Antiproliferative Effect of Fucoxanthin on Caco-2 (human colorectal carcinoma) and HepG2 (human hepatoma) Cells

S. Mohamadnia[1, 2], H. Foroutanfar[2], O. Tavakoli[1, *], M. Ali Faramarzi[2]
1. School of Chemical Engineering, College of Engineering, University of Tehran, Tehran 14176, Iran
2. Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran
otavakoli@ut.ac.ir

Abstract
Fucoxanthin is a major pigment in edible brown algae, has many properties (antioxidant, antibacterial, anticancer, antiobesity, antiinflammatory, etc.) due to its unique structure. Fucoxanthinol (fucoxanthin metabolite) has also anticancer effect. It is hydrolyzed from dietary fucoxanthin in the gastrointestinal tract before absorption in the intestine. Rcently, owing to a lot of side effects that is caused from chemical medicine used in chemotherapy, researchers attention turned to natural compound with anticancer activity e.g. fucoxanthin. The anticancer activity of fucoxanthin was assesed in the current study. To this end, microalga was cultivated in synthetic seawater enriched with f/2 medium. In the late of exponential phase the cells were harvested and three concentration of total carotenids (50, 250, 2500 µg/ml) and fucoxanthin (4, 10, 20 µg/ml) were prepared for measuring the anticancer activity on Caco-2 and HepG2 cells line through MTT [3-(4,5 dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide] assay. The results showed that total carotenoid and purified fucoxanthin at 24-hour treatment with 20 and 2500 µg/ml concentration respectively, has fairely effective anticancer activity on the Caco-2 and HepG2 cells line. By considering the attained results, chemotherapy and chemical drugs used in cancer treatment, can be replaced by purified fucoxanthin which is a hopeful supplementary diet in food and pharmaceutical industries.

Keywords: Fucoxanthin, *Tisochrysis lutea*, Antiproliferative effect, Caco-2, HepG2

Introduction
Cancer is one of the most pioneer cause of death and a numerous number of people worldwide contract cancer every year. Surgical removal, radiotherapy or chemotherapy used to treat the cancer which they are attended by severe side effects. Some researchers are started to search for natural antitumor compounds with anti-proliferative and antitumor activities that are more useful and less risky that could be used as alternative and complementary methods of therapy. For this reason, scientists are interested in to use marine algae. Golden-brown algae are the principle source of foucoxanthin.
Fucoxanthin is an allenic carotenoid that has shown multiple beneficial effects on human health. Today, widespread applications of fucoxanthin in food industry, pharmaceutical, and medical purposes are increasing. It has shown multiple beneficial effects on human health by

anti-cancer, anti-obesity, antioxidant, anti-inflammatory, anti-diabetic, and anti-angiogenic activities [1]. Based on present researches, fucoxanthin has anticancer effects. Anticancer effects of fucoxanthin and its metabolit (fucoxanthinol) on colorectal cancer cell lines was reported by Takahashi et al. (2015). They represented that these bioactive compounds from brown algae can be used as chemotherapeutic agents [2]. Anticancer properties of fucoxanthin was approved by Martin et al. (2015) in various field of cancer cell lines (liver cancer, bladder cancer, prostate cancer, breast cancer, leukemia, cervical cancer, osteosarcoma cancer and skin cancer). They exhibit cancer cells development inhibition through fucoxanthin and its metabolites neglecting to sex differentiate [3].

Among golden-brown algae, the microalga *Tisochrysis lutea* is the best candidate for comercial production of fucoxanthin due to broad range of light intensity and temperature conditions in a fewer days of cultivation (i. e. 7–8 days with specific growth rate p=0.082 h^{-1}) at 25 °C as an optimum growth temperature. Also, no cell wall, causes an easy extraction in the downstream processing. Then, the anticancer aspect of total carotenoid (50, 250, 2500 µg/ml) and purified fucoxanthin (4, 10, and 20 µg/ml) which are extracted from *T. lutea* were assesed after 24h incubation time.

