Original Research Paper

# The Bioactivity of Fucoxanthin from *Undaria pinnatifida in Vitro*

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**Abstract:** Fucoxanthin, a marine carotenoid, can be found in marine algae and some marine invertebrates, has remarkable biological properties for human health. It is known that fucoxanthin has anti-cancer activity on some human cancer cells such as human colon cancers, leukemia cells, prostate cancer cells, breast cancer cells. In this study, the fucoxanthin was extracted from *Undaria pinnatifida,* after purification by silica gel ion exchange column and TLC, the purity of fucoxanthin was reached to 95%. For anti-oxidant activity, the extracted fucoxanthin from *Undaria pinnatifida* had strong properties on hydroxyl radical scavenging activity ($EC_{50}$ = 7.03 µM) and ferrous metal ions chelating activity ($EC_{50}$ = 30.22 µM). For immunomodulatory activitiy, the fucoxanthin can activate proliferation of RAW 264.7 and protect it from DOX-induced and LPS-stimultaed cell damage. For anti-cancer activity, the extracted fucoxanthin could inhibit proliferation of HT1080, MDA-MB-231 and U-2 OS cells. Therefore, the fucoxanthin could be used as potential functional food additives in food industry.

**Keywords:** *Undaria Pinnatifida*, Fucoxanthin, Anti-Oxidant Activity, Immunomodulatory Activitiy, Anti-Cancer Activity

## Introduction

Fucoxanthin, a nature carotenoid, is generally found in edible algae such as Chrysophyceae, Bacillariophyceae, Prasinophyceae, Phaeophyceae and a number of marine invertebrates such as tunicates and shellfish (Stancher *et al*., 1998; Matsuno *et al*., 1986). Fucoxanthin together with β-carotene is considered as one of the most abundant carotenoids in nature, which is found in edible brown algae such as *Undaria pinnatifida* (Omenn *et al*., 1996). Also, Fucoxanthin supplies more than 10% of the expected entirely products of carotenoids in nature. Fucoxanthin and chlorophyll α are bound to some proteins to assemble fucoxanthin-chlorophyll α-protein complexes (FCPc) in the thylakoid and work as harvesting light and transferring pigment (Kim *et al*., 2011).

Fucoxanthin is reported to induce apoptosis in prostate cancer cells (Kotake *et al*., 2005), colon cancer cells (Hosokawa *et al*., 2004) and leukemia cancer cells (Kim *et al*., 2010) through down-regulated Bcl-2 expressions. Furthermore, fucoxanthin inhibits the growth of tumor cells through inducing cell cycle arrest at the G1 phase by MAPKs regulations, p21[WAF1/Cip1], cyclin D and reactive oxygen species generation inactivated the Bcl-xl pathway on prostate cancer DU145 cells, colon adenocarcinoma WiDr, hepatic carcinoma HepG2 and leukemia cancer HL-60 cells, respectively (Liu *et al*., 2012; Satomi, 2012; Xiao *et al*., 2012). Our previous research indicated that the anticancer activity of fucoxanthin on human bladder cancer T24 cells was related to arrest at G0/G1 phase via increasing the expression level of p21 at low doses. Furthermore, high doses fucoxnathin induced apoptosis on T24 through down-regulating the expression level of mortalin, which is reported as a member of heat shock protein 70

(Zhang *et al.*, 2008; Wang *et al.*, 2014). However, the inhibition effects of fucoxanthin on human fibrosarcoma HT1080, human adenocarcinoma MDA-MB-231 and human osteosarcoma U-2 OS cells are still unclear.

*Undaria pinnatifida,* an important economic brown algae, is taken as traditional food in Asian countries, especially Japan and Korea. The worldwide annual production of *Undaria pinnatifida* exceeds millions of tons, amount of which comes from China. In Japan, about 79,000 tons of cultured kombu is discarded during food processes. *Undaria pinnatifida* contains a large amount of fucoxanthin and would be regarded as an excellent source of fucoxanthin.

In this study, the fucoxanthin was extracted from *Undaria pinnatifida,* in order to reutilize the waste food material and develop the more bioactivity of fucoxanthin *in vitro*, the antioxidant activities on hydroxyl free radical scavenging activity and ferrous metal ions chelating activity, the immunomodulatory activitiy on macrophage RAW 264.7 cell and the anti-cancer activities on HT1080, MDA-MB-231 and U-2 OS cancer cells of fucoxanthin were measured.

## Material and Methods

### Alage Sample

*Undaria pinnatifida* (dried sample) was purchased from supermarket in Tsukuba Japan.

### Chemicals and Reagents

Ascorbic acid, hydrogen peroxide and Ethylenediaminetetraacetic Acid (EDTA) were purchased from Wako Pure Chemical, Osaka, Japan. SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) and penicillin-streptomycin solution were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). A Cell Proliferation Kit (MTT) was purchased from Roche (Tokyo, Japan). Doxorubicin (DOX) was purchased from TopoGEN, Inc. (Florida, USA). Lipopolysaccharide (LPS) from *E. coli* 055 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All of the chemical reagents were analytical grade.

