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Full Length Research Paper

Isolation of fucoxanthin and fatty acids analysis of *Padina australis* and cytotoxic effect of fucoxanthin on human lung cancer (H1299) cell lines

Irwandi Jaswir[1]*, Dedi Noviendri[1,2], Hamzah Mohd. Salleh[1], Muhammad Taher[3] and Kazuo Miyashita[4]

[1]Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, Gombak, 53100 Kuala Lumpur, Malaysia.
[2]Research Center for Marine and Fisheries Product Processing and Biotechnology, Ministry of Marine Affairs and Fisheries, Republic of Indonesia, Petamburan VI, 10260, Central Jakarta, Indonesia.
[3]Department of Pharmaceutical Technology, Faculty of Pharmacy, International Islamic University Malaysia, Kuantan, 25200 Pahang, Malaysia.
[4]Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate 041 8611, Japan.

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Fucoxanthin has been successfully isolated from species of Malaysian brown seaweed, namely *Padina australis*. The purity of the fucoxanthin is >98% as indicated by high performance liquid chromatography analysis. This seaweed also contains a considerable amount of unsaturated fatty acids. Thirteen fatty acids were detected with gas chromatography. However, fatty acid methyl ester (FAMEs) of eicosapentanoic acid (C20:5n-3), arachidonic acid (C20:4n-6), linoleic acid (C18:2n-6) and alpha-linolenic acid (C18:3n-3) contents of *P. australis* were found to be 2.06, 9.50, 6.37, and 2.83%, respectively. For saturated fatty acids, palmitic acid (C16:0) was found to be the major fatty acid with about 23.97%. Furthermore, data obtained from the methyl thiazolyl tetrazolium (MTT) assay indicated that fucoxanthin reduced the viability of H1299 cell lines, showing an IC_{50} value of 2.45 mM.

Key words: Brown seaweed, *Padina australis*, fucoxanthin, fatty acid methyl ester, H1299 cell lines, human lung cancer, MTT assay.

INTRODUCTION

About 6000 species of seaweeds have been identified and are grouped into different classes. Seaweed can be classified as brown seaweed (Phaeophytes), green seaweed (Chlorophytes) and red seaweed (Rhodophytes) (Chandini et al., 2007; Dawczynski et al., 2007). These seaweed are excellent sources of bioactive compounds such as carotenoid, dietary fibre, protein, vitamins (Holt, 2008), essential fatty acids and minerals (Bhaskar and Miyashita, 2005; Sugarawa et al., 2002).

Interest in seaweed lipids has been on the rise, owing to the recognition of important bioactive molecules like conjugated fatty acids and pigments (especially fucoxanthin), that have profound physiological effects in the treatment of tumors and other cancer related problems (Hosokawa et al., 2004; Kohno et al., 2002, 2004). Fucoxanthin occurs in great abundance in brown seaweed, but is absent in higher plants (Ballard et al., 1989). Furthermore, pigments in seaweeds have important nutraceutical properties, including: Antioxidant and biological response modifying qualities (Holt, 2008). In addition, polyunsaturated fatty acids (PUFAs) are reported to share more than 30% of total fatty acids in diatom or brown algae (Nomura et al., 1997).

In the present study, we extracted and purified the fucoxanthin and investigated the fatty acid content of

*Corresponding author. E-mail: irwandi@iium.edu.my.

Abbreviations: **FAMEs**, Fatty acid methyl esters; **MTT**, methyl thiazolyl tetrazolium.

Malaysian brown seaweed, namely *Padina australis.* Furthermore, we analyzed the effect of fucoxanthin on human lung cancer (H1299) cell lines. To the best of our knowledge, there have been no reports on extraction, purification of fucoxanthin, investigation of the fatty acid content and cytotoxic effect of fucoxanthin from Malaysian brown seaweeds (tropical area) on H1299 cell lines.

