

## Cytoprotective effect of fucoxanthin isolated from brown algae *Sargassum siliquastrum* against H<sub>2</sub>O<sub>2</sub>-induced cell damage

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**Abstract** In this study, the cytoprotective effect of fucoxanthin, which was isolated from *Sargassum siliquastrum*, against oxidative stress induced DNA damage was investigated. Fucoxanthin, a kind of carotenoid, was pretreated to the medium and the protective effect was evaluated via 2',7'-dichlorodihydrofluorescein diacetate, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide, and comet assays. Intracellular reactive oxygen species were over produced by addition of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), but the production was significantly reduced by the treatment with fucoxanthin. The fucoxanthin strongly enhanced cell viability against H<sub>2</sub>O<sub>2</sub> induced oxidative damage and the inhibitory effect of cell damage was a dose-dependent manner. Furthermore, a protective effect against oxidative stress-induced cell apoptosis was also demonstrated via nuclear staining with Hoechst dye. These results clearly

indicate that fucoxanthin isolated from *S. siliquastrum* possesses prominent antioxidant activity against H<sub>2</sub>O<sub>2</sub>-mediated cell damage and which might be a potential therapeutic agent for treating or preventing several diseases implicated with oxidative stress.

**Keywords** Fucoxanthin · *Sargassum siliquastrum* · Cell damage · Oxidative stress · Reactive oxygen species

### Introduction

Reactive oxygen species (ROS), which include free radicals such as superoxide anion radical (O<sub>2</sub><sup>-</sup>), hydroxyl radicals (-OH) and non free-radical species such as H<sub>2</sub>O<sub>2</sub> and singlet oxygen (<sup>1</sup>O<sub>2</sub>), formed during normal metabolic processes, which can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. Free radical scavengers and antioxidants can reduce lipid peroxidation and the generation of ROS. The importance of antioxidants in human health has become increasingly clear due to spectacular advances in understanding the mechanisms of their reaction with oxidants. Furthermore, interest in employing antioxidants from natural sources to increase the shelf-life of foods is considerably enhanced by consumer preference for natural ingredients and concerns about the toxic effects of synthetic antioxidants [1–3].

Algae, as photosynthetic organisms, are exposed to a combination of light and high oxygen concentrations what induces the formation of free radicals and other oxidative reagents. The absence of structural damage in the algae leads to consider that these organisms are able to generate the necessary compounds to protect themselves against oxidation [4]. In this respect, algae can be considered as an

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important source of antioxidant compounds that could be suitable for protecting our bodies against the ROS formed e.g. by our metabolism or induced by external factors (as pollution, stress, UV radiation, etc.).

Fucoxanthin is found in edible brown algae and, along with  $\beta$ -carotene, is one of the most abundant carotenoids found in nature [5]. There have recently been several reports that fucoxanthin showed biological activities such as radical scavenging, antiproliferative, antiobesity, anti-tumor, and antiangiogenic activities [6–10]. Nonetheless, the protective effects of cell damages related to antioxidant activities of fucoxanthin have not yet been reported. Accordingly, the present study isolated the marine natural carotenoid fucoxanthin from *Sargassum siliquastrum* and evaluated its cytoprotective effects against  $H_2O_2$ -induced cell damage.

## Materials and methods

### Materials

The marine alga *S. siliquastrum* was collected along the coast of Jeju Island, Korea, between October 2005 and March 2006. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water, and maintained in a medical refrigerator at  $-20\text{ }^\circ\text{C}$ . Therefore, the frozen samples were lyophilized and homogenized with a grinder prior to extraction.

### General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. The UV and FT-IR spectra were recorded on a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrometer and a SHIMAZU 8400s FT-IR spectrometer, respectively. NMR spectra were recorded on a Varian INOVA 400 MHz NMR spectrometer.  $CD_3OD$  was used as a solvent for the NMR experiments, and the solvent signals were used as an internal reference. ESI and HREI mass spectra acquired using a Finnigan Navigator 30086 and JMS-700 MStation high resolution mass spectrometer system, respectively. The HPLC was carried out on a Waters HPLC system equipped with a Waters 996 photodiode array detector and Millennium32 software using  $C_{18}$  column (J'sphere ODS-H80, 150 mm  $\times$  20 mm, 4  $\mu\text{m}$ , YMC Co.).