### Cell Lines and Cell Culture

The human cancer cell line HT1080, MDA-MB-231 and U-2 OS and the murine macrophage cell line RAW 264.7 were obtained from the Riken Cell Bank (Tsukuba, Japan). All of the cell lines were kept in DMEM medium, which containing 10% fetal bovine serum, 100 U $mL^{-1}$ penicillin and 100 µg $mL^{-1}$ of streptomycin and placed in a humidified 5% $CO_2$ atmosphere (ESPEC $CO_2$ Incubator) at 37°C. After

culturing for 2-3 days, the cells were reach to the logarithmic phase and then used for experiments.

### Extraction and Purification of Fucoxanthin

The flow chart was showed in Fig. 1. The dried *Undaria pinnatifida* samples were pulverized into powder using a grinder. The *Undaria pinnatifida* powder (50 g) was extracted by 80% methanol (500 mL) for three times at room temperature for 24 h. After extraction, the methanol extract was filtered and evaporated to dryness under vacuum at 40°C. The methanol extract was subsequently dissolved with 10 mL 99% chloroform and then fractionated using silica column chromatography with the stepwise elution of a chloroform-methanol mixture (100:1-1:1) to separate active fractions. During silica column chromatography experiment, modified conventional methods to adjust random flow rate of stepwise elution, as shown in Fig. 2. Then the Thin-Layer Chromatography (TLC) method was used to isolate fucoxanthin fraction with fucoxanthin standard substance (Wako, Japan). After TLC method, the fucoxanthin fraction was evaporated to dryness under vacuum at 40°C. Then the dried powder was weighted by an electronic balance (PM 400, Japan). Fucoxanthin dissolved in 20% DMSO and stored at -80°C.

### Hydroxyl Free Radical Scavenging Activity

The method of determining hydroxyl free radical scavenging activity was base on a literature procedure with a few modifications (Smirnoff and Cumbes, 1989). Different concentrations of fucoxathin extract solutions (1 mL) added into the reaction mixture, which contained 0.5 mL $FeSO_4$ (1.5 mM), 0.35 mL of $H_2O_2$ (6 mM) and 0.15 mL of sodium salicylate (20 mM). Ascorbic acid (Vitamin C) was used as the positive control. Negative control without any antioxidant or sample was considered as 100% solvent. The reaction mixtures were incubated for 1 h at 37°C and then the absorbance was measured at 562 nm. The scavenging activity was calculated as:

$$\% \text{ HO} \bullet \text{scavenged} = \left[ 1 - \frac{A_1 - A_2}{A_0} \right] \times 100\%$$

where, $A_0$ was the absorbance of the solvent control, $A_1$ was the absorbance of the sample or positive control and where as $A_2$ was the absorbance of the reagent (without sodium salicylate), respectively.

### Ferrous Ion Chelating Activity

The determination of ferrous ion chelating activity of fucoxanthin was according to a literature procedure with a few modifications (Yuan *et al.*, 2008). Different concentrations of fucoxathin extract solutions (1 mL) were mixed with 50 µL of ferrous chloride (2 mM) and

0.2 mL of ferrozine (5 mM), shaken well, kept at room temperature for 10 min and then the absorbance of mixtures were determined at 562 nm. EDTA (ethylenediaminetetraacetic acid) was used as the positive control. Negative control without any antioxidant or sample was considered as 100% solvent. The ferrous ion chelating activity was calculated as:

$$\text{Chelating rate} = \text{Chelating rate} = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100\%$$

where, $A_0$ was the absorbance of the control (without sample and any antioxidant) and $A_1$ was the absorbance of the sample and positive control, $A_2$ was the absorbance of the reagent (without ferrozine), respectively.

### Effect of Fucoxanthin on Macrophages Proliferation

The effect of fucoxanthin on the proliferation of RAW 264.7 cells was estimated using the MTT assay kit purchased from Roche (Tokyo, Japan). According to the MTT kit manufacturer's instructions, $5 \times 10^4$ cells $mL^{-1}$ of RAW 264.7 cells were seeded in 96-well plate and pre-incubated at 37°C in a 5% $CO_2$ atmosphere for 24 h. Then RAW 264.7 cells were exposed to different concentrations of fucoxanthin extract solutions (5, 10, 20 and 40 μM) for 24 h, 48 h or 72 h. Then, the medium was removed, 100 μL Phosphate Buffered Saline (PBS)

and 10 μL of MTT solution were added to each well for 4 h of incubation and then, 100 μL of the solubilization solution in the kit was added to each well and mixed the contents of each well thoroughly using a pipette, whereafter kept the 96-well plate in a humidified atmosphere for 12 h. A microplate reader (BIO-RAD Model 550, Bio-Rad Laboratories, Hercules, California, USA) was used to measure the spectrophotometrical absorbance of the sample at wavelength of 570 nm.