MATERIALS AND METHODS

Plant material (brown seaweed) used in this study was *P. australis.* It was freshly collected from Port Dickson, Seremban, Negeri Sembilan Malaysia in August 2009. The sample was washed thoroughly with freshwater to remove the salt and sand attached to the surface (Soo-Jin et al., 2008, 2009), then stored frozen at -80°C and thawed before using it for analysis (Noviendri et al., 2011).

Total lipid content

The methods of Terasaki et al. (2009) were adopted to determine total lipid. All the extractions were carried out under dim light, and air in the extraction vessel was replaced with nitrogen to prevent any possible degradation of carotenoids or lipids (Noviendri et al., 2011).

Analysis of fucoxanthin content by HPLC

The methods of Terasaki et al. (2009) were adopted for fucoxanthin content determination by HPLC. All HPLC analyses were carried out using a Hitachi L-7000 HPLC system (Hitachi, Tokyo, Japan) equipped with a pump (L-7000), auto-sampler (L-7200) and a photo diode-array spectrophotometric detector (Hitachi L-7455). Fucoxanthin content in seaweed TL was determined by reversed-phase HPLC (RP-HPLC) with methanol-acetonitrile (7:3 v/v) as the mobile phase at a flow rate of 1.0 ml/min (Maeda et al., 2005, 2006 , 2007). All RP-HPLC analyses were carried out at 28°C using a RP column (Develosil-ODS, UG-5, 5.0 µm particle size, 250 x 4.6 mm i.d.; Nomura Chem, Co., Seto, Aichi, Japan) (Maeda et al., 2006, 2007) protected with a guard column (10 x 4.0 mm i.d.) having the same stationary phase. Briefly, an aliquot of TL was dissolved in the mobile phase, filtered with a 0.22 µm membrane filter, and an aliquot of the filtered sample was submitted to HPLC analysis. The detection wavelength was set at 450 nm for detecting fucoxanthin (Yan et al., 1999; Mori et al., 2004; Maeda et al., 2006; Cheng-Ling et al., 2009; Nakazawa et al., 2009). A standard curve prepared using authentic standard was used for quantification of fucoxanthin content in seaweed samples. Standard fucoxanthin (>99% purity established by HPLC) was prepared from wakame (*Undaria pinnatifida*) lipids as described previously (Hosokawa et al., 1999). Fucoxanthin content in seaweed samples were expressed as $mg.g^{-1}$ dry weight of seaweed sample. The amount of fucoxanthin was quantified from the peak area by using a standard curve with purified fucoxanthin (Maeda et al., 2006; Noviendri et al., 2011).

Extraction and purification of fucoxanthin

Extraction of fucoxanthin

A slight modification of the method described by Haugen et al. (1992) was adopted for the extraction and purification of fucoxanthin (Noviendri et al., 2011a, b). Cold acetone-methanol (7:3 v/v) was added to a 1 L flask containing dried and ground brown seaweed (Haugan and Liaaen-Jensen, 1989). These mixtures were homogenized on ice for 10 to 15 min, and then the mixtures were filtered through a filter paper. The steps were repeated at least three times. The acetone : methanol extracts were pooled and left at room temperature, under N_2 and in the dark until the extract became colorless. The extract was evaporated to dryness at 30 to 35°C on a rotary evaporator, and the residue was dissolved in methanol. The reconstituted residue was partitioned in a separation funnel between n-hexane and 90% (v/v) aqueous methanol for three times. The hexane phase was discarded. Fucoxanthin from the aqueous phase was moved to diethyl ether. The diethyl ether phase was evaporated to dryness on a rotary evaporator. The residue was re-dissolved in minimum amount of benzene for the purification step (Haugan et al., 1992; Haugan and Liaan-Jensen, 1994; Noviendri et al., 2011, b).

