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Evaluation of Carotenoid Extract from *Dunaliella salina* against Cadmium-Induced Cytotoxicity and Transforming Growth Factor *β1* Induced Expression of Smooth Muscle *α*-Actin with Rat Liver Cell Lines

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ABSTRACT

The carotenoid extract from *Dunaliella salina* was identified and quantitated. The effects against cadmium (Cd)-induced hepatotoxicity and transforming growth factor *β*1 (TGF-*β*1)-induced activation of hepatic stellate cells (HSCs) were evaluated. All-*trans*-*β*-carotene (474.82 mg/g extract) and 9- or 9′-*cis*-*β*-carotene (425.64 mg/g extract) were major carotenoids in the extract, accounting for 90.30% of total carotenoids (997.17 mg/g extract). Other carotenoids were all-*trans*-lutein (22.77 mg/g extract), all-*trans*-zeaxanthin (39.26 mg/g extract), 13- or 13′-*cis*-*β*-carotene (17.18 mg/g extract), all-*trans*-*α*-carotene (9.26 mg/g extract) and 9- or 9′-*cis*-*α*-carotene (8.24 mg/g extract). The algal extract had a higher effect ($p < 0.05$) than all-*trans*-*β*-carotene in the reduction of cytotoxicity for $CdCl_2$-treated Clone 9 cells and expression of smooth muscle *α*-actin (*α*SMA) for TGF-*β*1-treated HSC-T6 cells. These effects were shown to be concentration-dependent.

Key words: cadmium, carotenoid, cytotoxicity, *Dunaliella salina*, HSC-T6 cells, Clone 9 cells, HPLC analysis, smooth muscle *α*-actin, *α*SMA, transforming growth factor *β*1, TGF-*β*1

INTRODUCTION

Dunaliella salina is unicellular and belongs to the class *Chlorophyceae* and the order *Volvocales*[1,2]. It is a type of halophile micro-algae that lacks a cell wall but has a mucus surface coat[2-5]. Algae gained commercial interest because it contains large amounts of *β*-carotene and is widely used in cosmetics and dietary supplements[6,7]. Carotenoids can reduce lipid peroxidation and singlet oxygen ($^1O_2$) formation[8,9]. Many reports indicate that carotenoids could lower the incidence of certain types of cancers, coronary heart disease and other degenerative diseases[10-15]. Raja *et al.*[2] found that *Dunaliella salina* could protect rats against fibrosarcoma. However, no thorough report has been conducted on the effect of algal carotenoid extract against cadmium (Cd)-induced cytotoxicity and transforming growth factor *β*1 (TGF-*β*1) induced activation of hepatic stellate cells (HSCs).

Cd is a toxic heavy metal, as well as an important industrial pollutant and food contaminant. It influences cellular functions such as enzyme activities, DNA repair systems, redox state of the cell and signal transduction. Cd also increases the risk of lung, prostate, kidney, liver, pancreatic and stomach cancer[16,17]. It induces both apoptosis and necrosis of the normal cells[18]. Shaikh *et al.*[19] reported that oxidative stress plays a major role in chronic Cd-induced hepatotoxicity, and antioxidants could protect animals against Cd-induced hepatotoxicity.

HSCs have important functions in the liver, such as the production of cytokines and growth factors, remodeling of extracellular matrix (ECM), and retinoid storage[20]. Nevertheless, after the liver has sustained

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injury or damage, HSCs are activated and transformed into proliferative myofibroblast-like cells that produce excessive collagen fiber in liver cirrhosis[21]. Therefore, suppression of HSC activation is believed to be a therapeutic target against liver fibrosis[22]. Cheng *et al.*[21] indicated that transforming the growth factor β1 (TGF-β1) is the most efficient single profibrogenic factor for the initiation and maintenance of fibrogenesis in the liver. It accelerates HSC activation, upregulates collagen expression, and decreases collagen degradation. Therefore, stimulation of HSC activation by TGF-β1 is considered to be the key fibrogenic response in hepatic fibrosis. TGF-β*1* has been known to induce the expression of smooth muscle α-actin (αSMA) in fibroblasts[21]. Horie *et al.*[23] illustrated that αSMA expression is a reliable marker for the identification of activated HSCs in human and rat liver.

In this study, carotenoid extract was prepared from *Dunaliella salina,* and normal rat liver Clone 9 cells and hepatic stellate HSC-T6 cells were used to evaluate the capacity of the algal extract against Cd-induced cytotoxicity and TGF-β1-induced αSMA expression, respectively.