### Extraction and isolation

The powdered *S. siliquastrum* (400 g) was extracted with 80% aqueous MeOH, and was evaporated under vacuo.

Then, the MeOH extract was partitioned with  $CHCl_3$ . The chloroform extract (9 g) was fractionated by silica column chromatography using stepwise elution with  $CHCl_3$ –MeOH mixture (100:1  $\rightarrow$  1:1) to afford separated active fractions. A combined active fraction was further subjected to a Sephadex LH-20 column saturated with 100% MeOH, and then finally purified by reversed-phase HPLC to give fucoxanthin (74.9 mg).  $^1H$  and  $^{13}C$  NMR data were assigned in Table 1 and the structure was illustrated in Fig. 1.

### Cell culture

Cells of a monkey kidney fibroblast line (Vero) were maintained at  $37\text{ }^\circ\text{C}$  in an incubator with humidified atmosphere of 5%  $CO_2$ . Cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100  $\mu\text{g}/\text{mL}$ ), and penicillin (100 U/mL).

### Intracellular ROS measurement

For detection of intracellular ROS, Vero cells were seeded in 96-well plates at a concentration of  $1 \times 10^5$  cells/mL. After 16 h, the cells were treated with various concentrations of fucoxanthin, and incubated at  $37\text{ }^\circ\text{C}$  under a humidified atmosphere. After 30 min,  $H_2O_2$  was added at a concentration of 1 mM, and then cells were incubated for additional 30 min at  $37\text{ }^\circ\text{C}$ . Finally, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; 5  $\mu\text{g}/\text{mL}$ ) was introduced to the cells, and 2',7'-dichlorofluorescein (DCF) fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer. The percentage of intracellular ROS scavenging activity was calculated in accordance with the following equation:

$$\text{Intracellular ROS scavenging activity(\%)} \\ = [1 - (C_1/C_0)] \times 100$$

where  $C_1$  is the fluorescence intensity of cells treated with  $H_2O_2$  and fucoxanthin, and  $C_0$  is the fluorescence intensity of cells treated with  $H_2O_2$  and distilled water instead of fucoxanthin.

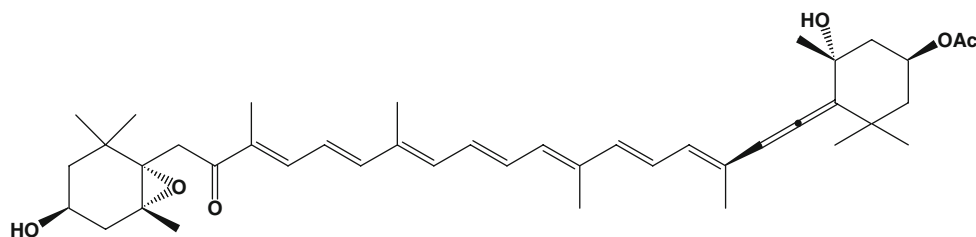
### Assessment of cell viability

Cell viability was then estimated via an 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in

**Table 1** NMR spectroscopic data for fucoxanthin in CDCl<sub>3</sub>

Position	<sup>13</sup> C	<sup>1</sup> H (mult. <i>J</i> = Hz)	Position	<sup>13</sup> C	<sup>1</sup> H (mult. <i>J</i> = Hz)
1	35.8		1'	35.2	
2	47.1	1.36 (1H, dd, <i>J</i> = 8.7, 14.2) 1.49 (1H, dd, <i>J</i> = 14.2)	2'	45.4	1.41 (1H, dd, <i>J</i> = 10.4, 14.9) 2.00 (1H, dd, <i>J</i> = 2.9, 14.9)
3	64.3	3.80 (1H, m)	3'	68.0	5.37 (1H, tt, <i>J</i> = 8.8, 12.0)
4	41.6	1.77 (1H, dd, <i>J</i> = 8.7, 14.2) 2.29 (1H, dd, <i>J</i> = 2.9, 17.8)	4'	45.2	1.53 (1H, dd, <i>J</i> = 10.4, 14.9) 2.29 (1H, dd, <i>J</i> = 2.9, 17.8)
5	66.2		5'	72.7	
6	67.1		6'	117.5	
7	40.8	2.59, 3.64 (2H, d, <i>J</i> = 20.4)	7'	202.4	
8	170.5		8'	103.4	6.04 (1H, s)
9	134.5		9'	132.5	
10	139.1	7.14 (1H, d, <i>J</i> = 12.8)	10'	128.5	6.12 (1H, d, <i>J</i> = 11.6)
11	123.4	6.58 (1H, m)	11'	125.7	6.71 (1H, t, <i>J</i> = 12.0)
12	145.0	6.66 (1H, t, <i>J</i> = 12.8)	12'	137.1	6.34 (1H, d, <i>J</i> = 11.6)
13	135.4		13'	138.1	
14	136.6	6.40 (1H, d, <i>J</i> = 11.6)	14'	132.2	6.26 (1H, d, <i>J</i> = 11.6)
15	129.4	6.67 (1H, m)	15'	132.5	6.71 (1H, t, <i>J</i> = 12.0, 14.2)
16	25.0	1.37 (3H, s)	16'	29.2	1.37 (3H, s)
17	28.1	0.95 (3H, s)	17'	32.1	1.06 (3H, s)
18	21.2	1.34 (3H, s)	18'	31.3	1.34 (3H, s)
19	11.8	1.80 (3H, s)	19'	14.0	1.80 (3H, s)
20	12.8	1.98 (3H, s)	20'	12.9	1.98 (3H, s)
			3'OAc, CH <sub>3</sub>	21.4	2.03 (3H, s)
			3'OAc, C=O	197.9	

