Studies on Extraction of Fucoxanthin and Its Potential Anti-obesity Effect

by

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Abstract

Fucoxanthin is an epoxy carotenoid with important beneficial bioactivities. In this study, a type of microalgae (diatom) *Thalassiosira weissflogii* was used as the feedstock for fucoxanthin extraction. Effects of solvent types, feedstock conditions, presence of antioxidants, and extraction time on fucoxanthin yield were investigated. Results suggested diatoms might be a more cost effective source for fucoxanthin extraction than brown algae. Wet diatoms can achieve high extraction yields over a much shorter period. Yield was 100.7 ± 5.8% of the average total available fucoxanthin in the diatoms after 10 min of extraction with acetone. Adding 0.3% of the antioxidant, butylated hydroxyanisole (BHA), during extraction may not increase the yields significantly in the short time, but it could prevent the further potential decomposition of fucoxanthin.

Several studies have suggested that fucoxanthin has anti-obesity, anti-diabetic, and anti-cancer properties. We sought to determine whether fucoxanthin, gavaged daily, would demonstrate anti-obesity and anti-diabetic properties in rats fed a high-fat/high-sucrose diet. To accomplish this, we performed an energy balance study in three groups of Wistar rats. Two groups of rats were fed a high-fat/high-sucrose diet. One of these groups was gavaged daily with fucoxanthin, while the other group was gavaged with vehicle. The remaining group was fed a low-fat diet and was gavaged daily with vehicle. Food intakes and body weights were determined daily for approximately 12 weeks. After 10-11 weeks on the respective diets, a subset of rats from each group was placed in metabolic cages for 3 days to determine energy expenditure, RQ, and physical activity. Lastly, all rats were given

an oral glucose tolerance test to determine how the diets and fucoxanthin treatment affected insulin sensitivity.

Results obtained in this study were contrary to the hypothesis that fucoxanthin has anti-obesity and anti-diabetic properties. In fact, we found that fucoxanthin-treated rats fed a high-fat/high-sucrose diet gained significantly more body fat than vehicle-treated rats fed the same diet. The increase in body fat associated with fucoxanthin treatment did not appear to be related to a change in the energy expenditure. Rather, the increase in body fat appeared to be due to a non-statistically significant increase in food intake and an increase in the energetic efficiency of the calories that were consumed. In addition, fucoxanthin-treated rats fed the high-fat diet also showed greater insulin resistance as compared to the vehicle-treated rats fed the high fat diet. No difference in insulin resistance was found between low-fat-fed and high-fat-fed rats treated with vehicle. The greater insulin resistance of fucoxanthin-treated rats may reflect greater amounts of overall fat gain or perhaps a greater amount of fat deposition in nonadipocytes tissues.

The present study suggests that caution should be exercised when considering whether fucoxanthin has anti-obesity and anti-diabetic bioactivities. The specific experimental conditions under which fucoxanthin is extracted and tested may greatly affect the outcome of such studies.

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List of Abbreviations

AA	Arachidonic Acid
AUC	Area Under the Curve
BAT	Brown Adipose Tissue
BHA	3-tert-butyl-4-hydroxyanisole
BMI	Body Mass Index
CD	Circular Dichroism Spectroscopy
DAD	Diode-array Detector
DHA	Docosahexaenoic Acid
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
EPI	Epididymal
FE	Fucoxanthin Extract
GHO	Global Health Observatory
HFD-V	High-fat Diet with Vehicle
HFD-F	High-fat Diet with Fucoxanthin
HPLC	High Performance Liquid Chromatography
HPTLC	High performance thin layer chromatography
IACUC	Auburn University Institutional Animal Care and Use Committee
LFD-V	Low-fat Diet with Vehicle
MS	Mass Spectrometry
NIH	National Institutes of Health

NMR	Nuclear Magnetic Resonance
OGTT	Oral Glucose Tolerance Test
PLE	Pressurized Liquid Extraction
RETRO	Retroperitoneal
RQ	Respiratory Quotient
TLC	Thin Layer Chromatograph
UCP1	Uncoupling Protein 1
VIS	Visible Spectroscopy
WAT	White Adipose Tissue
WHO	World Health Organization

1. Introduction

1.1 General Problem Statement

Fucoxanthin is a major carotenoid that exists in the Phaeophyta, a division of algae that have chlorophyll masked by brown pigment, and most other heterokonts such as diatoms. Several recent metabolic and nutritional studies have suggested that fucoxanthin exerts some possible health benefits and has the potential to be used as a functional food or medicine (Hosokawa et al., 2009). Recent research has suggested that fucoxanthin might have multi-benefits on human health, including anti-obesity, anti-diabetic, anti-cancer, and anti-oxidation properties. The anti-obesity effect of fucoxanthin has been demonstrated in several studies using murine models (Maeda et al., 2005, 2007, 2009; Woo et al., 2009; Jeon et al., 2010). Fucoxanthin promotes expression of uncoupling protein 1 (UCP1) in white adipose tissue, which was proposed to accelerate fat oxidation within fat cells of white adipose tissue (Maeda et al., 2005). It also inhibits intracellular lipid accumulation during adipocyte differentiation of murine 3T3-L1 cells (Maeda et al., 2006). Furthermore, a study using humans suggested that fucoxanthin promotes weight loss, reduces body and liver fat content, and improves liver function tests in obese non-diabetic women (Abidov et al., 2010). An anti-cancer effect is another important potential health benefit of fucoxanthin. Fucoxanthin inhibits growth of human neuroblastoma GOTO cells (Nishino et al., 1992) and human leukemia cells (Hosokawa et al., 1999). In addition, fucoxanthin reduces the viability of human colon cancer cells (Hosokawa et al., 2004), and induces apoptosis through caspase-3 activation in human prostate cancer cells (Kotake-Nara et al., 2001, 2005). Besides potential anti-obesity and anti-cancer properties, fucoxanthin has also been

suggested to oppose the development of diabetes (Maeda et al., 2007, 2009), as well scavenge free radicals and quench singlet oxygen (Sachindra et al., 2007).

Functional foods are defined as foods that have health-promoting or disease-preventing properties beyond that of the basic nutrients they supply. In this regard, fucoxanthin could be considered as a functional food that has been suggested to have anti-obesity and anti-diabetic properties. It is attracting more and more attention from researchers in food science, food engineering, and human nutrition. Although fucoxanthin is rich in several edible seaweeds, such as brown algae, diatoms, and several other algal classes, its content is too low to directly use them as functional food or medicine. Therefore, extraction and enrichment are indispensable steps for the application of fucoxanthin as a functional food or a dietary supplement.

Obesity is a chronic metabolic disorder that results from the imbalance between energy intake and energy expenditure. It is characterized by an enlarged fat mass and possibly elevated lipid concentrations in blood. The Global Health Observatory (GHO) reported that the worldwide prevalence of obesity has nearly doubled between 1980 and 2008. This is a serious public health concern because people who are obese are at an increased risk for various physical, mental, and emotional health problems, including coronary heart disease, stroke, some forms of cancer, impaired glucose tolerance, insulin resistance, atherosclerosis, eating disorders, and low self-esteem. Obesity has become a major public health problem and is at epidemic levels in the United States. Two thirds of adults in the United States are considered either overweight (BMI between 25 and 30) or obese (BMI greater than 30). Few drugs are available for the treatment of obesity. Currently, the Food and Drug Administration (FDA) has only approved 3 drugs for long-term

treatment of obesity. Orlistat (Xenical and Alli) is an inhibitor of pancreatic lipase, which lowers that amount of fat that is absorbed, Lorcoserin (Belviq), an appetite suppressant, and a combination of phentermine-topiramate that goes by the name Qsymi, also an appetite suppressant. Some, previously approved agents for the treatment of obesity have been taking off the market due to life-threatening side-effects like heart attack and stroke (sibutramine) or heart value problem and pulmonary hypertension (fen/phen). Therefore, there has been high demand to find new therapeutic agents that are safe and effective in preventing or treating obesity. A potential source for these therapeutic agents could come from functional foods, like fucoxanthin.