MATERIALS AND METHODS

I. *Dunaliella salina Sample*

Spray dried powder of *Dunaliella salina* algae cultivated in Taiwan was purchased from Gong Bih Enterprise Co., Ltd. (Wunlin, Taiwan).

II. *Chemicals and Standards*

All-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin standards were obtained from Sigma Co. (St. Louis, MO, U.S.A.). Solvents for the extraction and determination of carotenoids, such as acetonitrile, methanol, methylene chloride, ethanol, acetone and *n*-hexane, were purchased from Merck Co. (Darmstadt, Germany). Deionized water was prepared by Ultrapure™ water purification system (Lotun Co., Ltd. Taipei, Taiwan). Ham's F-12 Medium, Waymouth MB 752/1 medium and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), *o*-phenylenediamine dihydrochloride (OPD), penicillin, phosphate-buffered saline (PBS), streptomycin, cadmium chloride ($CdCl_2$) and TGF-β1 were obtained from Sigma Co. (St. Louis, MO, U.S.A.).

III. *Extraction of Carotenoid from Dunaliella salina*

The method reported by Hu *et al.*[24] was used. 10 g of *Dunaliella salina* powder were extracted with 250 mL of hexane/acetone/ethanol (2 : 1 : 1, v/v/v) at 25°C for 24 h in a shaker. Saponification was then performed by adding 10 mL of 40% methanolic KOH at 25°C for 16 h. After filtering, the extract was transferred to a separatory funnel and washed with 250 mL of distilled water for 3 times. The solvent was removed by a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) to yield the carotenoid extract. 5 μg of the extract was dissolved in 1 mL of methanol/methylene chloride (1 : 1, v/v) for analysis. The extract was stored in the dark at -80°C prior to analysis.

IV. *Determination of Carotenoids*

The high performance liquid chromatography (HPLC) method used was based on that reported by Hu *et al.*[24] and Inbaraj *et al.*[25]. A *PrimeLine*™ Gradient Model 500G HPLC pump system (Analytical Scientific Instruments, Inc., El Sobrante, CA, U.S.A.) with a S-3210 photodiode-array detector (PDA) (Schambeck SFD GmbH, Bad Honnef, Germany) was used. A YMC C30 column (250 × 4.6 mm, 5 μm) (Waters Co., Milford, MA, U.S.A.) and a gradient solvent system consisting of (A) methylene chloride and (B) methanol-acetonitrile-water (84 : 14 : 2, v/v/v) were used for the separation of carotenoids. The gradient condition was as follows: (A) was increased from 10 to 25% from 0 to 15 min and kept at 25% from 15 to 40 min. The flow rate was set at 1 mL/min and the UV spectra were recorded from 210-650 nm at a rate of 1.00 spectrum/s. The identification of carotenoids in the algal extract was performed by comparing the retention times and absorption spectra of unknown peaks with those obtained from reference standards, and adding carotenoid standards to the sample for co-chromatography. The identification of *cis*-forms of carotenoids was based on the spectral characteristics and *Q*-ratios (the height ratio of the *cis* peak to the main absorption peak) reported in literature[24-29]. The peak purity of carotenoids was determined by the S-3210 PDA automatically. For the calculation, the peak maximum (apex) and the half-function values on the left (upslope) and the right (downslope) of the peak were compared.

V. *Cell Viability Assay*

In this study, normal rat liver Clone 9 cells and rat hepatic stellate HSC-T6 cells were used. These cells have been used in various hepatotoxicity and hepatofibrosis tests, respectively. The Clone 9 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and maintained in Ham's F-12 medium containing heat-inactivated 10% FBS and penicillin (100 units/mL). The HSC-T6 cells isolated from male retired breeder Sprague-Dawley rats, as described by Friedman[30], were maintained in Waymouth MB 752/1 medium supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/mL)-streptomycin (100 mg/mL) medium. The experimental methods were based on those reported by Bruscalupi *et al.*[16] and Lin and Chou[31]. These two kinds of cells were washed with PBS, re-suspended in the

Journal of Food and Drug Analysis, Vol. 18, No. 5, 2010

medium at a density of 5×10^4 cells/mL and then seeded in each well of 24-well flat-bottom plates (1 mL/well), separately. After these cells were incubated in 5% CO_2/air at 37°C for 24 h, 1 μL of DMSO solution containing the algal carotenoid extract or all-*trans*-*β*-carotene was added to obtain the final levels of 0, 5, 10 and 20 μg/mL, respectively. For the blank control well, 1 μL of DMSO was added. Subsequently, the cells were incubated for 4 h. $CdCl_2$ (final concentration of 5 μM) or TGF-*β*1 (2 ng/mL) was added to each well and the cells were incubated for another 20 h. The cell growth was counted by MTT assay[32] as follows: 1 mL of MTT (0.5 mg/mL) in PBS was added to each well and incubated for 4 h at 37°C. The metabolized product of MTT was dissolved by isopropanol. The proportion of surviving cells was determined at 563 nm.