In this study, we extracted the totoal carotenoids, next the fucoxanthin was seperated and purified by column chromatography. Finally, the anticancer propertie of this natural source as a potential medicine was estimated.

Experimental procedure

Tisochrysis lutea CCAP 927/14 was purchased from the Culture Collection of Algae and Protozoa (CCAP, Oban, Scotland). *T. lutea* was cultivated in 1000 mL flasks (500 mL medium volume) at 24 ± 1 °C and pH=7.9±0.1. Light was provided by 60 W fluorescent lamps under 60 µmol m^{-2} s^{-1} illumination intensity with (16:8) light: dark conditions in phytotron. Mixing was performed by aeration with 0.45-µm filtered air at 5 L min^{-1} by an air pump. This study has been carried out in department of pharmaceutical biotechnology, faculty of pharmacy and biotechnology research center, Tehran University of Medical Sciences, 2019.

Figure 1: *Tisochrysis lutea* cultivated in f/2 medium.

Growth analysis

Algal performance was determined in terms of cell number using a hemocytometer after Lugol dying on a microscope until the culture reached the late stationary phase. Each sample was numerated in three replicates and the mean was used as the algal density for each

replicate. Biomass productivity of microalgae were determined by sampling 5 mL of each culture through pre-weighed whatman glass microfiber filters (diameter 25 mm, pore size 2.5 µm) and washed twice with deionized water followed by drying in an oven at 75 °C until achieving a constant weight. Therefore, the biomass productivity was obtained using Eq. (1) [4]:

$$\text{Biomass productivity (mg L}^{-1}\text{ day}^{-1}) = (w_f - w_0)/(t_{tot}) \quad (1)$$

W_0 and W_f are the biomass concentration (mg L^{-1}) after inoculation and end of the cultivation (t_{tot}=6 days), respectively.
The specific growth rate (µ) was obtained during exponential phase according to Eq. (2):

$$\mu\ (\text{day}^{-1}) = (\ln X_2 - \ln X_1)/(t_2 - t_1) \quad (2)$$

where X_2 and X_1 are the cell density (cells mL^{-1}) at time t_2 and t_1 (day), respectively.

Pigments analysis

Microalga sample was harvested by centrifugation at 5000×g for 5 min at 4 °C. The supernatant was removed, and biomass was washed off with distilled water. Finally, it was freeze-dried at –70 °C for 2 days. *T. lutea* does not have cell wall, therefore all pigments, particularly fucoxanthin could be extracted easily using conventional solvents (e.g. ethanol as the main solvents recommended in numerous articles [5] without any cell disruption. All extraction process was carried out triplicate, at room temperature under dim light in order to eliminate pigment degradation.
According to described method in [6] total carotenoid were extracted and the contents were quantified by microplate reader at 452 nm through Eq. (3).

$$\text{Total carotenoid (mg l}^{-1}) \quad A_{452} \quad 3.86 \quad \frac{V_e}{V_t} \quad (3)$$

4 mL ethanol was added to 100 mg freeze-dried microalgae and the samples were then vortexed and allowed to stand for five minutes. This process was repeated once, and the extract supernatants collected and were filtered through 0.42-µm filter and used for HPLC analysis of fucoxanthin [5]. The extracts were analyzed by an HPLC system equipped with a PDA Detector 2800 and a Pump 1000. Samples (20 µL) were injected into a Eurospher 100 RP & C18 column (5 µm, 250 mm × 4.6 mm) from Knauer (Berlin, Germany). Isocratic mobile phase was methanol/water at a ratio of (95:5 v/v). Fucoxanthin was monitored at 450 nm. The retention times of fucoxanthin (at flow rate of 1 mL min^{-1}) were 7.7 min.