Data were obtained using 400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C

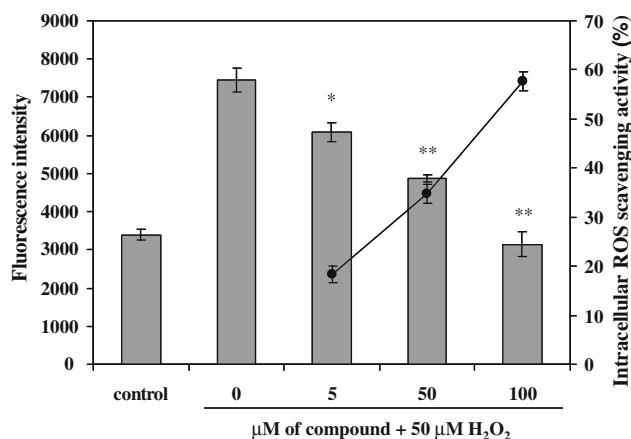
**Fig. 1** Chemical structure of fucoxanthin

viable cells [11]. The cells were seeded in 96-well plate at a concentration of  $1 \times 10^5$  cells/mL. After 16 h, the cells were treated with fucoxanthin at difference concentrations. Then, 10  $\mu$ L of H<sub>2</sub>O<sub>2</sub> (1 mM) was added to the cell culture medium, and incubated for 24 h at 37 °C. MTT stock solution (50  $\mu$ L; 2 mg/mL) was then applied to the wells, to a total reaction volume of 200  $\mu$ L. After 4 h of incubation, the plates were centrifuged for 5 min at 800 $\times$ g, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150  $\mu$ L of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the

formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

#### Determination of DNA damage by comet assay

Comet assay was performed to determine the oxidative DNA damage [12]. The cell suspension was mixed with 75  $\mu$ L of 0.5% low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose. After solidification of the agarose, slides were covered with another 75  $\mu$ L of 0.5% LMA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and

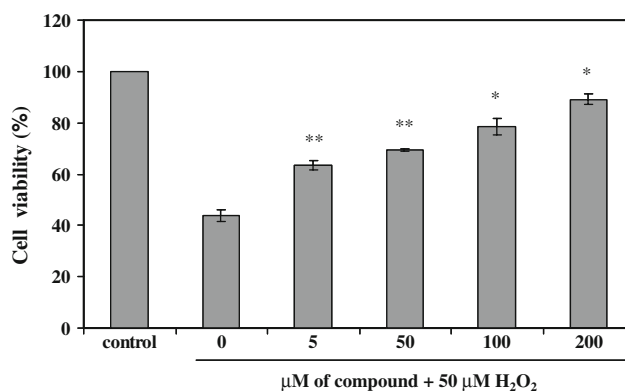


**Fig. 2** Effect of fucoxanthin isolated from *S. siliquastrum* on scavenging ROS. The intracellular ROS generated was detected by DCFH-DA assay. *Dark filled square* indicates fluorescence intensity; *Dark filled circle* indicates intracellular ROS scavenging activity. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. \* $p < 0.05$ , \*\* $p < 0.01$

1% sodium lauryl sarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4 °C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na<sub>2</sub>EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4 °C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4 °C, and then treated with ethanol for another 5 min before staining with 50 μL of ethidium bromide (20 μg/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, UK) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity; 50 cells from each of two replicate slides).

#### Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis [13, 14]. The cells were placed in 24-well plates at a concentration of  $1 \times 10^5$  cells/mL. Sixteen hours after plating, the cells were treated with various concentration of fucoxanthin, and further incubated for 1 h prior to expose to H<sub>2</sub>O<sub>2</sub> (1 mM). After 24 h, 1.5 μL of Hoechst 33342 (stock 10 mg/mL), a DNA-specific fluorescent dye, were added to each well, followed by 10 min of incubation at 37 °C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera, in order to examine the degree of nuclear condensation.



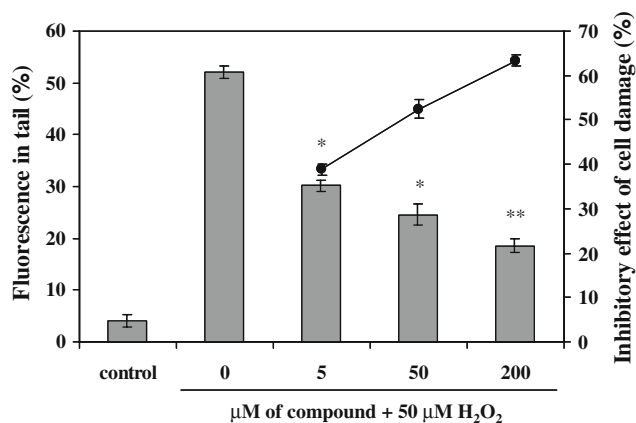
**Fig. 3** Protective effect of fucoxanthin isolated from *S. siliquastrum* on H<sub>2</sub>O<sub>2</sub> induced oxidative damage of vero cells. The viability of cells on H<sub>2</sub>O<sub>2</sub> treatment was determined by MTT assay. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. \* $p < 0.05$ , \*\* $p < 0.01$

#### Statistical analysis

The data are expressed as the mean  $\pm$  standard error. A statistical comparison was performed via the SPSS package for Windows (Version 10).  $p$ -Values of  $<0.05$  were considered to be significant.