### The Protective Activity of Fucoxanthin on Macrophages

About $5 \times 10^4$ cells $mL^{-1}$ of RAW 264.7 cells were incubated in a 96-well plate at 37°C in a 5% $CO_2$ atmosphere. After 24 h incubation, various concentrations of fucoxanthin extract solutions (5, 10, 20 and 40 μM) were added into each well and the cells were incubated with DOX (5 μM) or LPS (1 μg $mL^{-1}$) for 24, 48 and 72 h. After drug exposure, the medium was removed, then 100 μL Phosphate Buffered Saline (PBS) and 10 μL of MTT reagent was added to each well for 4 h of incubation and then, 100 μL of the solubilization solution in the kit was added to each well and mixed the contents of each well thoroughly using a pipette and the plate was incubated in a humidified atmosphere for overnight. A microplate reader (BIO-RAD Model 550, Bio-Rad Laboratories, Hercules, California, USA) was used to measure the spectrophotometrical absorbance of the sample at wavelength of 570 nm.
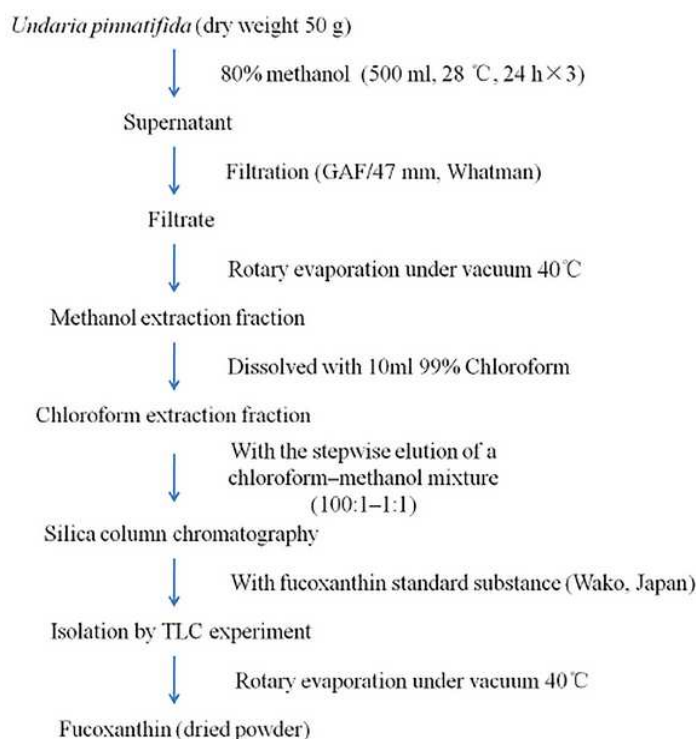


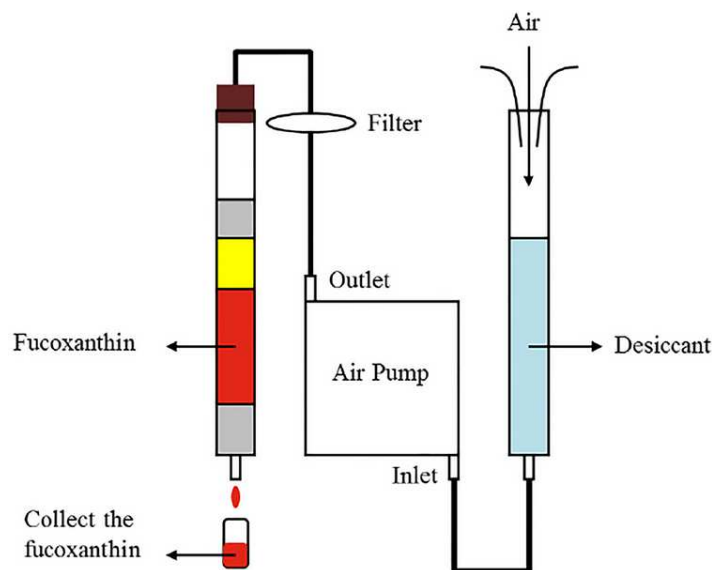Fig. 1. Extraction scheme of fucoxanthin from *Undaria pinnatifida*