VI. *αSMA Expression Assay*

*α*SMA expression was measured by a cell enzyme-linked immunosorbent assay (ELISA), according to the method of Horie *et al.*[23]. The HSC-T6 cells treated with various concentrations of samples and TGF-*β1* were incubated with monoclonal anti-*α*SMA antibody (DAKO, Carpinteria, CA) at room temperature for 1 h. The second antibody used was HRP-conjugated goat anti-mouse IgG (DAKO Carpinteria, CA, U.S.A.). After adding OPD under dim lighting for 20 min, the reaction was terminated by adding 1 N sulfuric acid. *α*SMA expression was detected at 490 nm.

VII. *Statistical Analysis*

All assays were performed in triplicate and the mean values were calculated. The data were subjected to analysis of variance (ANOVA) and Duncan's multiple range test to assess differences between means. Significant difference was affirmed at a level of $p < 0.05$.

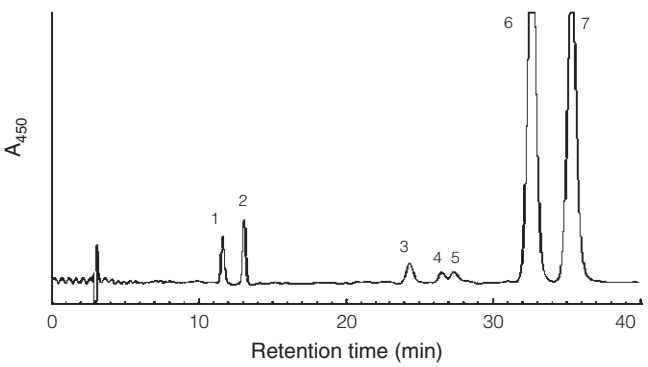

Figure 1. HPLC chromatogram of carotenoid extract from *Dunaliella salina*. Refer to Table 1 for the identification of peaks.

RESULTS AND DISCUSSION

I. *Content of Carotenoids in the Algal Extract*

The yield of the algal extract was 28.69%. The HPLC chromatogram of the extract from *Dunaliella salina* was shown in Figure 1. There were 7 compounds in the extract and all of their peak purities were higher than 90% (Table 1). By matching peaks 1, 2, 4 and 6 with reference standards, they were identified as the all-*trans* forms of lutein, zeaxanthin, *α*-carotene and *β*-carotene, respectively. The remaining peaks (3, 5 and 7) were identified by comparing their spectral characteristics and *Q*-ratio values with those presented in literature[24-29]. Peak 3 was identified as 13- or 13′-*cis*-*β*-carotene, according to Inbaraj *et al.*[25] and Lin and Chen[28]. Peak 5 was identified as 9- or 9′-*cis*-*α*-carotene, according to Inbaraj *et al.*[25] and Böhm *et al.*[27]. Peak 7 was identified as 9- or 9′-*cis*-*β*-carotene, according to Inbaraj *et al.*[25] and Liu *et al.*[26].

The total concentration of carotenoids in the algal extract was 997.17 mg/g, indicating that the extract consisted of mainly carotenoids. All-*trans*-*β*-carotene

Table 1. Identification of all-*trans* and *cis* forms of carotenoids in the algal extract

Peak No.	Compound	Retention Time (min)	λ (nm)	*Q*-ratio [a]	Peak Purity (%)
1	All-*trans*-Lutein	11.63	333 [c] 423 445 [b] 470	0.12	96.4
2	All-*trans*-Zeaxanthin	13.08	344 [c] 426 453 [b] 473	0.10	97.2
3	13- or 13′-*cis*-β-Carotene	24.32	344 [c] 423 447 [b] 475	0.46	93.4
4	All-*trans*-α-Carotene	26.52	344 [c] 422 446 [b] 474	0.11	92.8
5	9- or 9′-*cis*-α-Carotene	27.35	344 [c] 422 450 [b] 475	0.19	90.8
6	All-*trans*-β-Carotene	32.43	348 [c] 428 454 [b] 478	0.10	96.2
7	9- or 9′-*cis*-β-Carotene	35.66	346 [c] 428 450 [b] 476	0.17	98.5