Purification of fucoxanthin

The benzene containing residue was loaded in a silica column (Silica 60G, Merck, 0.040 - 0.063 mm). Elution was initially performed with n-hexane (100%) to remove chlorophyll and carotenoids apart from fucoxanthin (Sangeetha et al., 2009). Elution was continued with n-hexane : acetone (6:4; v/v) to recover fucoxanthin (Hosokawa et al., 1999). Finally, residual fucoxanthin was eluted with acetone solvent. The acetone and hexane : acetone (6:4; v/v) fractions containing fucoxanthin were combined and evaporated to dryness by a rotary evaporator (Noviendri et al., 2011, b).

Further purification of fucoxanthin

The residue from the combined acetone and hexane : acetone (6:4; v/v) evaporation step was re-dissolved in methanol. The concentration of fucoxanthin was checked by HPLC preparative (ODS double column, 1 ml/min at 450 nm, methanol and acetonitrile were the mobile phase). All procedures were carried out under dim yellow light to minimize degradation and isomerization of fucoxanthin by light irradiation (Sugarawa et al., 2002).

Fatty acid contents

Fatty acid (FA) analysis was accomplished by injecting FA methyl esters (FAMEs) into a gas chromatography (GC) system (Shimadzu GC-14B; Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame-ionization detector (FID) and a capillary column (Omegawax-320; 30 m x 0.32 mm i.d.; Supelco, Bellefonte, PA). The carrier gas was helium at a flow rate of 50 Kpa (Bhaskar et al., 2004). The detector, injector and column temperatures were 260, 250 and 200°C, respectively. Briefly, to an aliquot of TL, 1 ml n-hexane and 0.2 ml 2 N NaOH in methanol were added, vortexed and incubated at 50°C for 30 min. Post incubation, 0.2 ml 2 N HCl in methanol solution was added, gently mixed to recover the upper n-hexane layer containing FAMEs. FA content in seaweed samples was expressed as weight percentage of total FAs (Terasaki et al., 2009; Noviendri et al., 2011).

Cytotoxicity assay of fucoxanthin

Cell culture

The human lung cancer lines (H1299) was obtained by Dr. Masaaki Ikeda, Department of Molecular and Craniofacial Embryology, Tokyo Medical and Dental University. This cell was cultured in Dulbecco's modified Eagles medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS) and antibiotics (1% penicillin, streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2.

Table 1. Total lipid and fucoxanthin content in *P. australis*.

Species	Dry weight (g)	Total lipid (mg/g dry wt)	Fucoxanthin content (mg/g dry wt)
P. australis	1.07 ± 0.01	10.70 ± 0.90	0.43 ± 0.07

The experiments were done in triplicate (n=3).

MTT assay

Cells were plated in 96 well culture plates (1×10^5 cells/well). After 24 h incubation, cells were treated with ethanol absolute (as control), 1.42; 2.84; 5.69; 11.37 and 22.75 mM of fucoxanthin (diluted by ethanol) for 48 h at 37°C. After incubation, medium was removed. Then, 3-(4-5-dimethylthiozol-2-yl)-2-5 diphenyl tetrazolium bromide (MTT) (dissolved in phosphate buffer saline (PBS) at 5 mg/ml) was added to all wells of an assay, and plate were incubated at 37°C for 4 h. Acid-isopropanol (100 µl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Microelisa reader, using a test wavelength of 570 nm, a reference wavelength of 630 nm (Mossman, 1983). Cell viability was expressed as percentage over the control (Srinivas et al., 2003). The experiments were repeated three times. The percentage of cell viability was calculated according to the equation described in Endrini et al. (2002), Moongkarndi et al. (2004) and Bakar et al. (2006):

Cell viability (%) = [OD sample (mean) / OD control (mean)] x 100%

The concentration required for inhibition of 50% of cell viability (IC_{50}) was calculated. The IC_{50} value in MTT assay was defined as the concentration of test compound resulting in a 50% reduction of absorbance as compared to untreated cells (Ruiz et al., 2008).

Statistical analysis

Mean and standard deviations were computed using Microsoft Excel software.