1.2 Overall aims

1. To determine whether fresh wet diatoms are a more efficient source for the extraction of fucoxanthin as compared with the traditional industrial raw material, brown algae. It is hypothesized that high extraction yield over a much shorter period of time can be achieved under the specific solvent and antioxidant conditions of industrial production.
2. To determine whether fucoxanthin derived from brown algae exerts anti-obesity and anti-diabetic effects in rats fed a high-fat/high-sucrose diet. It is hypothesized that the daily gavage of fucoxanthin will prevent the increased accumulation of body fat due to a high fat diet and prevent the induction of insulin resistance.

1.3 Significance of the problem

The prevalence of obesity in the United States has increased dramatically over the past three decades. Currently, approximately two-thirds of adults in the United States are either overweight or obese. Obesity increases the risk of coronary heart disease, stroke, some

forms of cancer, and type 2 diabetes. Because of the high incidence of obesity and its association with several life-threatening chronic diseases, obesity has become a serious public health problem. Few drugs are available for the treatment of obesity. Currently, the Food and Drug Administration (FDA) has only approved 3 drugs for long-term treatment of obesity. However, administration of these drugs has limited effectiveness and often leads to undesirable side effects. Some, previously approved agents for the treatment of obesity have been taking off the market due to life-threatening side-effects like heart attack and stroke (sibutramine) or heart value problem and pulmonary hypertension (fen/phen). Therefore, identifying safe and effective anti-obesity agents would be of great benefit in combating the obesity epidemic.

Fucoxanthin is a compound derived from seaweed (brown algae) that has been suggested to have anti-obesity and anti-diabetic biological activities. In addition, fucoxanthin has also been suggested to have anti-tumor, anti-angiogenic, antioxidant, and radical scavenging properties. It is a natural component extracted from algae and it has fewer known negative side effects than other anti-obesity therapeutic agents. In this regard, fucoxanthin may be considered a functional food that has the potential to be used as an anti-obesity and anti-diabetic agent. There is no study on the extraction technology of fucoxanthin to fulfill the market demand. Optimization of the extraction procedures of fucoxathin would have value to the companies that produce the extracted fucoxanthin. In addition, the mechanism of its anti-obesity and anti-diabetic effects is not clear. An energy balance study has not been performed on high-fat-fed animals that have been gavaged daily with fucoxanthin.

2. Review of literature

2.1 Fucoxanthin

Fucoxanthin is a major carotenoid that is found in the Phaeophyta, a division of algae that have chlorophyll masked by brown pigment, and most other heterokonts such as diatoms. Several recent metabolic and nutritional studies have suggested that fucoxanthin exerts some possible health benefits and has the potential to be used as a functional food or medicine (Hosokawa et al., 2009). Recent research has suggested that fucoxanthin might have multi-benefits on human health, including anti-obesity, anti-diabetic, anti-cancer, and anti-oxidation properties.

Fucoxanthin is an orange pigment. The molecular weight is 658.91 g/mol. It is a major carotenoid with a molecular formula of $C_{42}H_{58}O_6$ (Figure 1), which is found as an accessory pigment in the chloroplasts of brown algae, and most other heterokonts, such as diatoms like prymnesiophytes, raphidophytes and chrysophytes, giving them a brown or olive-green color. Fucoxanthin has a unique structure, including allenic, conjugated carbonyl, epoxide, and acetyl groups in its structure. Fucoxanthin absorbs light primarily in the blue-green to yellow-green part of the visible spectrum, peaking at around 510-525 nm by various estimates and absorbing significantly in the range of 450 to 540 nm (Wikipedia). Like other carotenoids, it could be highly susceptible to heat, light, and oxygen. In acidic solutions, and under dim light and room temperature without heat, fucoxanthin is stable; under weak acid, weak alkali conditions, a reversible change of color takes place from deep orange to light orange; under strong alkali, strong light or heat conditions, the structure of fucoxanthin is destroyed. The thermostability of fucoxanthin was evaluated at 80°C and 100°C by Oryza Oil and Fat Chemical Co. (Japan). The result shows that fucoxanthin is

relatively stable at 80°C for one hour. However, to some extent, it degrades at 100°C for one hour. Therefore, it is recommended that fucoxanthin should be processed at temperatures under 100°C.

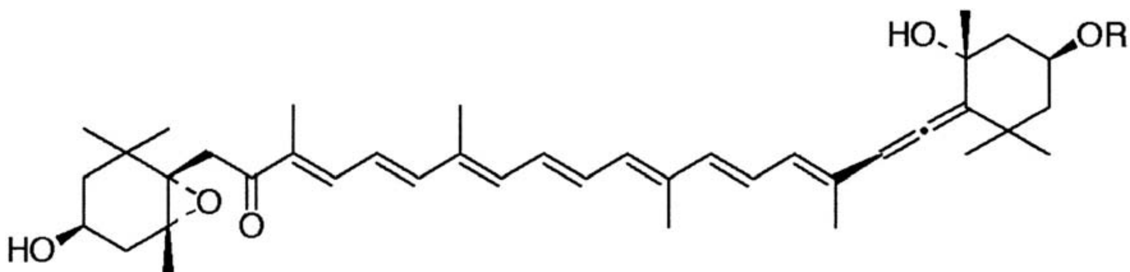

Fig. 1 Structure of fucoxanthin (R=COCH3) and fucoxanthinol (R=H)

Fucoxanthin is a type of non-provitamin A carotenoid and it belongs to xanthophyll, any of a group of carotenoid yellow pigments. Fucoxanthinol is a major metabolite from fucoxanthin. Several geometrical isomers (*cis* isomers) of fucoxanthin also exist in nature. It has also been conclusively shown that freshwater fish do not accumulate fucoxanthin, although their natural feed (e.g., diatoms and aquatic insects) contains fucoxanthin.

2.2 The Resource of Fucoxanthin

Algae are a large and diverse group of simple, typically autotrophic organisms. Their sizes range from single cells of picophytoplankton - the smallest of which are less than 1 μm - to seaweeds, the largest of which are more than 50 meters long (Shinichi et al., 2011). Algae are being used for producing commercial polysaccharides in many countries, such as carrageenan. These polysaccharides are mainly used as a food ingredient. There are 580 structurally different carotenoids described, and more than 100 of these are found in algae

(Synnnve Liaaen-Jensen, 1989). Algae pigments can be roughly divided into three classes: chlorophyll, carotenoid, and phycobiliprotein. The carotenoids are among the most widespread and important ones. Some unicellular green algae, under appropriate conditions, become red due to the accumulation of high concentrations of “secondary” carotenoids. Two examples of this are, *Dunaliella spp.* and *Haematococcus pluvialis*, which are cultured extensively as sources of β-carotene and astaxanthin, respectively. Because of their ubiquitous occurrence, different functions and interesting properties, carotenoids are the subject of interdisciplinary research in chemistry, biochemistry, biology, medicine, physics, and many other branches of science.

2.2.1 Large-size algae

Large-size algae (macroalgae), commonly known as seaweed, have many commercial and industrial uses, but due to their size and specific requirements, they are not easily cultivated on a large scale and are most often taken in the wild. The most common one is the Phaeophyta or brown algae, which are a large group of mostly marine multicellular algae. They play an important role in marine environments both as food, and for the habitats they form. Worldwide there are about 1500-2000 brown seaweed species. Some members of the division are used as food for humans; such as Laminaria japonica, which are commercially important species as a part of the staple diet in Japan, Korea, and China. The demand from only these three countries provide the basis of an industry that worldwide harvests 6 million metric tons of wet seaweed per annum with a value of around five billion dollars (FAO Fisheries and Aquaculture Department, 2002).