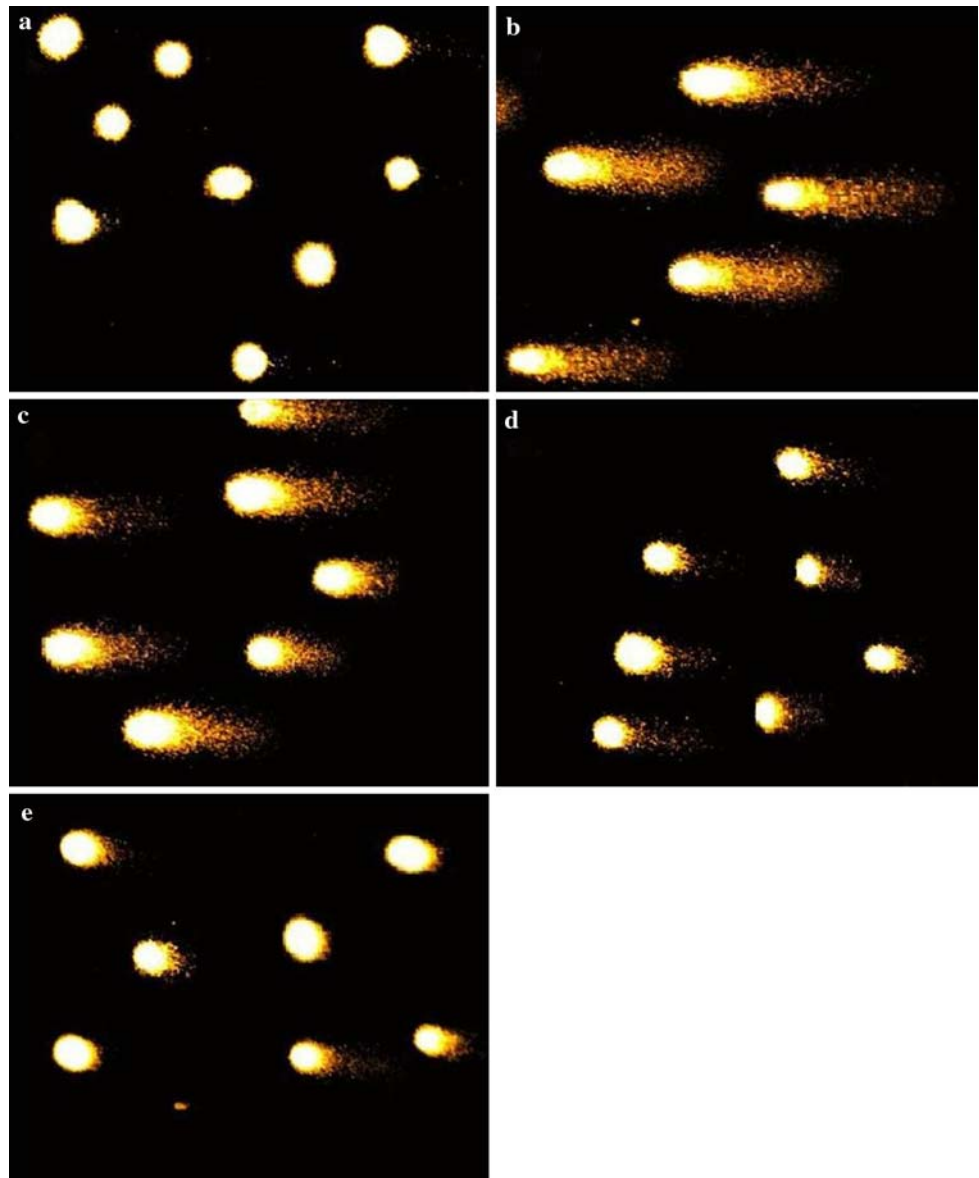
#### Results and discussion

Cells are protected from ROS-induced damage by a variety of endogenous ROS-scavenging enzymes, chemical compounds, and natural products. Recently, interest has been increased in the therapeutic potential of natural products to be used as natural antioxidants in the reduction of such free



**Fig. 4** Inhibitory effect of different concentration of fucoxanthin isolated from *S. siliquastrum* on H<sub>2</sub>O<sub>2</sub> induced DNA damage. The damaged cells on H<sub>2</sub>O<sub>2</sub> treatment was determined by comet assay. *Dark filled square* indicates percentage of fluorescence in tail; *Dark filled circle* indicates inhibitory effect of cell damage. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. \* $p < 0.05$ , \*\* $p < 0.01$

**Fig. 5** Photomicrographs of DNA damage and migration observed under fucoxanthin isolated from *S. siliquastrum* where the tail moments were decreased. **a** control, **b** cells treated with 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , **c** cells treated with 5  $\mu\text{M}$  compound + 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , **d** cells treated with 50  $\mu\text{M}$  compound + 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , **e** cells treated with 200  $\mu\text{M}$  compound + 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$