[a] *Q*-ratio: Height ratio of the *cis* peak to the main absorption peak
[b] The major absorption, λ
[b, c] The λ values used for *Q*-ratio calculation

(474.82 mg/g extract) and 9- or 9′-*cis*-*β*-carotene (425.64 mg/g extract) were the major carotenoids in the extract, accounting for 90.30% of the total carotenoids. The content of all-*trans*-lutein, all-*trans*-zeaxanthin, 13- or 13′-*cis*-*β*-carotene, all-*trans*-*α*-carotene and 9- or 9′-*cis*-*α*-carotene were 22.77, 39.26, 17.18, 9.26 and 8.24 mg/g, respectively (Table 2).

Our data corroborated the results of García-Conzález *et al*.[5], Hu *et al*.[25] and Ben-Amotz *et al*.[33]. Previous studies also indicated that *Dunaliella salina* contained a substantial amount of 9- or 9′-*cis*-*β*-carotene and all-*trans*-*β*-carotene, which were the major carotenoids. In this study, 4 all-*trans* and 3 *cis* forms of carotenoids were observed in *Dunaliella salina* cultivated in Taiwan.

Table 2. The content of caroteinoid in the algal extract

Compound	Content (mg/g extract) [a]
All-*trans*-Lutein	22.77 ± 5.37
All-*trans*-Zeaxanthin	39.26 ± 4.26
13- or 13′-*cis*-β-Carotene	17.18 ± 2.52
All-*trans*-α-Carotene	9.26 ± 0.94
9- or 9′-*cis*-α-Carotene	8.24 ± 1.02
All-*trans*-β-Carotene	474.82 ± 10.51
9- or 9′-*cis*-β-Carotene	425.64 ± 12.12
Total amount	997.17

[a]. All values are means ± SD obtained by triplicate analyses.

II. *Effect of the Algal Extract on Viability of Clone 9 Cells Treated with Cd*

Bruscalupi *et al*.[16] indicated that concentrations of Cd between 0.1 and 5 μM resulted in an increased inhibitory effect on the viability of rat hepatocytes. This study referred to their method to induce cytotoxicity in Clone 9 cells using 5 μM of $CdCl_2$, which is the suitable concentration for the functional evaluation of the algal extract. As all-*trans*-*β*-carotene is a major compound in the algal extract and also a commercial carotenoid, it was used for comparison in the experiments. Figure 2 showed that there was 43.9% survival of Clone 9 cells treated with $CdCl_2$ alone. When the algal extract or all-*trans*-*β*-carotene was added, followed by $CdCl_2$, mortality of the cells was lowered significantly. The survival of Clone 9 cells increased with the concentrations of the algal extract and all-*trans*-*β*-carotene. The algal extract resulted in higher cell viability than all-*trans*-*β*-carotene. The highest survival of Clone 9 cells was 66.3% when 20 *μ*g/mL of algal extract was used. At the same concentration, the survival rate of the cells treated with all-*trans*-*β*-carotene was 60.3%. The difference was statistically significant (Figure 2).

Shaikh *et al*.[19] demonstrated that chronic Cd administration increased lipid peroxidation in rat liver. However, co-administration of antioxidants (*n*-acetylcysteine or vitamin E) and Cd could reduce Cd-induced lipid peroxidation and protect rats against hepatotoxicity. Carotenoids could also act as antioxidants to suppress lipid peroxidation[8]. Our results showed that all-*trans*-*β*-carotene as well as the carotenoid extract from *Dunaliella salina* lowered Cd-induced hepatotoxicity effectively. The algal extract had a higher effect than all-*trans*-*β*-carotene. Hu *et al*.[24] demonstrated that the carotenoid extract from *Dunaliella salina* had a higher antioxidant capacity than all-*trans*-*β*-carotene. Similarly, Jimenez and Pick[10] and Leivin *et al*.[34] described that 9- or 9′-*cis*-*β*-carotene had more efficiency against oxidative damage. Ben-Amotz and Fishler[35] indicated that 9- or 9′-*cis*-*β*-carotene is a potential compound for chemoprevention of oxidation. In addition to all-*trans*-*β*-carotene, the algal extract also contained abundant 9- or 9′-*cis*-*β*-carotene and other trace carotenoids (Table 2), which might improve the effect of the algal extract against Cd-induced hepatotoxicity.