Fig. 2. Procedure of Silica column chromatography experiment with modification

## *Inhibitory Effect of Fucoxanthin on Cancer Cells*

HT1080, MDA-MB-231 and U-2 OS cells were cultured in DMEM to logarithmic phase at 37°C in a 5% $CO_2$ atmosphere for 2-3 days. Fucoxanthin Standard (Sigma, St. Louis, USA) was dissolved in Dimethylsulfoxide (DMSO) from Sigma (Sigma, St. Louis) and stored at -20°C. HT1080, MDA-MB-231 and U-2 OS cells were seeded in 96-well plate at $5 \times 10^4$ cells $mL^{-1}$, respectively. After 24 h incubation, cells were exposed to different concentrations of fucoxanthin extract solutions (5, 10, 20 and 40 μM) for 24, 48 or 72 h. Then, the medium was removed, 100 μL Phosphate Buffered Saline (PBS) and 10 μL of MTT solution were added to each well for 4 h of incubation and then, 100 μL of the solubilization solution in the kit was added to each well and mixed the contents of each well thoroughly using a pipette and the plate was incubated in humidified atmosphere for overnight. A microplate reader (BIO-RAD Model 550, Bio-Rad Laboratories, Hercules, California, USA) was used to measure the spectrophotometrical absorbance of the sample at wavelength of 570 nm.

## Results

### *Extraction and Purification of Fucoxanthin*

By using silica column chromatography and TLC, the fucoxanthin fraction from Undaria pinnatifida can be purified. After purification, the purity of fucoxanthin fraction was reached to 95% and the fucoxanthin was dissolved in 99.5% DMSO to prepare the below experiments.

## *Anti-Oxidant Activity of Fucoxanthin*

It is important to remove hydroxyl radical for living systems. Hydroxyl radical can destroy almost all types of macromolecules, such as carbohydrates, lipids, amino acids and nucleic acids, which is extremely dangerous to human (Gulcin, 2006; Ke *et al*., 2009). Therefore, the developing of antioxidant which could resist hydroxyl radical with good scavenging capacity becomes very important. The hydroxyl radical scavenging capacity of an extract is directly contributed its antioxidant activity (Babu *et al*., 2001). Figure 3 depicts the scavenging activity of a hydroxyl radical. The scavenging ratio of Vc (Vitamin C, positive control) and fucoxanthin correlated well with increasing concentrations, increasing from 67.41 to 98.75%, 21.43 to 53.45% when the concentration increased from 5 to 40 μM. Meanwhile $EC_{50}$ value of scavenging activity of fucoxanthin and ascorbic acid is 30.22 and 6.1 μM. The scavenging activity of fucoxanthin was lower than that of Vc, but still showed the potent scavenging activities. The results indicated that fucoxanthin exhibits potent antioxidant effects on hydroxyl radical activity.

It is reported that antioxidant effect on chelating of metal ions is related to copper promote oxidative damage and the transition metals iron at different levels (Saiga *et al*., 2003). As shown in Fig. 4 the metal chelating activity of EDTA (positive control) and fucoxanthin increased with dose-dependent manner. Compared with EDTA, the chelating activity of the samples on ferrous ion was weaker. The result showed that fucoxnanthin had negligible $Fe^{2+}$ chelating activity and the maximal chelating activities of fucoxanthin was 54% at 40 μM. Meanwhile, $EC_{50}$

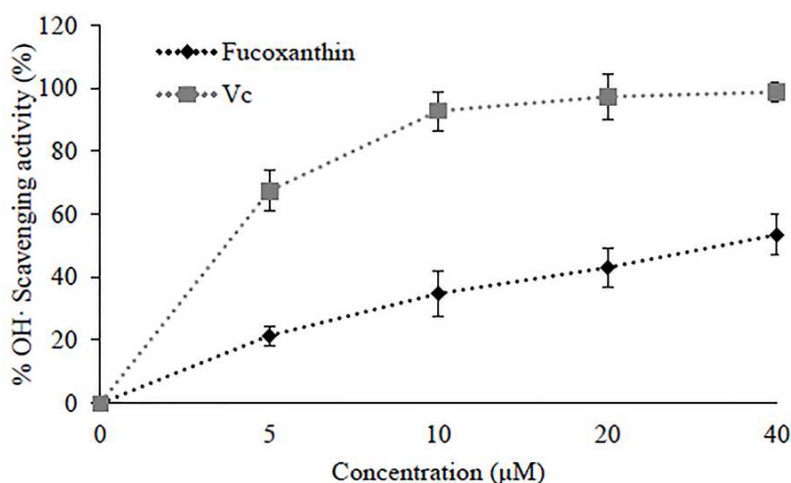value of chelating activity of fucoxanthin and EDTA    is 7.03 and 0.45 µM, respectively.