RESULTS AND DISCUSSION

Fucoxanthin and total lipid contents of the seaweeds were determined. The quantitative data on total lipid and fucoxanthin of *P. australis* is presented in Table 1. Results show that *P. australis* contained a considerable amount of fucoxanthin and total lipid. The fucoxanthin and total lipid content of *P. australis* were (0.43 ± 0.07 and 10.70 ± 0.09 mg/g dry-weight, respectively). The percentage of total lipid and fucoxanthin content of *P. australis* were 1.1 and 0.04%, respectively. Fucoxanthin content of *P. australis* in this study was lower than the results obtained from two brown seaweeds such as *Laminaria digita* (2.4%) and *Padina tetrastromatica* (1.7%) (Dhargalkar and Pereira, 2005), but the percentage of lipid in *P. australis* was higher than that of *Sorghum vulgare* (0.45%) (Plaza et al., 2008). Furthermore, the fucoxanthin contents of *Sargassaceae* such as *Sargassum thunbergii*, *Sargassum fusiforme* and *Sargassum confusum* (1.8 ± 1.0; 1.1 ± 0.6; and 1.6 ± 0.8, respectively) from Hakodate, Japan (Terasaki et al., 2009) were higher than fucoxanthin content of *P. australis* from Malaysia determined in this study. However, the fucoxanthin contents of this brown seaweed in the present study was higher than that of the three brown seaweeds, *Leathesia difformis*, *Sphaerotrichia divaricata* and *Desmarestia viridis* (0.3 ± 0.1; 0.2 ± 0.1 and 0.1 ± 0.1 mg/g dry weight, respectively) from Hakodate, Japan (Terasaki et al., 2009).

After SiO_2 column chromatography using n-hexane/acetone (6:4, v/v) as mobile phase to separate fucoxanthin, the isolated fucoxanthin from *P. australis* showed 94.8% purity. Furthermore, the purity of fucoxanthin after purification with ODS-double column HPLC showed 98.1% purity (Figure 1A and B). Maeda et al. (2007) reported that *wakame* lipid extraction with acetone followed by salicic acid column chromatography using n-hexane/acetone (7:3, v/v) as the mobile phase was successful for fucoxanthin separation. When this separation step was repeated three times, the recovered fucoxanthin showed purity >78% on HPLC. In addition, the HPLC chromatograms of purified fucoxanthin from *P. australis* showed only one major peak with a retention time of about 7.8 min (Figure 1B).

In this study, thirteen fatty acids were identified in the extracts of Malaysian brown seaweeds. The fatty acid compositions of the brown seaweeds are shown in Table 2, whereas Figure 1C shows a typical chromatogram of the FAMEs of *P. australis*. In this study, the most abundant fatty acid was C16:0 (palmitic acid), which in *P. australis* accounted for 23.97% of all fatty acids. This result is comparable to other brown seaweeds such as, *Hormosira banksii*, *Ralfsia* sp., *Dictyota dichomota* (Johns et al., 1979), *Stilophora rhizodes, Entonema parasiticum, Pylaiella littoralis, Corynophlaea umbellate*, *Cystoseria crinita*, (Dembitsky et al., 1990), *Saccorhiza polyscides*, *Himanthalia elongate* and *Laminaria ochroleuca* (Sánchez-Machado et al., (2004), *Sargassum marginatum* (Bhaskar et al., 2004), *Laminaria* sp. (Dawczyski et al., 2007), *Sargassum binderi* and *Laminaria duplicatum* (Noviendri et al., 2011) where the single most abundant fatty acid was palmitic acid. The predominant fatty acids found in the brown seaweed in the present study were palmitic, palmitoleic, stearidonic, arachidonic, linoleic and oleic acids (23.97; 14.45; 9.73; 9.50; 6.37 and 5.88%, respectively). Stearic (0.77%), arachidic (0.43%) and lauric (0.31%) acids were found at very low levels.