III. *Effect of the Algal Extract on αSMA Expression of HSC-T6 Cells Treated with TGF-β1*

2 ng/mL of TGF-*β*1 was used in this study because TGF-*β*1 did not affect HSC-T6 cell growth and could induce higher expression of αSMA under this concentration. All-*trans*-*β*-carotene and the algal extract (5-20 μg/mL) also did not influence cell growth (data not shown). Figure 3 showed that all-*trans*-*β*-carotene and the algal extract reduction of TGF-*β*1-induced *α*SMA expression of HSC-T6 cells was concentration-dependent. At 20 μg/mL, the algal extract had significantly higher effect (decreased 21.8% of the *α*SMA expression compared with controls) than all-*trans*-*β*-carotene (decreased 18.2% of the *α*SMA expression compared with controls).

Chen *et al*.[36] demonstrated that the antioxidant, (-)-epigallo-catechin-3-gallate (ECG), could inhibit activated HSC growth. Ali-Riza *et al*.[37] found that antioxidants, vitamin E, vitamin C, and their combination retard hepatic fibrosis in rats. They further indicated that oxidative stress plays an important role in the pathogenesis of hepatic fibrosis. Our results also showed that the carotenoid extract from *Dunaliella salina* could inhibit HSC activation. The algal extract should have the capacity to prevent liver fibrosis.

CONCLUSIONS

The carotenoid extract of *Dunaliella salina* was found to contain abundant all-*trans*-*β*-carotene and 9- or 9′-*cis*-*β*-carotene, and other five minor carotenoids. The algal extract was demonstrated to significantly decrease

Journal of Food and Drug Analysis, Vol. 18, No. 5, 2010

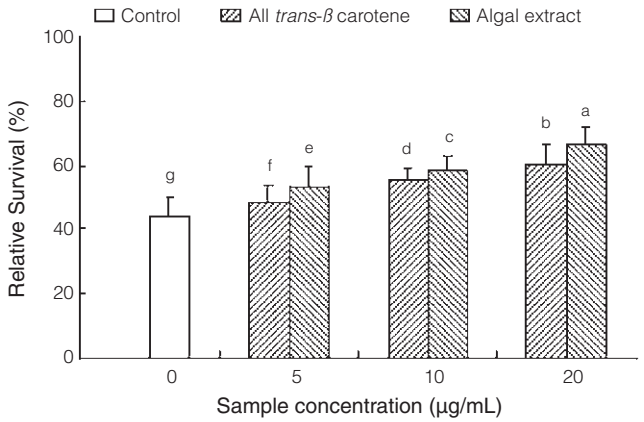

Figure 2. Effects of the algal extract and all *trans-β*-carotene on the survival of Clone 9 cells with Cd-induced cytotoxicity. Clone 9 cells were treated with various concentrations of samples and 5 μM of $CdCl_2$. Their survival was determined by MTT assay. Each value is expressed as mean ± SD (n = 3). Means with different letters were significantly different at the level of $p < 0.05$.

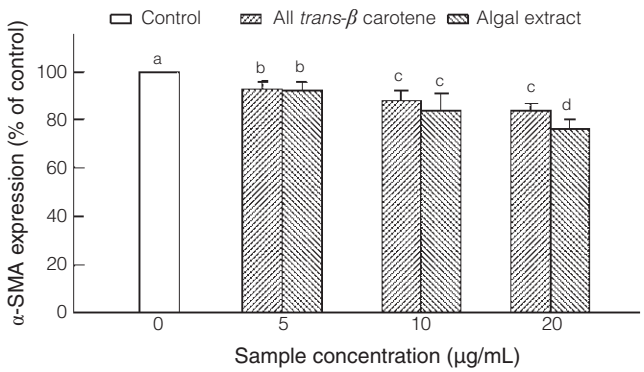

Figure 3. Effects of the algal extract and all *trans-β*-carotene on *α*SMA expression of HSC-T6 cells induced by TGF-*β*1. HSC-T6 cells were treated with various concentrations of samples and 2 ng/mL of TGF-*β*1. The percentage of *α*SMA expression was determined by ELISA assay. Each value is expressed as mean ± SD (n = 3). Means with different letters were significantly different at the level of $p < 0.05$.

Cd-induced cytotoxicity in liver cells and TGF-*β*1-induced HSC activation. It also produced higher effects compared to all-*trans*-*β*-carotene. We concluded that in addition to all-*trans*-*β*-carotene, other carotenoids in the extract, especially 9- or 9′-*cis*-*β*-carotene, might contribute to the commendable efficacy. *Dunaliella salina* has the potential to be used for liver protection.

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