Most brown algae contain the pigment fucoxanthin, which is responsible for the distinctive greenish-brown color that gives them their name. The total global seaweed production in the year 2005 was around 1.3 million metric tons by capture and 14.8 million metric tons by aquaculture. Fucoxanthin is rich in the brown algae such as Undaria pinnatifida and Laminaria japonica Aresch, which are the common source for fucoxanthin extraction. As the traditional feedstock for fucoxanthin extraction, large-size algae, including brown algae, have some disadvantages in the industrial production. In the growing stage, they need deep seawater surroundings, and require an abundance of minerals, pathogen- and pollution-free water, and low water temperature to thrive.

Table 1. Total Carotenoid (mg/g) and Fucoxanthin/ Fucoxanthinol (mg/g) Content in Selected Brown Seaweeds (Barrow and Shahidi, 2007)

No.	Species	Total Carotenoid	Fucoxanthin	Fucoxanthinol
1	*Undaria pinnatifida* (young thallus)	–	0.32	–
2	*U. pinnatifida* (commercial-dried)	–	0.33	–
3	*U. pinnatifida* (female gametophyte)	–	1.64	–
4	*U. pinnatifida* (male gametophyte)	–	2.67	–
5	*Scytosiphon lamentaria* (young thallus)	–	0.24	–
6	*S. lamentaria* (germlings)	–	0.56	–
7	*Petalonia binghamiae* (young thallus)	–	0.43	–
8	*P. binghamiae* (germlings)	–	0.58	–
9	*Laminaria religiosa* (young thallus)	–	0.24	–
10	*Ecklonia radiata*	6.85	1.65	0.24
11	*Carphophyllum maschalocarpum*	6.21	1.17	–
12	*C. plumosum*	5.68	1.44	0.41
13	*Harmosira banksii* (coastal rocks)	6.48	–	–
14	*H. banksii* (mangroves)	3.75	–	–
15	*Cystophora retroflexa*	4.71	0.46	0.62
16	*Sargassum sinclairii*	9.79	0.54	–
17	*Fucus serratus*	0.80	0.56	–

Note: Content on wet weight basis for species from numbers 1 to 9 and on dry weight basis for species listed in numbers 10-16

2.2.2 Micro-size algae

Micro-size algae (microalgae), also referred to as phytoplankton, microphytes, or planktonic algae constitute the majority of cultivated algae. Recently, increased research attention is being paid to marine micro-algae as sources of bioactive compounds, including carotenoids. Fucoxanthin used to be an index for analyzing the composition of phytoplankton assemblages. Marine microalgae are at the base of the entire aquatic food chain. Therefore, it is not surprising that the microalgae play a vital role in the rearing of aquatic animals. One of the main reasons for the interest in microalgae is to obtain pure fucoxanthin and to make use of other components in cultivated microalgae, such as *Bacillariophyta* (diatoms). There are several reports on the fatty acid, lipid, amino acid, and sugar composition of almost all microalgae used in mariculture for determining the nutritional value of the microalgae as food for animals in mariculture (Brown et al., 1997; Volkman et al., 1989).

Compared with macroalgae, microalgae can achieve high growth rate, high reproduction rate, and high photosynthetic efficiency. They can be cultivated under highly controllable conditions and have a very short harvesting cycle. Microalgae have been reported to double their biomass within 24 h when commercially cultured. In the production stage, brown algae need more pretreatment, such as pulverization, and they produce a lot of waste themselves after extraction. On the contrary, high surface area-to-volume ratio of microalgae allows easy solvent penetration for high extraction efficiency.

The diatom *Thalassiosira weissflogii* is a commercially available microalga that has been commonly used to feed shrimp and shellfish larvae in hatcheries and in aquaculture. It was selected in this work as a model to investigate fucoxanthin extraction from microalga.

2.3 Extraction, purification and analysis of Fucoxanthin

The established extraction and purification procedures employed by various researchers are similar (Barrow and Shahidi, 2007). An organic solvent is first used to extract fucoxanthin from algae, and then silica gel column chromatography and/or thin layer chromatograph (TLC) is used to purify the sample. It is important to perform the whole process under low temperatures and dark environment conditions to minimize the degradation of fucoxanthin. To identify and quantify fucoxanthin, visible spectroscopy (VIS), mass spectrometry (MS), high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), circular dichroism spectroscopy (CD) or a combination of these is typically used.

2.3.1 Extraction and purification

Wang et al. (2005) reported an isolation method of fucoxanthin from rhizoid of *Laminaria japonica Aresch*. In this work, fucoxanthin was extracted with dimethylsulfoxide (DMSO), and recovered from the DMSO extract by ethyl acetate partition and subsequent evaporation. Isolation parameters, including solvent volume and extraction time, were optimized. The quantity and quality of the extracted fucoxanthin were determined by absorption spectra and fluorescence emission spectra. The extraction solvent, DMSO, is one of the least toxic organic chemicals known. The median lethal dose (LD-50s) of DMSO (oral, dermal, and inhalation) has a much lower acute toxicity than ethanol, acetone, and other common solvents. This method is simple and easy to use; however, the purity of fucoxanthin in the extract is relatively low (63%).

Shang et al. (2010) optimized the extraction conditions for fucoxanthin from brown algae *Eisenia Bicyclis* through a pressurized liquid extraction (PLE) method and statistical experimental design. The procedure was optimized by a Plackett–Burman design as a first step to screen the most important variables in the extraction of fucoxanthin. Subsequently, a central composite design was used to obtain the optimum conditions of the selected factors for fucoxanthin extraction. Two factors, temperature and ethanol concentration, significantly influenced the extraction efficiency of fucoxanthin at a 95% level. The maximum predicted value of fucoxanthin extraction was 0.42 mg/g at 110°C and 90% ethanol.

Hosokawa et al. (2009) soaked fresh *Undaria pinnatifida* in two volumes (v/w) of methanol solution for two days. This extraction was repeated twice. The methanol solution was then filtered and evaporated to obtain the methanol extracts. Water and ethyl acetate were added to these extracts, and then the ethyl acetate layer was obtained with a separatory funnel. The orange-colored fucoxanthin fraction was separated from the ethyl acetate-soluble fraction by means of preparative silica gel thin layer chromatography (TLC) developed with chloroform: methanol:water (65:25:4, v/v/v). Further purification was carried out with preparative silica gel TLC developed with n-hexane: acetone (6:4, v/v).

Maeda et al. (2007) extracted fucoxanthin-containing lipids from commercial *Undaria pinnatifida* dried seaweed using acetone extraction. Fucoxanthin was purified from the lipid extracts by silica gel column chromatography with n-hexane/acetone (7:3, v/v).

When using micro-size algae as the source of fucoxanthin, as compared with the large-size algae, the methods by which the algae cells are fragmented and the pigments releasing are also considered, in addition to the choice of extraction agent, extraction time,

temperature, and so on. The main methods of alga cells fragmentation are ultrasonication and freezing and thawing. Wang et al. (2007) investigated the crush of seventeen species of alga by ultrasonication and freezing and thawing, and their fragmenting effects was determined by the fragmentation efficiency and antimicrobial activity, to chose the optimal method for different alga. The results showed that the fragmentation efficiency of all of tested algae cells was more than 90％ after 12 minutes of treatment using the ultrasonic method. Moreover, the fragmentation efficiency of some of these samples was more than 99％ after only being treated for 3 minutes. The results of using the freezing and thawing method indicated that it was not as efficient as the ultrasonication method for some species of algae.

Wright and Jefferey (1987) successfully used reverse-phase HPTLC plate to separate the fucoxanthin. The system comprised a Merck RP-8 bonded silica plate, developed for 30 min with methanol: water = 9:1 (v/v). Reverse-phase RP-8 HPTLC plate separated three fucoxanthin pigments, fucoxanthin (R_f value = 0.43), 19'-hexanoyloxyfucoxanthin (R_f value = 0.40) and 19'-butanoyloxyfucoxanthin-like pigment (R_f value = 0.48), from extracts of cultures of *Pavlova lutheri, Emiliania huxleyi* and *Pelagococcus subviridis* and mixtures of these (Fig. 2A, B, C, D). All 3 pigments were found in the East Australian Current strain and 2 Antarctic strains of *Phaecystis pouchetii*, although the proportion of the fucoxanthin pigments varied (Fig. 2E, F, G). The Antarctic concentrate also contained the three fucoxanthin pigments (Fig. 2H).