The PUFA content of this brown seaweed (33.91%) was much higher than the SFA (28.94%), and MUFA

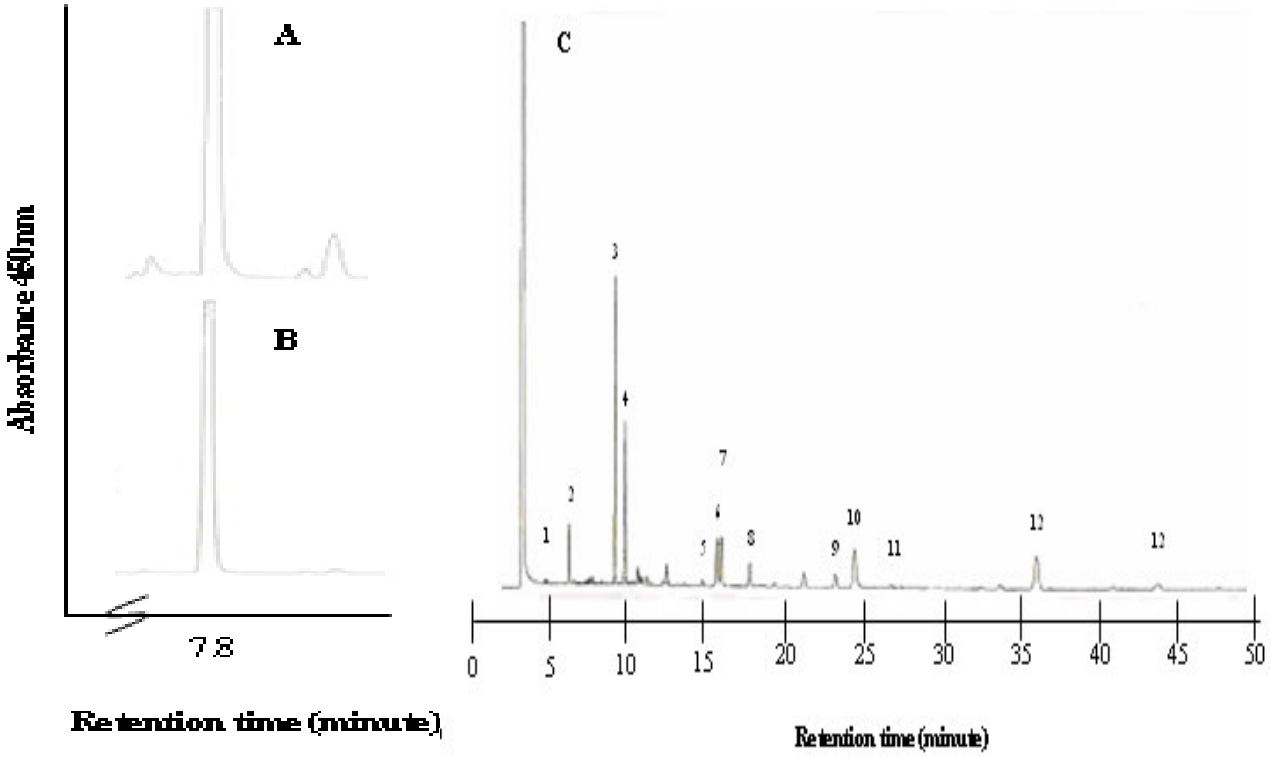

Figure 1. Chromatogram of HLPC results of fucoxanthin after SiO_2 open column (A), and after HPLC-ODS-double column (B). Chromatogram of the FAMEs of *P. australis* by gas chromatography (C).

Table 2. Fatty acid profiles of *P. australis*.

