueous extract triggered HSC cell cycle arrest at the G2/M phase, which resulted in apoptosis. Cell population with hypodiploid was determined by flow cytometric analysis; annexin-V staining reconfirmed the progression of apoptosis. In conclusion, these findings revealed the potential antifibrotic action of blue−green algae, confirming their promise as a possible therapeutic treatment for liver fibrosis.

ABBREVIATIONS USED

HSC, hepatic stellate cells; MEM, minimal essential medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline.

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Received for review December 5, 2004. Revised manuscript received March 10, 2005. Accepted March 17, 2005. This work was supported by NSC 92-2113-M-260-012, Taiwan National Science Council; TCVGH-NCNU 937903A, Taichung Veterans General Hospital and National Chi-Nan University.

JF0479517