Fig. 3. Scavenging activity of fucoxanthin on hydroxyl free radical. Each value represents mean ± SE of at least three independent experiments and each experiment was performed in triplicate. *p<0.05, **p<0.01 and ***p<0.001



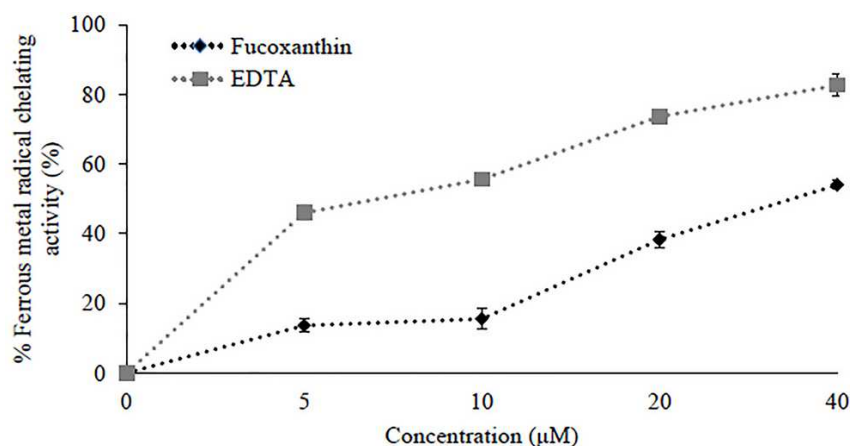Fig. 4. Chelating activity of fucoxanthin on ferrous metal radical. Each value represents mean ± SE of at least three independent experiments and each experiment was performed in triplicate. *p<0.05, **p<0.01 and ***p<0.001
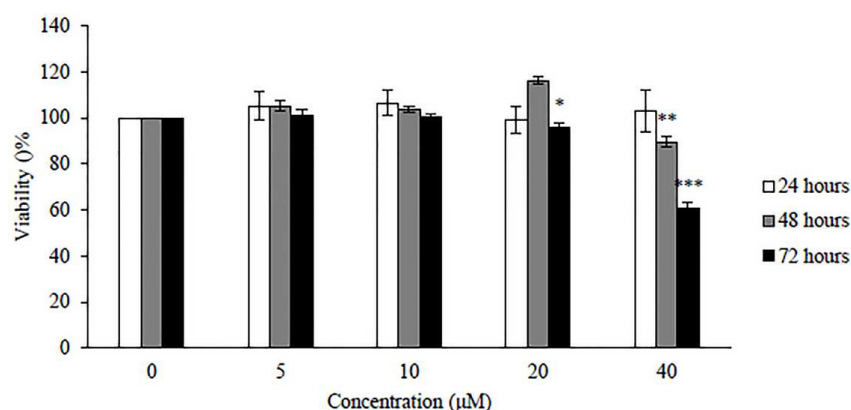


Fig. 5. Proliferation activity of fucoxanthin on macrophage RAW 264.7 cells. Each value represents mean ± SE of at least three independent experiments and each experiment was performed in triplicate. *p<0.05, **p<0.01 and ***p<0.001

## Immunomodulatory Activitiy of Fucoxanthin

The stimulating effect of fucoxanthin was tested. The results showed the exposure of fucoxanthin activated the proliferation of the macrophages (Fig. 5). At the concentration of 20 μM for 48 h, the stimulated effect reached a maximum, was 116.37%. To compare with 24 h and 48 h treatments, the cell viability of RAW 264.7 was decreased into 61.02%, especially at high dose of fucoxanthin. Actually higher concentrations (40 μM) decreased the proliferation rate of the macrophages and this may be related to the immunologic paralysis caused by the high dosage. It was suggested that high dose of fucoxanthin possessed stimulated effect on the proliferation of the macrophage RAW 264.7 cells with low cytotoxicity. Previous study demonstrated that fucoxanthin have the inhibition of the cancer cell proliferation (Kotake *et al.*, 2005).

Doxorubicin (DOX) is an anti-cancer drug for cancer chemotherapy. It is an anthracycline antibiotic, which works through intercalating DNA (Singal and Iliskovic, 1998). In this study, DOX treatment resulted in a decrease of the macrophages survival rate, however fucoxanthin protected the cytotoxicity of DOX on RAW 264.7 cells and increase the proliferation into maximum of 155.06% at 40 μM for 24 h. 24 h exposure of fucoxanthin was the most efficiency treatment time in this study (Fig. 6). In the presence of fucoxanthin, the macrophages viability was significantly increased and in a dose-dependent manner.

LPS Treatment led to a decrease of the macrophages survival rate. However, fucoxanthin protected the RAW 264.7 cells from LPS with dose-dependent manner. For 24 h with 40 μM fucoxanthin treatment, the cell viability of RAW 264.7 increased into 159.48±2.11% (Fig. 7). Therefore, fucoxanthin significantly protected RAW 264.7 cells from LPS-induced cell damage.