Fatty acid	Species (*P. australis*)
Saturated fatty acids (SFA) (%)	
C12:0 (Lauric acid)	0.31
C14:0 (Myristic acid)	3.46
C16:0 (Palmitic acid)	23.97
C18:0 (Stearic acid)	0.77
C20:0 (Arachidic acid)	0.43
Total	28.94
Monounsaturated fatty acids (MUFA) (%)	
C16:1n-7 (Palmitoleic acid)	14.45
C18:1n-9 (Oleic acid)	5.88
Total	20.33
Polyunsaturated fatty acids (PUFA) (%)	
C18:2n-6 (Linoleic acid)	6.37
C18:3n-6 (Gamma-linolenic acid)	3.42
C18:3n-3 (Alfa- linolenic acid)	2.83
C18:4n-3 (Stearidonic acid)	9.73
C20:4n-6 (Arachidonic acid)	9.50
C20:5n-3 (Eicosapentaenoic acid)	2.06
Total	33.91
Others (Un-identified)	16.82
Identified-total	83.18

Table 3. Summary of saturated and unsaturated fatty acid contents (%) of *P. australis.*

Fatty acid content (%)	Species (*P. australis*)
Total Saturated fatty acids (SFAs)	28.94
Total monounsaturated fatty acids (MUFAs)	20.33
Total polyunsaturated fatty acids (PUFAs)	33.91
PUFA/SFA	1.17
Total n-3	14.62
Total n-6	19.29
n6/n3	1.32

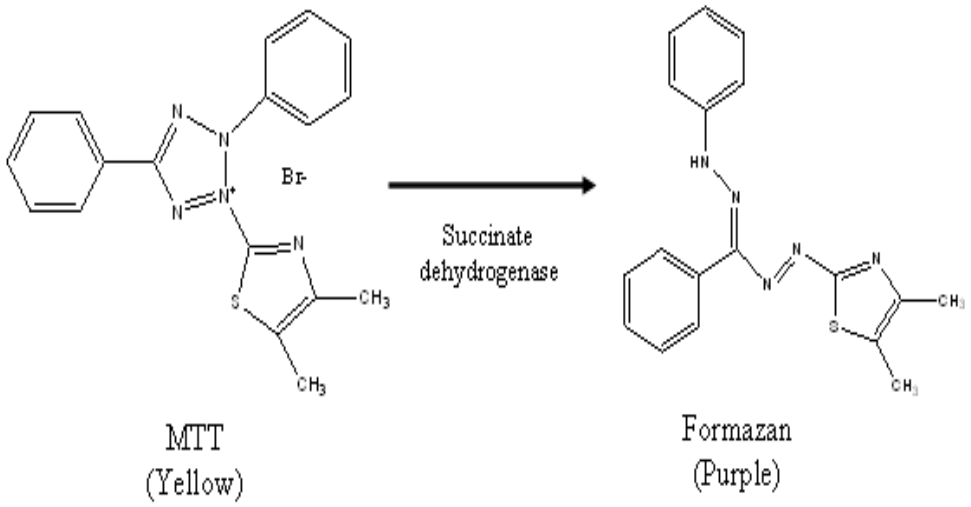

Figure 2. Metabolization of MTT to formazan salt by viability cells (Roche, 2005; Brescia and Banks, 2009).

contents are lowest (20.33%). Furthermore, the concentration of n-3 and n-6 PUFA of *P. australis* in this study were 14.62 and 19.29, respectively (Table 3). PUFA, especially the n-3 and n-6 PUFA, have been considered as essential fatty acids and have been shown to have curative and preventive effect on cardiovascular diseases, neurodevelopment in infants, cancers and fat glycemic control (Corner, 1997).

Seaweed products represent an important source of long-chain PUFA (n-3; n-6) that is fundamental for the formation of important structural lipids and elements of cell membranes (Dawczyski et al., 2007). The seaweed studied in this work (*P. australis*) has n6:n3 ratio (1.32%). Sánchez-Machado et al. (2004) reported that *Himanthalia elongata* and *U. pinnatifida* have n6:n3 ratio of 0.81 and 0.49%, respectively. Furthermore, Noviendri et al. (2011) reported that *Sargassum binderi* and *S. duplicatum* have n6:n3 ratio 0.87 and 1.73%, respectively. The n6:n3 ratio is beneficial for health due to several sources of information suggesting that human consume diet in which the ratio of omega-6 to omega-3 essential fatty acids (EFA) was about 1, whereas in Western diets, the ratio is 15/1 to 16.7/1. Excessive amounts of n-6 PUFA and a very high n6:n3 ratio will promote the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune disease, whereas increased levels of n-3 PUFA (a low n6:n3 ratio) exert suppressive effects (Simopoulus, 2002). The intake of food rich in n-3 PUFA can have a positive influence on the composition of blood lipids and can therefore be used for the prevention of arteriosclerosis (Murata et al., 2002).