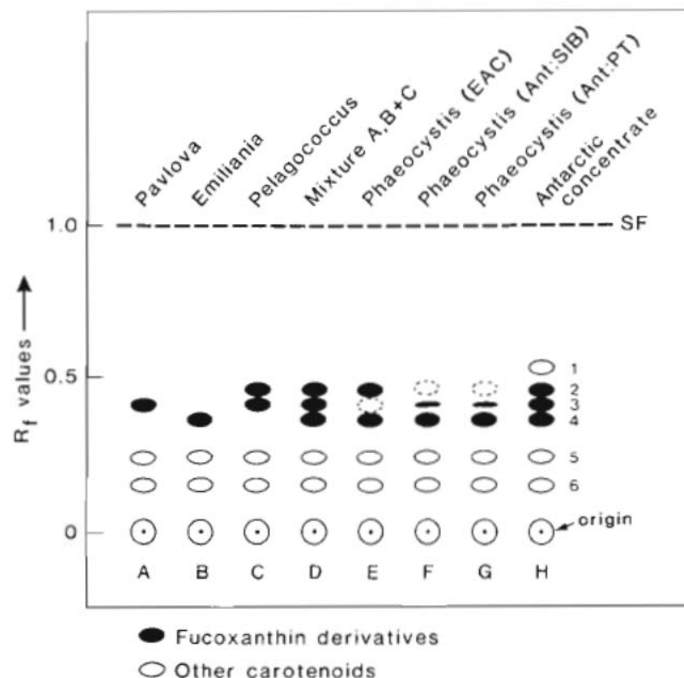

Fig. 2 Separation of major carotenoids from various algal species and the Antarctic concentrate on the reverse-phase bonded-silica HPTLC plate (Merck. RP-8).

Note - (A) Pavlova lutheri; (B) Emiliania hwdeyi; (C) Pelagococcus subviridis; (D) mixture of A, B & C; (E, F, G) Phaeocystis pouchetii: East Australian Current strain (EAC), and Antarctic strains (S. I. Blackburn [Ant:SIB] and P. Thomas [Ant:PT] respectively); (H) Antarctic concentrate. 1: peridinin (red); 2: 19'-butanoyloxyfucoxanthin-like (yellow-orange); 3: fucoxanthin (red-orange); 4: 19'-hexanoyloxyfucoxanthin (yellow-orange); 5: diadinoxanthn (yellow); 6: diatoxanthin (pale orange)

2.3.2 Analysis of fucoxanthin

Since fucoxanthin content is very low even in fucoxanthin-rich sources (at mg/g levels), the fucoxanthin extract is a complicated mixture, containing lipids and other carotenoids. Notable amount of impurities may still exist even after multi-step purifications. Therefore,

high sensitive and selective analysis techniques are highly preferred in fucoxanthin identification and quantitation analysis. A variety of modern analytical instruments, including visible spectroscopy (VIS), mass spectrometry (MS), high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), circular dichroism spectroscopy (CD) and their combinations, are typically employed to identify and quantify fucoxanthin in extracted samples.

Since it is a photosynthetic pigment, VIS spectrum is an essential characteristic of fucoxanthin. Haugan et al. (1992) reported VIS absorption peaks of fucoxanthin at 330, 445 and 471 nm with a %III/II of 6 in an HPLC analysis with a mobile phase of hexane: isopropyl acetate: 1-propanol: N-ethyldiisopropylamine (83.9:14:2:0.1). The term % III/II describes spectral fine-structure. Conjugated ketocarotenoids have in general reduced spectral fine-structure. In hexane, all-trans-fucoxanthin (1e) had a %III/II value = 40. For the other geometrical isomers, values from 0-35% were recorded. Fucoxanthin VIS absorptions in different organic solvents were also been measured. Absorption peaks at 420, 444 and 467 nm with a %III/II of 6 were observed in acetone, while peaks at 329, 423, 448, 476 nm with a %III/II of 40 were detected in hexane. Similar results were obtained by Hosokawa et al. (1999). Strand et al. (1998) recorded absorption peaks at 447 and 470 nm with a %III/II of 3 using a HPLC online detector. The mobile phase they used was composed of hexane, isopropyl acetate, acetone and methanol (76:17:7:0.1).

Wright and Jeffrey (1987) purified fucoxanthin and its derivatives from four kinds of plankton (*Pavlova lutheri* , *Emiliania huxleyi, Emiliania huxleyi* and *Phaeocystis pouchetii*). The pigments were isolated with TLC systems and examined with a recording

spectrophotometer to obtain absorption spectra (Fig. 3). These absorption data are in accordance with the yellow-orange color of fucoxanthin.

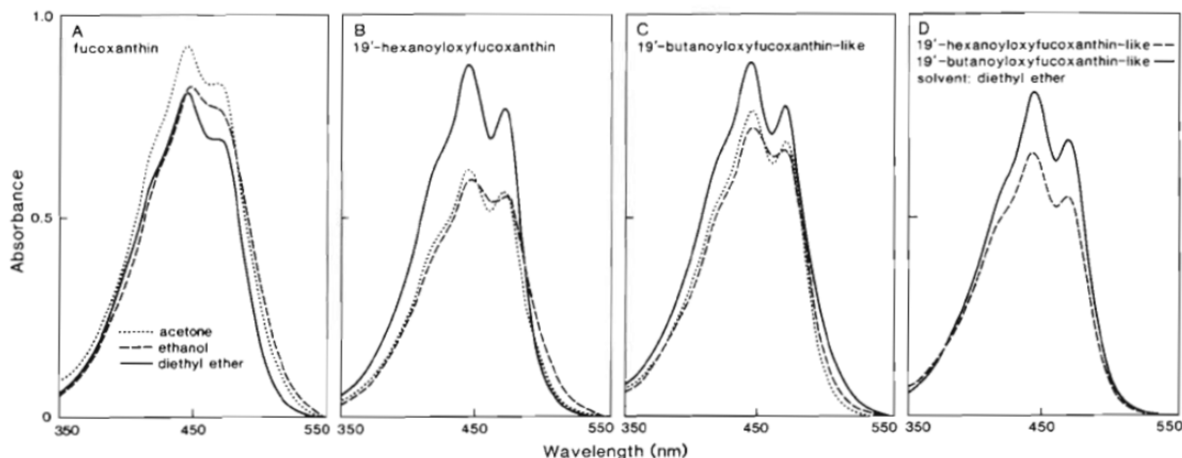

Fig. 3 Visible absorption spectra of fucoxanthin pigments in acetone, ethanol and diethyl ether. Note: (A) Fucoxanthin from *Pavlova lutheri*; (B) 19'-hexanoyloxyfucoxanthin from *Emiliania huxleyi*; (C) 19'- butanoyloxyfucoxanthin-like pigment from *Pelagococcus subviridis*; (D) the 19'-hexanoyloxyfucoxanthin-like and 19'- butanoyloxyfucoxanthin-like pigment from *Phaeocystis pouchetii* in diethyl ether.

HPLC is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of a mixture. In fucoxanthin studies, HPLC is typically coupled with a VIS detector to achieve high sensitivity and selectivity. Van Leeuwe et al. (2006) optimized the analysis method of algae pigments by applying water-packing of the sample during injection, resulting in improved peak shape and peak separation without dilution. The gradient solvent system in this HPLC method is adapted to the micro-sized algae because

the composition of pigments in the microalgae is much more complex than in the macroalgae. A comparison of three representative previous studies is listed in Table 2.