## Anti-Cancer Activity of Fucoxanthin

Fucoxanthin showed a significant inhibition effect on the proliferation of HT1080 cells from 24 to 72 h treatment. HT1080 cells were sensitive to fucoxanthin treatment from 5 μM to 40 μM. For 24 h treatment, the cell viability was decreased from 47.24 to 31.50% with increasing the concentration of fucoxanthin, which indicated fucoxanthin inhibited HT1080 cells for 24 h treatment with dose-dependent manner. For 48 h treatment, the cell viability was decreased to 46.05, 54.46, 45.61 and 41.26%, respectively. For 72 h treatment, the cell viability was decreased to 49.44, 56.49, 48.12 and 35.50%, respectively (Fig. 8). The most significant inhibition effect of fucoxanthin on HT1080 was for 24 h with 40 μM. These results indicated that the most efficiency treatment time and treatment concentration was 24 h and 40 μM.

For 24 h fucoxanthin treatment, Fig. 9 showed the lowest cell viability and fucoxanthin reduced cell viability of MDA-MB-231 cells with dose dependent manner. The cell viability was decreased from 73.45 to 62.77% upon exposure to fucoxanthin (5 μM to 40 μM). For 48 h fucoxanthin treatment, the cell viability was decreased to 75.40, 81.94, 76.13 and 70.42%. For 72 h fucoxanthin treatment, the cell viability was decreased to 81.48, 75.86, 71.84 and 71%. These results indicated that the most efficiency treatment time and treatment concentration was 24 h and 40 μM, however, the inhibition effect of fucoxanthin on MDA-MB-231 cells was weak.

As shown in Fig. 10, cell growth was gradually inhibited after 24, 48 and 72 h treatment with fucoxanthin. U-2 OS cell proliferation was decreased to 72.90% upon 72 h exposure to 40 μM fucoxanthin. Therefore, the inhibition effect of fucoxanthin on U-2 OS cells was week.
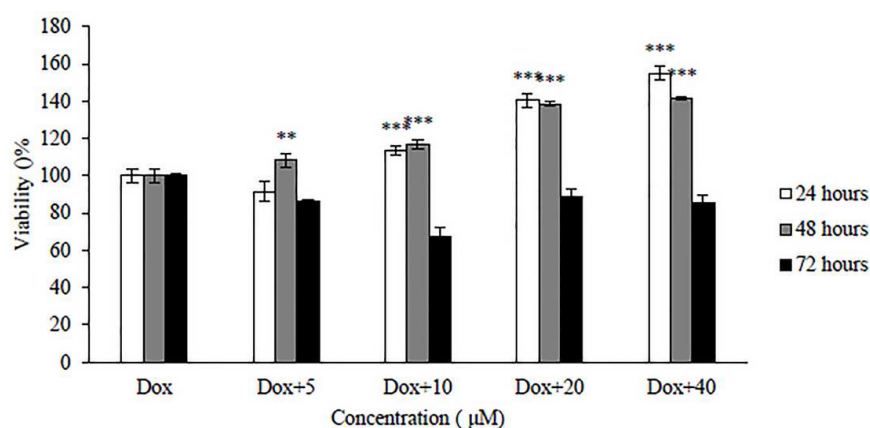


Fig. 6. Protective effect of fucoxanthin on DOX-induced RAW 264.7 cells. Each value represents mean ± SE of at least three independent experiments and each experiment was performed in triplicate. *p<0.05, **p<0.01 and ***p<0.001
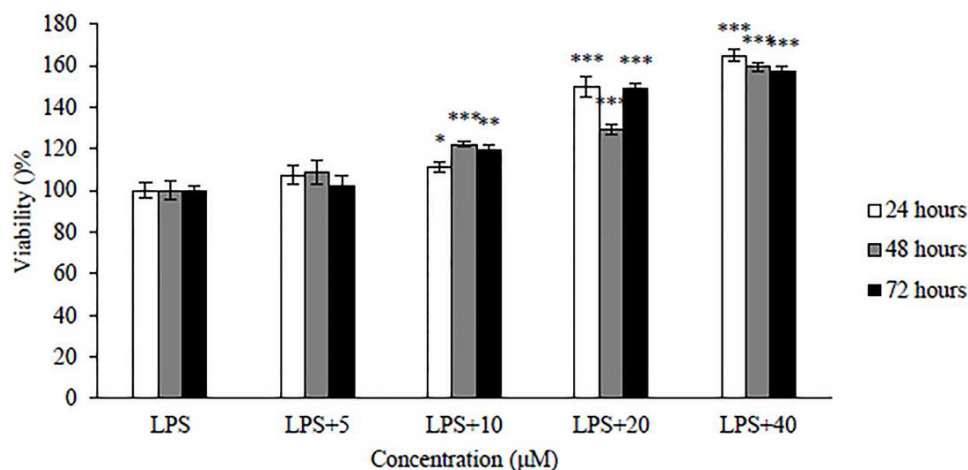
Fig. 7. Protective effect of fucoxanthin on LPS-stimulated RAW 264.7 cells. Each value represents mean ± SE of at least three independent experiments and each experiment was performed in triplicate. $*p<0.05$, $**p<0.01$ and $***p<0.001$
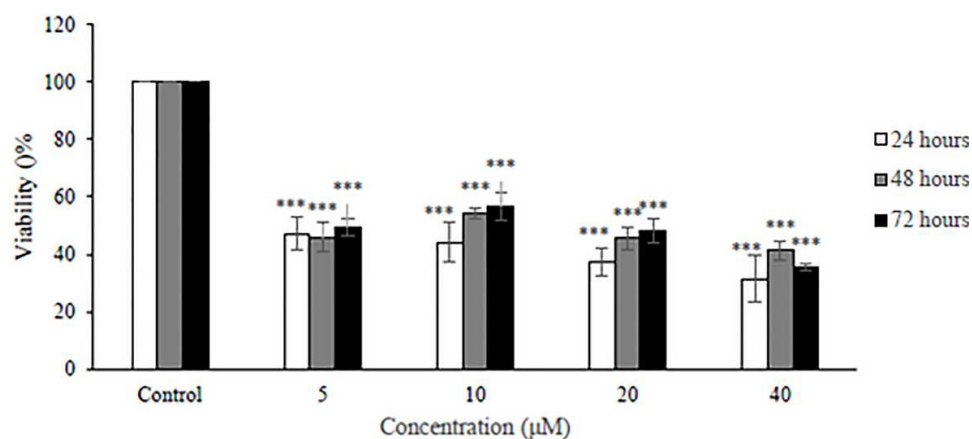