In this study, the anticancer activity of the fucoxanthin was investigated using a 3-(4-5-dimethylthiozol-2-yl)-2-5 diphenyl tetrazolium bromide assay on human lung cancer line, H1299. On the other hand, the cytotoxicity effect of fucoxanthin was determined by using MTT assay. MTT is a yellow water-soluble tetrazolium dye which is reduced by live cells to a purple formazan product insoluble in aqueous solutions. MTT assay is based on the reduction of soluble tetrazolium salt, by a mitochondrial enzyme (succinate-dehydrogenase) in living cells. The assay is based on the cleavage of the yellow tetrazolium salt, MTT to purple formazan crystals by metabolic active cells (Roche, 2005). The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored, formazan product (Yedjou et al., 2006). Mitochondrial dehydrogenase cleaves the tetrazolium ring and converts the MTT to an insoluble purple formazan (Figure 2), and the number of formazan produced is directly proportional to the number

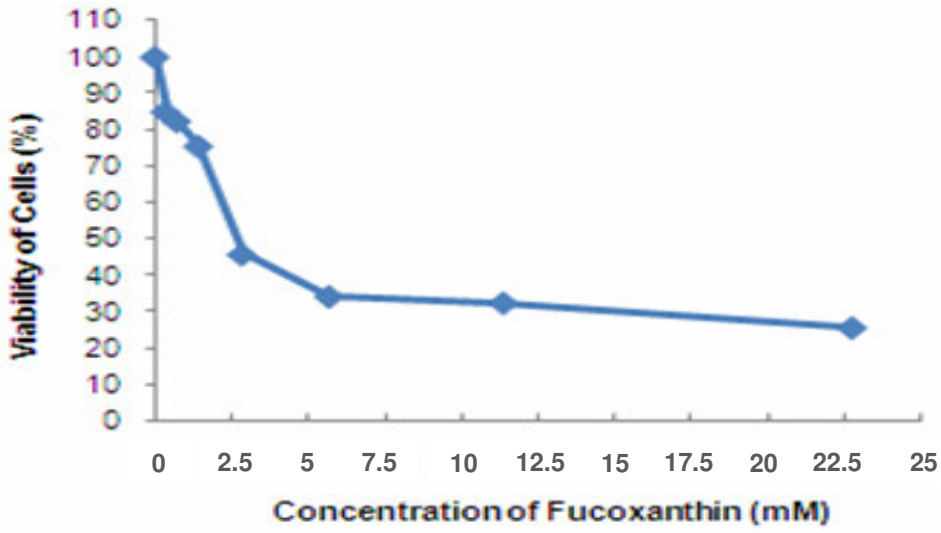

Figure 3. Viability of human lung cancer cells (H1299) treated with fucoxanthin.