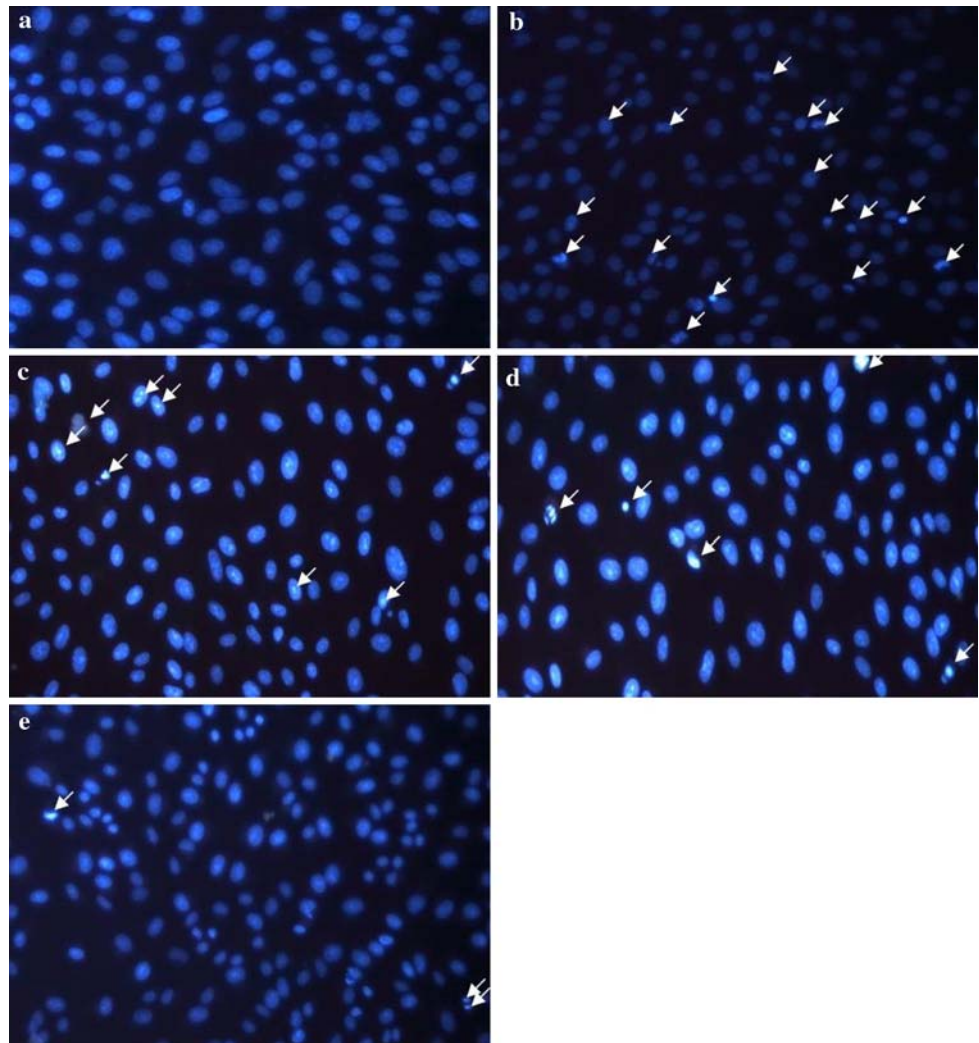
radical-induced tissue injuries, thereby suggesting that many natural products may prove to possess therapeutically useful antioxidant compounds [15–17].

$\text{H}_2\text{O}_2$  has been extensively used as an inducer of oxidative stress in in vitro models. It readily crosses the cellular membranes giving rise to the highly reactive hydroxyl radical, which has the ability to react with macromolecules, including DNA, proteins, and lipids, and ultimately damage to whole cell [18, 19]. Several studies have shown that oxidative stress is a major cause of cellular injuries in a variety of human diseases including cancer, neurodegenerative, and cardiovascular disorders [20–22]. Recently, many researchers have made considerable efforts to search natural antioxidants. Many studies have revealed that seaweeds have potential to be used as a candidate for natural antioxidant. Potent antioxidative compounds have

already been isolated from seaweeds and identified as phylophoeophytin in *Eisenia bicyclis* (arame), fucoxanthine in *Hijikia fusiformis* (hijiki), phlorotannin in *Ecklonia stolonifera*, bromophenols in *Polysiphonia urceolata*, and sulfated polysaccharides [6, 23–27]. Among these antioxidants, fucoxanthine, a kind of carotenoid, showed radical scavenging activity. In this study, we investigated the antioxidant effects of natural carotenoid fucoxanthin, after the administration of  $\text{H}_2\text{O}_2$  in cell lines.

2',7'-Dichlorodihydrofluorescein diacetate was used as a probe for ROS measurement. DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterase to nonfluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent DCF. It is well known that  $\text{H}_2\text{O}_2$  is the principal ROS responsible for the oxidation of DCFH to DCF [28]. In the present study,

**Fig. 6** Protective effect of fucoxanthin isolated from *S. siliquastrum* against  $H_2O_2$ -induced apoptosis in vero cells. Cellular morphological changes were observed under a fluorescence microscope after Hoechst 33342 staining. **a** untreated, **b** 500  $\mu M$   $H_2O_2$ , **c** 5  $\mu M$  compound + 500  $\mu M$   $H_2O_2$ , **d** 50  $\mu M$  compound + 500  $\mu M$   $H_2O_2$ , **e** 200  $\mu M$  compound + 500  $\mu M$   $H_2O_2$ . Apoptotic bodies are indicated by arrows



we investigated scavenging activity of fucoxanthin on intracellular ROS and the results are illustrated in Fig. 2. Fluorescence intensity of control cells was recorded as 3398 while  $H_2O_2$  treated cells showed 7452. However, the addition of fucoxanthin to the cells mixed with  $H_2O_2$  reduced intracellular ROS accumulation to 6079, 4867, and 3149 at 5, 50, and 100  $\mu M$ , respectively. Moreover, the scavenging activity of fucoxanthin on intracellular ROS was dose-dependently increased as 18.4, 34.7, and 57.7% at 5, 50, and 100  $\mu M$ , respectively. Hence these results indicate that fucoxanthin has efficient antioxidant properties which can be developed into a potential bio-molecular candidate to inhibit ROS formation of cellular.