Mass Spectrometry (MS) is an analytical technique used to identify, as well as quantify chemicals in a sample. A high-resolution mass-to-charge number ratio (M/Z value) of the molecular ion can be used to calculate the molecular formula, and the ions of the fractions are helpful to deduce the molecular structure. The intensity of selected ion(s) can be used for quantitative analysis. The calculated M/Z value of the fucoxanthin molecule ($C_{42}H_{58}O_2$) is 658.4234. Hosokawa et al. (1999) reported an experimental value of 658.4249 (m/z). Molecular ions and fraction ions detected in previous research and their M/Z values are listed in Table 3. The fragmentation pattern is consistent with the presence of two water molecules and an acetoxy-group in the parent compound (Bonnett et al. 1969). Figure 4 shows the mass spectrum of fucoxanthin (MassBank Record: CA000057).

Nuclear magnetic resonance (NMR) and circular dichroism spectroscopy (CD) are important tools for fucoxanthin identification. They are especially helpful to distinguish the geometrical isomers. Fine molecular structure change will cause changes of the chemical environment of the atoms in the molecule, resulting in notable movements of the NMR chemical shifts. CD can be used to detect optical property changes caused by geometrical changes of optically active chiral molecules. Haugan et al. (1992) measured ^{1}H NMR chemical shifts of *all-trans* fucoxanthin (the natural fucoxanthin, shown in Figure 5) and its six geometrical isomers, as well as CD properties of five isomers in them (Figure 6). Based on the results, the structure of the six isomers was successfully determined. The ^{13}C NMR chemical shifts of fucoxanthin and their assignments are listed in Table 4 (Hosokawa et al., 1999).

Table 2. HPLC analysis parameters

	Sugawara et al. (2002)	***Van Leeuwe et al. (2006)***	***Carreto et al. (2008)***
Column	C18	C18	C8
Detector	UV-VIS	DAD	DAD
Detection wavelength	400 to 750 nm	450 nm	440 nm
The mobile phase contents	Acetonitrile: methanol: water (75:15:10, v/v/v), containing 1 g/L ammonium acetate	A: methanol: water (0.5M ammonium acetate) (v/v) = 85:15;B: acetonitrile/water (v/v) = 90:10;C: ethyl acetate. (Gradient elution)	A: methanol: acetonitrile: aqueous pyridine solution (0.25 M, pH adjusted to 5.0 with acetic acid) (50:25:25, v/v/v); B: methanol: acetonitrile: acetone (20:60:20, v/v/v). A linear gradient from 0% to 40% B was pumped for 22 min, followed by an increase to 95% at minute 28 and isocratic hold at 95% B for further 10 min.
Flow rate	1.0 ml min^{-1}	0.8 ml min^{-1}	—
Retention time	12.5min	6.75min	19.5min

Table 3. MS (m/z) of Fucoxanthin

Ion	Bonnett et al. (1969)		Haugan et al. (1992)	
	m/z	Intensity%	m/z	Intensity%
M	658.4201	—	658	23
M-18	640.4113	—	640	37
M-18-18	622.4018	—	622	26
M-18-60	580.3908	—	580	21
M-80	—	—	578	7
M-18-18-60	—	—	562	13
M-18-80	—	—	560	6