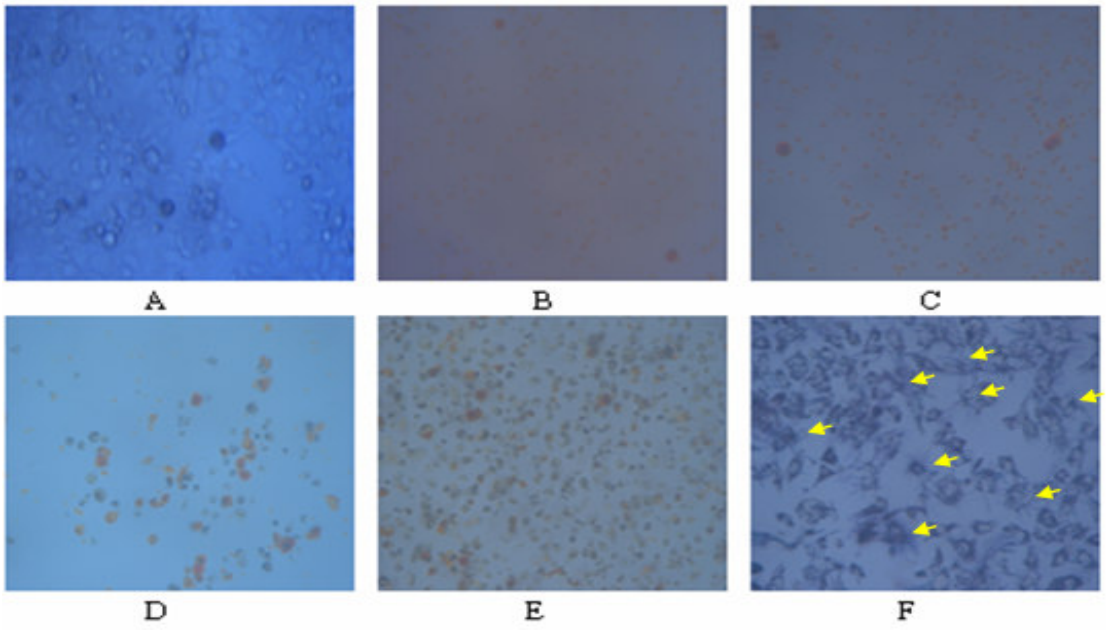

Figure 4. Morphological changes induced by fucoxanthin in cancer cells. Human cancer cells, H1299 (lung cancer) was seeded in 96-well plates. Cells were visualized in an inverted microscope after 48 h and photographed (magnification: 20x). H1299 untreated cells (control: absolute ethanol without fucoxanthin was used) (A), H1299 cells treated with fucoxanthin (22.75 mM) (B), (11.37 mM) (C), (5.69 mM) (D), (2.84 mM) (E) and (1.42 mM) (F). The crystals of formazan in living cells are indicated by yellow arrows.

of cells (Endrini et al., 2002; Saravanan et al., 2003; Lee et al., 2004; Theiszova et al., 2005). Growth inhibitory effect of fucoxanthin in lung cancer cells (H1299) was monitored by determining the viability of the cells based on their ability to reduce MTT (Figure 3).

Fucoxanthin inhibited the growth of H1299 cells in a dose-dependent manner. Fucoxanthin dose-dependently suppressed cell growth at concentration of 2.84; 5.69; 11.37 and 22.75 mM, 48 h after treatment, the end time point of this study as compared with the control. The 50% inhibitory concentration (IC_{50}) of fucoxanthin on cell growth on 48 h was 2.45 mM. The IC_{50} value (the drug concentration causing 50% inhibition of the tumor cells) was used as parameter for cytotoxicity (Smit et al., 1995). Furthermore, the phenotypic characteristics of fucoxanthin treated cells were evaluated by microscopic inspec-

tion of overall morphology. The size of cells decreased because of treatment of fucoxanthin as compared to control cells (untreated cells). The treatment of cells with fucoxanthin 22.75 to 2.84 mM (Figure 4B to E), for 48 h resulted in formation of nuclear condensation, which was clearly evident in inverted microscope. The effect of fucoxanthin disappeared on H1299 cell lines for concentration of 1.42 mM (Figure 4F). In this case, the living cells can react with MTT solution. Active mitochondria of living cells can cleave MTT to produce formazan crystals, the amount of which is directly correlated to the living cell number (Hu et al., 2007). Reduction in the number of cells by particular agent (cytotoxicity) can generally be explained by cell killing and/or inhibition of cell proliferation (Endrini et al., 2002). The morphological characteristics of fucoxanthin treated cells and control are shown in Figure 4.

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