Fig. 8. Inhibition of fucoxanthin on HT1080 cells. Each value represents mean ± SE of at least three independent experiments and each experiment was performed in triplicate. $*p<0.05$, $**p<0.01$ and $***p<0.001$


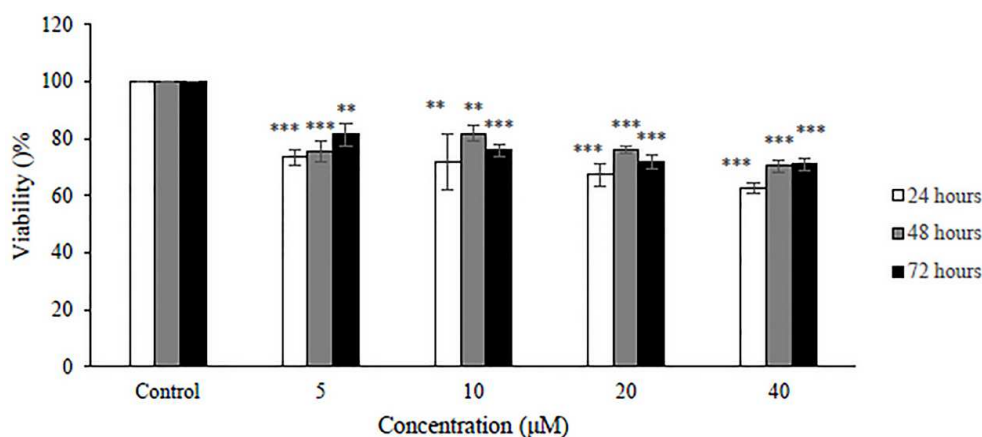
Fig. 9. Inhibition of fucoxanthin on MDA-MB-231 cells. Each value represents mean ± SE of at least three independent experiments and each experiment was performed in triplicate. $*p<0.05$, $**p<0.01$ and $***p<0.001$
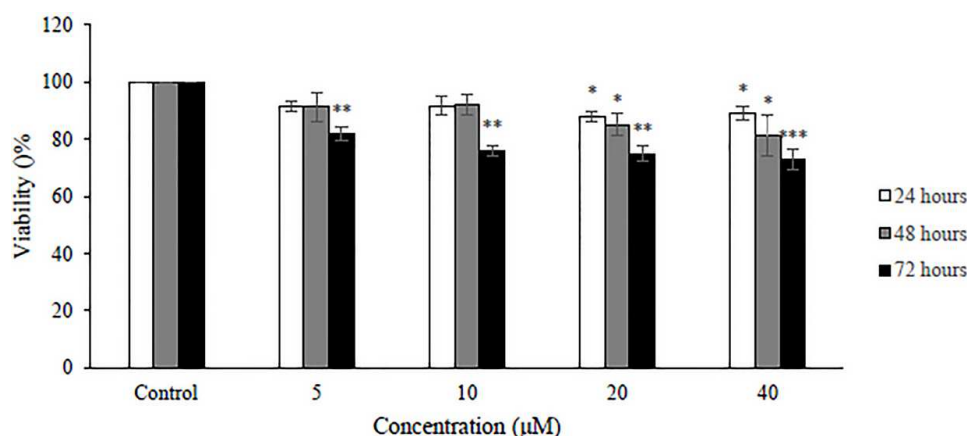
Fig. 10. Inhibition of fucoxanthin on U-2 OS cells. Each value represents mean ± SE of at least three independent experiments and each experiment was performed in triplicate. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

## Discussion

Fucoxanthin exhibited potent hydroxyl radical scavenging activity and ferrous metal ions chelating activity. The antioxidant activity of fucoxanthin varied in a dose-dependent manner and higher concentration resulted in fucoxanthin with higher antioxidant activity.