Cell line culture and MTT assay

Caco-2 and HepG2 cells line were obtained from Pastour Institute Center. Cancer cells line was cultured at complete culture medium (RPMI) and normal cells line was cultured at DMEM culture medium (Gibco company USA) as supplemented with 10% fetal bovine serum (FBS), 1% antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin) and then incubated for 6, 24 and 48 h at 37 ˚C in 5% CO2 and humidity of 80% for cells attachment MTT method was used to assess the capacity of fucoxanthin extract on cell viability in cancer cells. Cancer cells were harvested by trypsinizing and re-suspending the cells in appropriate media supplemented with serum and plated in 96 well culture plates (Cell quantity: density of 1.0×10^5 cells/well) and incubated for 24 hours. Cells were then exposed to three different concentrations of purified fucoxanthin (4, 10, and 20 µg/ml) and total

carotenoid (50, 250, 2500 µg/ml) extracted from the microalga *Tisochrysis lutea* and incubated at incubation times (6, 24 h) at 37 ˚C and 5% CO_2 atmosphere. After finishing incubation time, MTT ([3-(4,5-dimethylthiazolyl)-2,5 diphenyl-tetrazolium bromide]) stock solution (5 mg/ml in phosphate buffer saline (PBS)) was added and again incubated for 3 h at 37 ˚C and 5% CO_2 atmosphere. Afterwards, solution of ethanol-DMSO, 1:1(v/v) was added to purple formazan crystals to dissolve it and absorbance was measured at 570 nm by ELISA reader (Synergy HTX Multi-Mode). All experiments were performed in triplicate. MTT assay is a chlorometric method based on changing tetrazolium salt MTT to formazan by dehydrogenases of viable cells at cell's mitochondria. The effect of the purified fucoxanthin and total extracts of microalgae on the cancer cell death was expressed as the percent of cell viability to negative control (cells treated only with 5% of DMSO) according to Eq. (4) [7].

$$\frac{A_{570}\text{Treated cells}}{A_{570}\text{control cells}} \quad 100 \quad \% \text{ Cell viability} \tag{4}$$

Results and Discussion

The growth of *T. lutea* was monitored in optimized f/2 medium until it reached to the stationary phase (Fig. 2). Then the biomass was harvested and total carotenoid were extracted. The fucoxanthin fraction of total carotenoid was purified in next step.

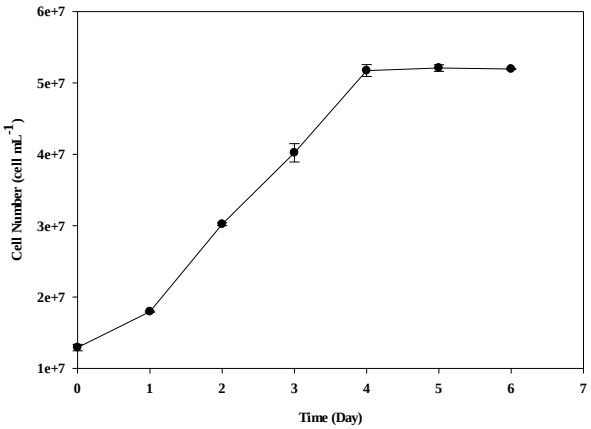

Figure 2: The growth curve of *Tisochrysis lutea*.

Potential cytotoxic effects of total carotenoid and fucoxanthin extracted from *T. lutea* on human colorectal carcinoma and human hepatoma cells line were evaluated. Fig. 3 presents the cells viability of cancer cells at various concentration treatments of total carotenoid and fucoxanthin from *T. lutea* at 24 h incubation time. As shown in Fig. 3(a), purified fucoxanthin significantly decreased Caco-2 and HepG-2 cells line vaiability approximately, to (60%) and (67%) at 20 µg mL^{-1}, respectively. The results are in accordance with other research at longer incubation time [8]. Fig. 3(b) exhibits the effect of total carotenoid extracted from *T. lutea* on viability of Caco-2 and HepG-2 cells line. According to the results, the concentration of 2500 µg mL^{-1} of total carotenoid inhibit the cell growth nearly to (84%) and (89%) , respectively, for Caco-2 and HepG-2 cells line after 24 h treatment time. Owing to almost high cytotoxic effect of purified fucoxanthin on cancer cells after 24 h, then, it can be comprehended that incubation time of 24 h, is the best incubation time for the treatment of two studied cells line.