To evaluate whether fucoxanthin protect from cellular damage induced by  $H_2O_2$ , cells were pretreated with fucoxanthin for 24 h in the absence or presence of oxidative stress. After then, cell viability was measured via MTT assay. As shown in Fig. 3,  $H_2O_2$  treatment without fucoxanthin decreased cell viability to 43.7%, while fucoxanthin prevented cells from  $H_2O_2$ -induced damage, restoring cell

survival to 63.6, 69.4, 78.5, and 89.2% at the concentrations of 5, 50, 100, and 200  $\mu M$ , respectively. Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is directly correlated to the living cell number [29]. The results of MTT assay showed that formazan content was reduced due to  $H_2O_2$  treatment, however, significantly increased with addition of fucoxanthin. These results suggest that fucoxanthin has ability to protect vero cells from oxidative stress-related cellular injuries.

The protective effect of fucoxanthin on DNA damages was also confirmed by comet assay and was presented as tail DNA percent. This assay can reflect different types of DNA damage, such as DNA single strand breakage, or incomplete DNA repairing and shows high sensitivity in detecting oxidative stress [30]. As shown in Fig. 4,  $H_2O_2$  treatment increased the tail moments in the cells versus the control cells. However, the increased tail moments caused by  $H_2O_2$  induced oxidative DNA damage were decreased in the cells treated with fucoxanthin. The inhibition effect

of fucoxanthin on DNA damage was 38.8, 52.5, and 63.4% at the concentrations of 5, 50, and 200  $\mu\text{M}$ , respectively. The DNA migration profiles of tested cells when treated with different concentrations of fucoxanthin are presented in Fig. 5. The DNA was completely damaged and the amount of tail DNA was significantly increased in the group treated with only  $\text{H}_2\text{O}_2$  (Fig. 5b), compared to the control (Fig. 5a). However, the amount of tail DNA was increasingly decreased with increased concentrations of fucoxanthin (Fig. 5c–e). In a previous study, Kang et al. [23] reported that some natural compounds from brown seaweeds have an ability to increase catalase located at peroxisome in cell that converts  $\text{H}_2\text{O}_2$  into molecular oxygen and water. In this study, we showed that fucoxanthin has prominent effect on the  $\text{H}_2\text{O}_2$ -induced DNA damage. The inhibition activities on the DNA damage and the decrease of the amounts of tail DNA might be related to the ability of fucoxanthin to increase catalase.

The protective effect of fucoxanthin on apoptosis induced by  $\text{H}_2\text{O}_2$  was investigated by nuclear staining with Hoechst 33342. Hoechst 33342 dye specifically stains DNA, and is widely used to detect the nuclear shrinkage such as chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies, which are all indications of apoptosis [31]. The microscopic photograph in Fig. 6, the negative control, which contained no compound or  $\text{H}_2\text{O}_2$ , possessed intact nuclei (Fig. 6a), and the positive control ( $\text{H}_2\text{O}_2$  treated cells) exhibited significant nuclear fragmentation, indicating apoptosis (Fig. 6b). However, the addition of fucoxanthin with  $\text{H}_2\text{O}_2$  reduced the apoptotic bodies (Fig. 6c–e), which suggested the ability of fucoxanthin to protect against nuclear fragmentation amidst oxidative stress.

In conclusion, the results obtained in the present study show that fucoxanthin isolated from *S. siliquastrum* can effectively inhibit intracellular ROS formation, DNA damage, and apoptosis induced by  $\text{H}_2\text{O}_2$ . Moreover, fucoxanthin showed evidence in cell survival rate. These results revealed that fucoxanthin can be used not only as the easily accessible source of natural antioxidants but also as an ingredient of the functional food and cosmetic agents related to the prevention and control oxidative stress.

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