Reactive oxygen species (ROS), such as the hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^-$) and hydroxyl radical (OH), are unwanted catabolites byproducts of normal aerobic metabolism. In order to keep the balance, ROS ordinarily exist in all aerobic cells, ROS could be converted to water by biochemical antioxidants (Ham *et al.*, 2006). Fucoxanthin has been reported to have high hydroxyl radical scavenging activity in previous research (Fung *et al.*, 2013). In this study, fucoxanthin showed antioxidant activity as followed sequence through compared $EC_{50}$ values, ferrous metal ions chelating activity > hydroxyl radical scavenging activity. To consider about the antioxidant stability of fucoxanthin, we suggested that freezing of fucoxanthin after purification and the temperature during extraction is quite prominent to prevent reducing antioxidant activity. Meanwhile, fucoxanthin showed potent ferrous metal ions chelating activity and hydroxyl radical scavenging activity, which are relative to prevent some diseases for human. Therefore, we suggested fucoxanthin can be a candidate antioxidant food supplement materials.

In this study, we detected the protective effect of fucoxatnin on LPS-stimulated and DOX-induced RAW 264.7 cells, which is important in the uptake of antigen, which is crucial in killing microbes, parasites and tumor cells. After phagocytic uptake, macrophages turn to be antigen-presenting cells with higher expression level of costimulatory molecules and then transmitted an interaction between T cells and macrophages (Hoebel *et al.*, 2003). In the present study, the results showed that fucoxanthin significantly increased the proliferation of RAW264.7 cells.

Our previous researches confirmed that fucoxanthin had inhibitory effect on human bladder cancer EJ-1 cells by inducing apoptosis (Zhang *et al.*, 2008) and the new data of our laboratory was reported that inhibitory effect of fucoxanthin on T24 cells was related to cell cycle arrest at G0/G1 phase through increasing p21 at low dosage and apoptosis via down-regulating the level of mortalin at high dosage (Wang *et al.*, 2014). In this study, the fucoxanthin also showed significant inhibition of human cancer cells, HT1080, MDA-MB-231 and U-2 OS cells and the mechanism should be discussed in future.

For the safety of fucoxanthin, previous researchers elucidated that fucoxanthin had few negative influences on normal and uninfected cells both *in vitro* and *in vivo* (Ishikawa *et al.*, 2008; Yamamoto *et al.*, 2011). Other previous literatures demonstrated when feeding 93% fucoxanthin to imprinting control region mice at dosage of 500, 1000 and 2000 mg/kg for one month, all of the experiment animals had no mortality and no abnormalities in gross appearance and also had no abnormal observation in liver, kidney, spleen and gonadal tissues induced by fucoxanthin (Beppu *et al.*, 2009; Iio *et al.*, 2011). These results indicated that fucoxanthin is a safe compound for mammals under these experiment conditions. In addition, fucoxanthin can be deacetylated into fucoxanthinol by lipase at intestinal epithelium and also can be deacetylated into fucoxanthinol during the uptake by differentiated cell lines and a tissue culture model. Furthermore, fucoxanthinol can be traced into the blood circulation system in mammals (Sugawara *et al.*, 2001; Yonekura *et al.*, 2010; Das *et al.*, 2010). Thus, the bioavailability of fucoxanthinol is higher than that of fucoxanthin in body.

## Conclusion

The bioactivity of fucoxanthin was evaluated in this study. Fucoxanthin extracted from *Undaria pinnatifida* had strong anti-oxidant activity on hydroxyl radical scavenging activity ($EC_{50}$ = 7.03 μM) and ferrous metal ions chelating activity ($EC_{50}$ = 30.22 μM). Fucoxanthin can activate proliferation of RAW 264.7 and protect it from DOX-induced and LPS-stimultaed cell damage. Fucoxanthin could inhibit proliferation of HT1080, MDA-MB-231 and U-2 OS cells. Therefore, this study can reutilize the food process waste to produce anti-oxidant and anti-cancer health food supplement or cancer therapy candidate drug, as well as we can solve the disposition of food process materials to avoid the environment pollution.

## Funding Information

## Author's Contributions

**Yiting Li:** Gave advice for the experiments and written the manuscript.

**Ye Liu, Yuepeng Wang and Ling Li:** Reviewed the manuscript.

**Yue Yu and Yang Zeng:** Feedbacks and suggestions

**Linbo Wang:** Designed the experiments and performed experiments, gave critical reviews and helped in writing the manuscript.

## Conflict of Interest

The author declares that they have no conflict of interest.

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