The concentration of total carotenoid and purified fucoxanthin on the percentage of cell death ($p<0.05$) are directly related together.

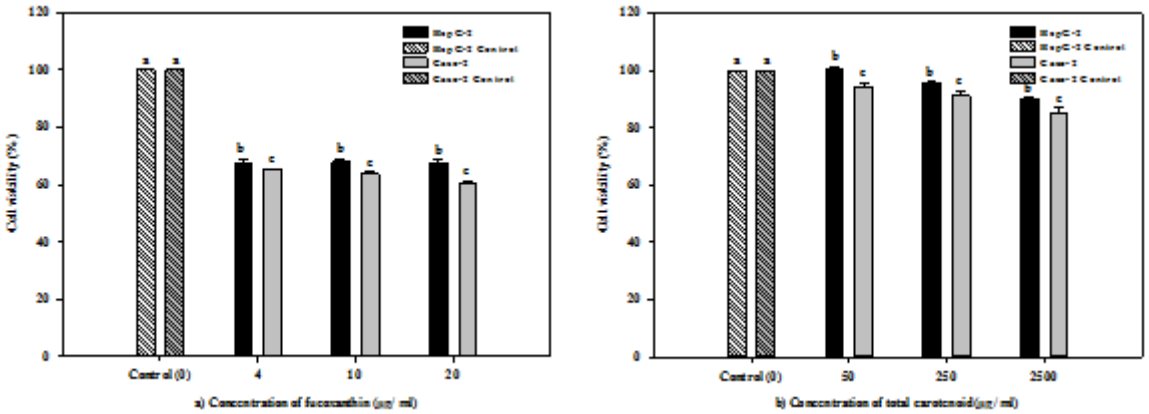

Figure 3: Percentages of cell viability at different concentrations of a) Fucoxanthin b) Total carotenoid from *T. lutea* at 24 h incubation time. Data are expressed as mean±standared deviation (n=3). Different letters (a, b, c) present significant mean differences between two cells line at a level of $p < 0.05$.

As wang et al. [9] examined the anticancer properties of biocompound, we also concluded that the cancer cells growth prevention by fucoxanthin or total carotenoid depends on the dose and time. Although, there is no report on toxicity effect of fucoxanthin on normal cells, but at very high concentration of fucoxanthin (about 2000 mg/kg) in it has opposite effect [10]. In fact, brown algae contain special bioactive compound (e.g. fucoxanthin and its metabolites) which give them higher anticancer effect in comparison to the other kind of algae [11, 12]. There are some researches on the influence of the usage of fucoxanthin contain sources, which emphesized the importance of this valuable pigment concentration and its antiproliferative effect. There are three point of view about the reaction of fucoxanthin in human body 1) Minimizing the propagation of target cells by consumin the fucoxanthin can diminish cell apoptosis, 2) It can contribute to morphological change on cells, and 3) DNA replication step prohibition and G0/G1 phase of the cell cycle is apprehended [7].

Conclusion

According to American Cancer Society estimation, 27 million new cancer disease will be presented by 2050, because of increasing global warming and numerous environmental issues. Marine algae is the best source of protein, carbohydrate, vitamine, anti- oxidant material, etc which can be used instead of chemical synthetic compounds with many side effects. The current study demonstrated the activity of purified fucoxanthin (4, 10, 20 µg mL^{-1}) and total carotenoid (50, 250, 2500 µg mL^{-1}) of *T. lutea* at 24 h incubation time on the viability of two cancer cells line. According to obtained results, 20 µg.mL^{-1} of purified fucoxanthin extensively prevents from cell growth at a short period of incubation rather than total carotenoid. This can be improved by lasting the incubation time or increasing the amount of fucoxanthin. The results suggest that the purified fucoxanthin or the diatery supplements containing the fucoxanthin extract of *T. lutea* may supply a novel therapeutic approach as the anticancer agent.

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