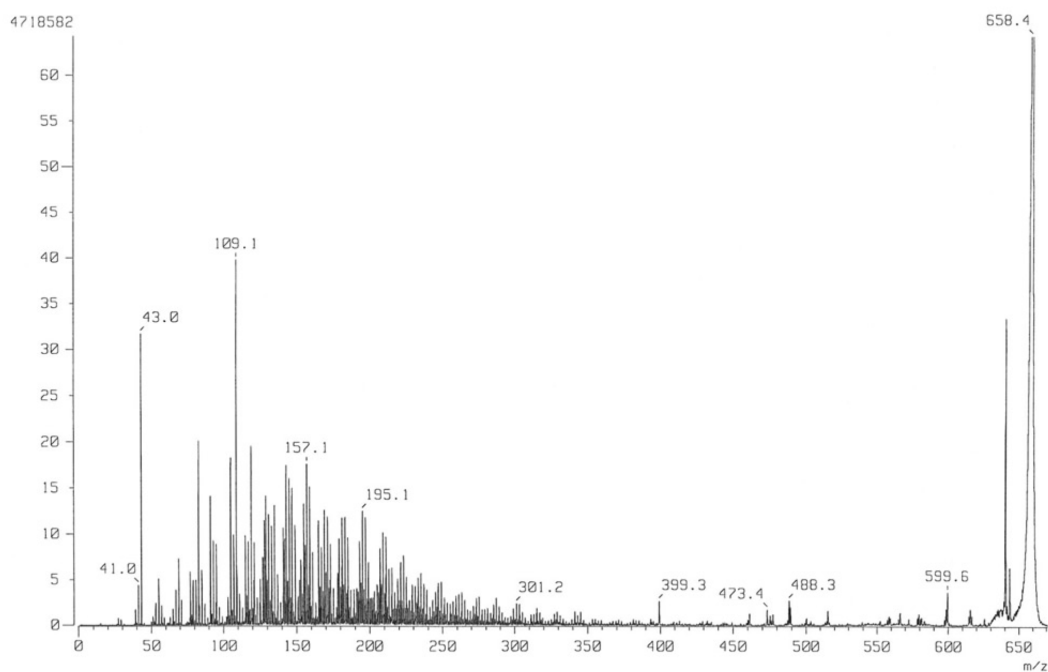

Fig.4 Mass Spectrum of fucoxanthin

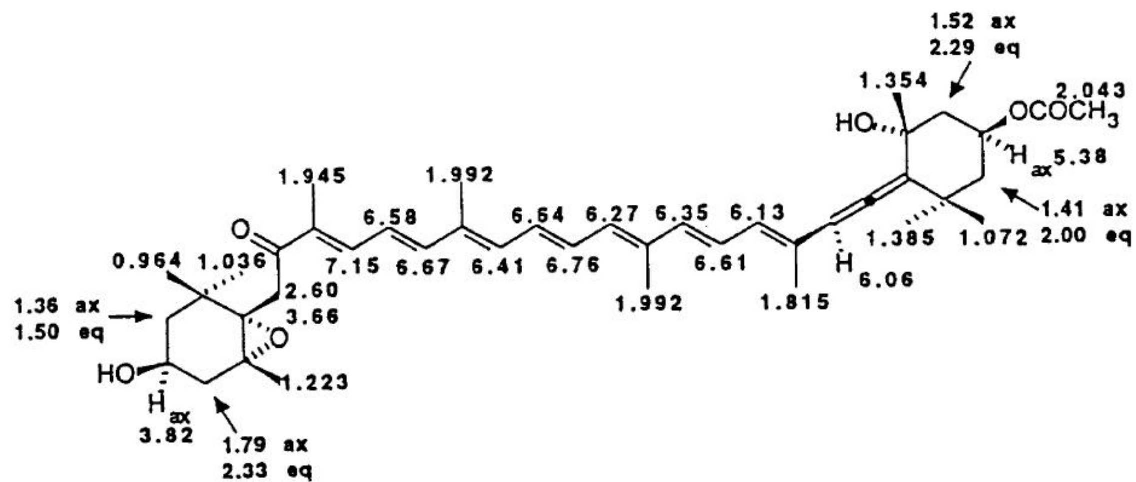

Fig. 5 ^{1}H NMR chemical shifts of *all-trans* fucoxanthin

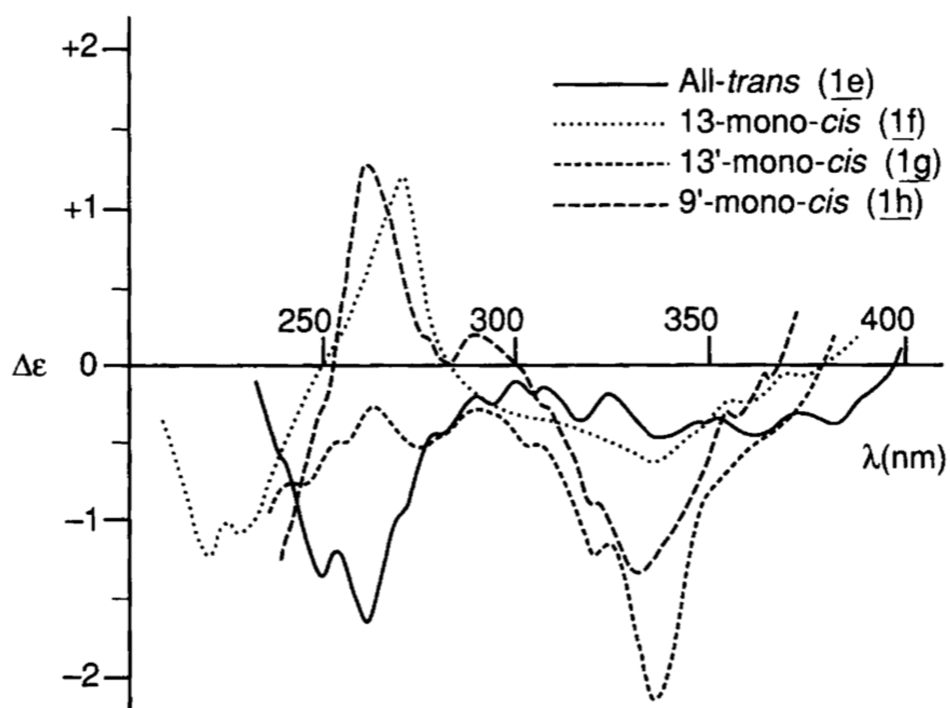

Fig. 6 CD spectra in EPA (diethyl ether-isopentane-ethanol 5:5:2) of fucoxanthin

Table 4. ^{13}C NMR Spectral data of fucoxanthin ($CDCl_3$) (Hosokawa et al. 1999)

No.C	1	2	3	4	5	6	7	8	9	10
δ value	35.8	47.1	64.3	41.7	66.1	67.1	40.8	170.6	134.6	139.0
No.C	11	12	13	14	15	16	17	18	19	20
δ value	123.4	145.0	135.4	136.6	129.4	25.1	28.2	21.2	11.8	12.7
No.C	1’	2’	3’	4’	5’	6’	7’	8’	9’	10’
δ value	35.2	45.5	68.0	45.5	72.7	117.6	202.3	103.4	132.5	128.6
No.C	11’	12’	13’	14’	15’	16’	17’	18’	19’	20’
δ value	125.7	137.1	138.0	132.1	133.	29.2	32.1	31.3	14.0	12.9
No.C			21’					22’		
Δ value			197.8					21.4		

2.4 Pharmacological Functions of Fucoxanthin

Fucoxanthin has been suggested to have several pharmacological effects on the body, including anti-obesity, anti-diabetic, anti-tumor, anti-angiogenic, and antioxidant properties. Also, as a natural compound extracted from algae it may have fewer negative side effects than other more traditional drugs used to treat these conditions. In this regard, fucoxanthin may prove to be a therapeutic option, serving as a functional food supplement, in conjunction with other types of treatments. Should fucoxanthin prove to the have therapeutic benefits, it would serve as the impetus for further industrial production of fucoxanthin.

2.4.1 Anti-obesity activity

Obesity is a chronic metabolic disorder that results from an imbalance between energy intake and energy expenditure. Consuming more calories than are being expended will, over time, increase the amount of fat that is in body. Thus, obesity is characterized by having more body fat than is considered normal. There are several methods for measuring the amount of body fat, including underwater weighing, dual emission X-ray absorptiometry (DEXA), bioelectrical impedance, and whole body air-displacement plethysmography . A cheap and simple estimate of body fat is the body mass index (BMI). Body mass index (BMI), defined as the weight in kilograms divided by the height in meters squared (kg/m2), is the most widely used estimate of obesity due to its low cost and simplicity. The World Health Organization (WHO) and the National Institutes of Health (NIH) have defined obesity as having a BMI greater than 30.0, while being overweight is defined as having a BMI between 25.0 and 29.9.

GHO reported that the worldwide prevalence of obesity has nearly doubled between 1980 and 2008. In 2008, 35% of adults aged 20+ were overweight (BMI $\geqslant$ 25) (34% men and 35% of women); 10% of men and 14% of women in the world were obese (BMI $\geqslant$30), compared with 5% for men and 8% for women in 1980. An estimated 205 million men and 297 million women over the age of 20 were obese – a total of more than half a billion adults worldwide. The highest rate of obesity has been reported in the Pacific Islands and the lowest rates have been seen in Asia (Nguyen and El-Serag, 2010).

Obesity has become a serious public health concern due to its high prevalence and because it leads to an increased risk for various physical, mental, and emotional health

problems, including adverse metabolic effects on blood pressure, cholesterol, triglycerides, and insulin resistance. Risks of coronary heart disease, ischemic stroke, and type 2 diabetes mellitus increase steadily with increasing BMI. Elevated BMI also increases the risk of cancer of the breast, colon, prostate, endometrium, kidney, and gall bladder. Mortality rates increase with increasing degrees of overweight.

Few drugs are available for the treatment of obesity. Currently, the Food and Drug Administration (FDA) has only approved 3 drugs for long-term treatment of obesity. Orlistat (Xenical and Alli) is an inhibitor of pancreatic lipase, which lowers that amount of fat that is absorbed, Lorcoserin (Belviq), an appetite suppressant, and a combination of phentermine-topiramate that goes by the name Qsymi, also an appetite suppressant. Some, previously approved agents for the treatment of obesity have been taking off the market due to life-threatening side-effects like heart attack and stroke (sibutramine) or heart value problem and pulmonary hypertension (fen/phen). Therefore, there has been high demand to find new therapeutic agents that are safe and effective in preventing or treating obesity. A potential source for these therapeutic agents could come from functional foods, like fucoxanthin.

Energy balance is dependent of two factors: energy intake (metabolizable calories consumed and absorbed), and energy expenditure. Basal metabolic rate, physical activity, and diet-induced thermogenesis are the contributors to energy expenditure. In rodents, one of the regulators of energy expenditure is brown adipose tissue (BAT), which establishes non-shivering thermogenesis through dissipation of excess energy as heat (Cannon and Nedergaard, 2004). BAT plays an important role in obesity control by controlling energy balance. The key player in this process is uncoupling protein-1 (UCP1),

which discharges the proton gradient generated by the electron transport system (ETS), thus uncoupling the ETS from oxidative phosphorylation. Rather than phosphorylating ADP to form ATP, the energy is dissipated as heat. Thus, searching for substances that upregulate UCP1 gene expression may be a worthy strategy for achieving obesity control through increased energy expenditure (Kumar et al., 1999). BAT can be recruited under certain conditions. For example, some studies have described remodeling mature white adipose tissue (WAT) into mitochondria-rich BAT cells with a high capacity for fatty acid oxidation (Cinti 2002; Mercader et al., 2006). Fucoxanthin, which is of marine origin, stimulates thermogenesis in BAT and promotes WAT to acquire the features of BAT in rodents (Figure 7) (Orci et al., 2004; Flachs et al., 2005; Maeda et al., 2007).

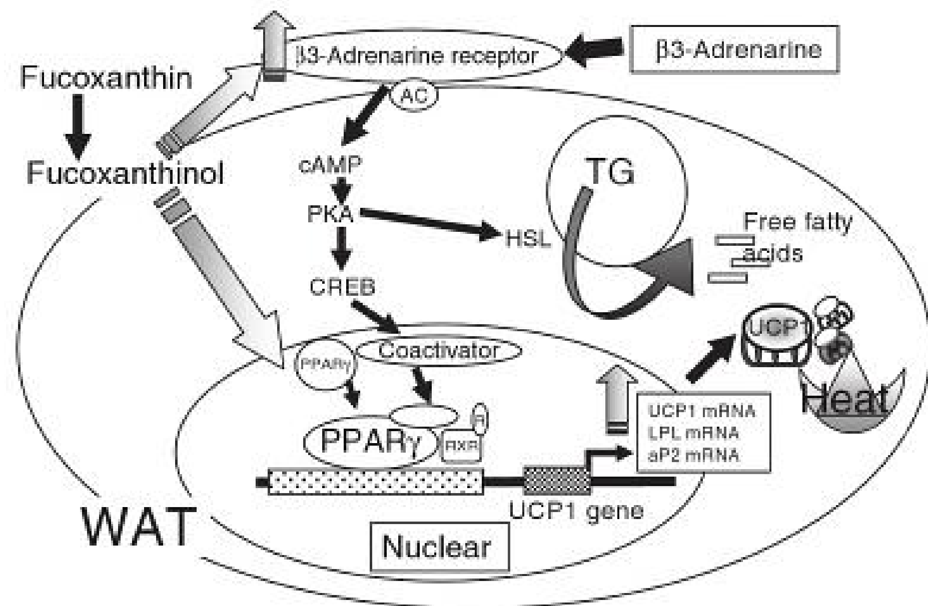

Fig. 7 Possible molecular mechanism for UCP1 expression in WAT of animals fed fucoxanthin

Maeda et al. (2005) found that fucoxanthin can upregulate the expression of UCP1 in WAT, which may increase energy expenditure and contribute to reducing body fat. As compared with the control group, fucoxanthin-fed mice had significantly decreased WAT weights. The WAT of the fucoxanthin-fed rats clearly expressed UCP1, while it did not in the control group. By adding 0.2% fucoxanthin to food, body weight gain was significantly reduced compared with that of the control mice ($P<0.05$), although there was no difference in the amount of food intake (Maeda et al., 2007). The reduction in body weight gain was consistent with the decrease in the weight of uterine, mesemteric, perirenal, and retroperitoneal fat pads. The weight of WAT normalized by body weight in the mice fed 0.2% fucoxanthin was significantly lower than in the control group. Further, the brown adipose tissue (BAT) weight normalized by body weight, which is related to energy expenditure, was increased in the mice fed 0.1 and 0.2% fucoxanthin compared with the control group. Other tissue weights were not affected by fucoxanthin.

When 3T3-L1 adipose cells were treated with fucoxanthin, fucoxanthinol, or neoxanthin, PPAR γ, a regulater of adipogenic gene expression, was down-regulated by these carotenoids in a dose-dependent manner (Maeda et al. 2006; Okada et al. 2008). Fucoxanthin and fucoxanthinol also decreased glycerol-3-phosphate dehydrogenase activity, an indicator of adipocyte differentiation. The effects of fucoxanthinol were stronger than fucoxanthin. These results suggest that fucoxanthin and fucoxanthinol inhibit the adipocyte differentiation of 3T3-L1 cells through down-regulation of PPAR γ. PPAR γ is a nuclear transcription factor that regulates adipogenic gene expression (Grimaldi, 2001), so it has an important role in the early stages of 3T3-L1 cell differentiation (Gregoire et al., 1998; Tontonoz et al., 1994), Affecting the regulation of PPAR γ would be one of the expected potential mechanisms underlying the anti-obesity effect of these dietary

carotenoids. This study suggests that fucoxanthinol may be an active compound for the antiobesity effect of fucoxanthin. Metabolites of fucoxanthin, like fucoxanthinol, may accumulate in the WAT of mice, and could be an effective natural compound used for prevention of obesity.

Fucoxanthin has been also suggested to affect fatty acid composition of tissues. Tsukui et al. (2009) examined the anti-obesity effect and enhancement of hepatic docosahexaenoic (DHA) and arachidonic acid (AA) contents in C57BL/6J mice fed fucoxanthin. Fucoxanthin was added to the basal diet AIN-93G at the rate of 0.025% and 0.05%. After 5 weeks on the experimental or control diet, liver weight and liver lipids content were not affected by fucoxanthin, but the body weight of mice fed 0.05% fucoxanthin was slightly lower (10.8%) than that of control mice. The fatty acid composition of total liver lipids was analyzed by gas-liquid chromatography. The fucoxanthin concentration of 0.05% in the diet increased DHA and AA content in the liver of C57BL/6J mice. Fucoxanthin is converted to fucoxanthinol in the gastrointestinal tract and is then metabolized to amarouciaxanthin A in the liver. The authors suggested these two metabolites of fucoxanthin might be the active compounds that mediate the increases in hepatic DHA and AA levels.

2.4.2 Anti-cancer activity

Besides having anti-obesity properties, some studies have suggested that fucoxanthin also has anti-cancer properties (Barrow and Shahidi, 2007; Kumar et al., 2013). Cancer is a class of diseases in which a group of cells display uncontrolled growth. These cells can invade and destroy adjacent tissues, and sometimes metastasize or spread to other

locations in the body via lymph or blood. These malignant properties of cancers differentiate them from benign tumors, which do not invade or metastasize.

The presence of cancer can be suspected on the basis of symptoms, or findings on radiology. Definitive diagnosis of cancer, however, requires the microscopic examination of a biopsy specimen. Most cancers can be treated. Possible treatments include chemotherapy, radiotherapy, and surgery. While cancer can affect people of all ages, and a few types of cancer are more common in children, the overall risk of developing cancer increases with age. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths (about 13% of all human deaths worldwide) are estimated to have occurred in 2008; rates are rising as more people live to an old age and lifestyles change in the developing world (Jemal Ahmedin et al., 2010).

Constipation, delirium, fatigue, nausea and vomiting, insomnia, are common problems for cancer patients. The growth and spread of cancer, and cancer treatment itself, contributes to these conditions. Dietary recommendations, which has garnered particular attention, to reduce the risk of developing cancer, including: (1) reducing intake of foods and drinks that promote weight gain, namely energy-dense foods and sugary drinks, (2) eating mostly foods of plant origin, (3) limiting intake of red meat and avoiding processed meat, (4) limiting consumption of alcoholic beverages, and (5) reducing intake of salt and avoiding moldy cereals (grains) or pulses (legumes). The vast majority of cancer risk factors are environmental or lifestyle-related, thus cancer is largely a preventable disease. Greater than 30% of cancer is preventable via avoiding risk factors including: tobacco, overweight or obesity, low fruit and vegetable intake, physical inactivity, alcohol, sexually

transmitted infections, and air pollution (Danaei et al., 2005; Wicki and Hagmann, 2011; Key 2011; Wang et al., 2014).

The effect of fucoxanthin on the viability of cancer cells and induction of apoptosis (cell death) has been examined. The anti-tumor activity of fucoxanthin is related to its unique molecular structure including an unusual allenic bond and a 5,6-monoepoxide (Meada et al., 2008). In anti-tumor activity, fucoxanthin plays a special role. Fucoxanthin is reported to be very effective in inducing apoptosis in human leukemia (Hosokawa et al., 1999; Kotake-Nara et al., 2005) and colon cancer (Hosokawa et al., 2004; Das et al., 2005) cells. Furthermore, neoxanthin and fucoxanthin exhibited the most potent growth retarding activity (Kotake-Nara et al., 2001) and reduced cell viability through apoptosis induction in different human prostate cancer cells (PC-3, DU 145 and LNCap) (Kotake-Nara et al., 2001). These results suggest that ingestion edible brown algae rich in fucoxanthin might have the potential to reduce the risk of prostate cancer.

Regarding the possible mechanism of fucoxanthin's anti-cancer effects, fucoxathin has been reported to induce apoptosis in human prostate cancer PC-3 (Kotake-Nara et al., 2005), colon cancer Caco-2 (Hosokawa et al., 2004), and leukemia HL-60 (Kim et al., 2009) cells through the down-regulation of Bcl-2 expression. Bcl-2 is an anti-apoptosis protein involved in the regulation of apoptosis. Additionally, many researchers have suggested that fucoxanthin inhibits the growth of tumor cells (hepatic carcinoma HepG2, colon adenocarcinoma WiDr, and prostate cancer DU145 cells) by inducing cell cycle arrest at the G1 phase by cyclin D, p21WAF1/Cip1, and MAPKs regulations, respectively (Das et al., 2008, 2005; Satomi and Nishino, 2009).

It has been noted that different isomers of fucoxanthin have different activity strengths regarding it effect of apoptosis in tumor cells. Nakazawa et al. (2009) found that the potent inhibitory effect of 13'-cis and 13'-cis isomers of fucoxanthin on HL-60 cells and Caco-2 cells could possibly be due to their higher apoptosis-inducing activity. The antiproliferative effect of the mixture of 13'-cis and 13'-cis isomers were stronger than all other geometrical isomers evaluated in the study.

2.4.3 Activity to decrease blood glucose and lipid concentrations

Obesity leads to adverse metabolic effects on blood pressure, lipid levels, and insulin resistance, and thus, poses a major risk for serious chronic diseases, including hypertension, type 2 diabetes, and cardiovascular disease. Some studies showed that while fucoxanthin reduces weight gain, it also affects the concentration of blood glucose in the body.

Maeda et al. (2007) suggested that dietary fucoxanthin decreases the blood glucose and plasma insulin concentrations of KK-Ay mice, along with down-regulating tumor-necrosis factor receptor (TNFR) mRNA. In addition, the combination of fucoxanthin and fish oil is more effective at attenuating the weight gain of WAT than feeding fucoxanthin alone. Woo et al. (2010) investigated the effects of fucoxanthin on lipid metabolism and blood glucose concentrations in C57BL/6N mice fed a high fat diet. It was found that fucoxanthin supplementation plays a beneficial role in not only regulating the plasma and hepatic lipids metabolism, but also in lowering blood glucose concentrations in high-fat-fed mice. Fucoxanthin supplementation significantly lowered the concentration of plasma triglyceride with a concomitant increase of fecal lipids in comparison to the high fat-fed

control group. Also, the hepatic lipid contents were significantly lowered in the fucoxanthin supplemented groups, which may have been due to the reduced activity of the hepatic lipogenic enzymes, glucose-6-phosphate dehydrogenase, malic enzyme, fatty acid synthase, and phosphatidate phosphohydrolase, and the enhanced activity of β-oxidation.

Fucoxanthin also lowered hemoglobin A_{1c} levels along with plasma resistin and insulin concentrations (Woo et al., 2010). This study demonstrated that fucoxanthin supplementation (0.05% and 0.2% in diet) markedly improved the plasma and hepatic lipid profiles and blood glucose concentration compared with the high-fat control (HFC) group in mice fed a high- fat diet, containing 39% of calories as fat. The blood glucose concentrations of the two fucoxanthin groups were significantly lower during the entire experimental period, which is consistent with the previous findings of Maeda et al.(2007) regarding the administration of the dietary combination of fucoxanthin and fish oil in obese/diabetic KK-Ay mice. Supplementation of 0.05% and 0.2% fucoxanthin significantly reduced the blood HbA_{1c} (by 30% and 32%), plasma insulin (by 36% and 50%) and resistin (by 24% and 26%) levels compared with the HFC group, although no change was observed in the plasma glucagon concentration. Further studies are required to clarify the mechanisms by which fucoxanthin elicits improvements in blood glucose and insulin concentrations.

2.4.4 Others

There have been several other reports of fucoxanthin showing different biological activities, such as anti-angiogenic activity (Sugawara et al., 2006), free radical scavenging and singlet oxygen quenching activity (Sachindra et al., 2007), inhibition activity of

replicative DNA polymerases (Murakami et al., 2002), suppression the inflammation of EIU by blocking the iNOS and COX-2 protein expression, and its anti-inflammatory effect on the eye (Shiratori et al., 2005) . Fucoxanthin also can be used in aquaculture and feeding poultry, which strengthens animal disease resistance and enhances the egg yolk color (Strand et al., 1998).

2.5 Conclusions and outlooks

Though fucoxanthin has shown many biological activities, very few functional foods of fucoxanthin currently existed in the market. The future studies of fucoxanthin will be in the following aspects. First is to optimize and establish the extraction technology to maximize the yield of fucoxanthin with high purity. Second, the multiple mechanisms of fucoxanthin exerting its many potential health benefits need to be elucidated. For botanist, there is a need to increase the production of fucoxanthin in the algae cultures by gene modification. Lastly, there is a need to know how to store fucoxanthin, while preventing degradation from oxidation and photolysis, and how to control the delivery of fucoxanthin in the body by microencapsulation.

2.6 Summary

The studies of this dissertation examined two aspects of fucoxanthin. In the first study, the extraction procedure of fucoxanthin from diatoms (*Thalassiosira weissflogii*) was optimized. This optimization will be useful to companies or other parties who wish to mass-produce fucoxanthin from algae.

In a second study, the anti-obesity and anti-diabetic effects of fucoxanthin were examined in Wistar rats fed a high-fat/high sucrose diet for 12 weeks. Changes in body fat were monitored by an energy balance study, which used a body composition analysis to determine the changes in body compartments over the time course of the study. Insulin resistance was determined at the end of the study by a glucose tolerance test. Rats were also monitored in real time using metabolic cages to determine energy expenditure, RQ, and physical activity. These results will contribute to the scientific framework exploring the utilization of fucoxanthin as an anti-obesity/anti-diabetic agent.

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3. Efficient Extraction of Fucoxanthin from diatoms

3.1 Abstract

Fucoxanthin is an epoxy carotenoid with important beneficial bioactivities. In this study, a type of microalgae (diatom) *Thalassiosira weissflogii* was used as the feedstock for fucoxanthin extraction. Effects on fucoxanthin yield of solvent types, feedstock conditions, presence of antioxidants, and extraction time were investigated. Results suggested diatoms might be a more cost-effective source for fucoxanthin extraction than brown algae. Wet diatoms can achieve high extraction yield over a much shorter period. Yield was 100.7 ± 5.8% of the average total available fucoxanthin in the diatoms during 10 min of extraction with acetone. Adding 0.3% of antioxidant, butylated hydroxyanisole (BHA) could not increase the yields significantly in the short time, but may prevent the further potential decomposition of fucoxanthin.

3.2 Introduction

Fucoxanthin is a non-provitamin A, epoxy carotenoid (Figure 1), which has visible spectrum peaks at around 450 nm and significant absorption in the range of 400-500 nm (Haugan et al., 1992; Hosokawa et al., 1999). Although fucoxanthin is rich in several edible seaweeds, such as brown algae, diatoms, and several other algal classes, its content is too low to directly use them as a functional food or medicine. Therefore, enrichment is an indispensable step for fucoxanthin application as a functional food or a dietary supplement. Solvent extraction is the most commonly used method to obtain certain compounds from different materials (Gil-Chávez et al., 2013). Mori et al. (2004) used methanol as the solvent to study the content of fucoxanthin in four edible brown algae. Maeda et al. (2007, 2009)

used acetone to extract fucoxanthin from dried powder of brown seaweed *Undaria pinnatifida* for their physiological research. Wang et al. (2005) reported that dimethyl sulfoxide (DMSO) is much more efficient than acetone to extract fucoxanthin from fresh *Laminaria japonica rhizoid*. They achieved a fucoxanthin recovery rate of 55% in 10 min, which increased to 88% when prolonging the extraction to 60 min. Roh and others (2008) used supercritical carbon dioxide with ethanol as a co-solvent to extract fucoxanthin and polyphenol from the brown seaweed *Undaria pinnatifida* and optimized the extraction conditions. Recently, Shang et al. (2011) applied a pressurized liquid method to fucoxanthin extraction from the brown alga *Eisenia bicyclis*. Important extraction parameters were screened with Plackett- Burman design and optimized with response surface methodology. The highest yield was achieved using a mixed solvent composed of 90% ethanol and 10% water at 110° C.

Nearly all published reports of fucoxanthin extraction were carried out using large-size algae as feedstock. Information on extraction of fucoxanthin from microalgae is sparse, although it is becoming a very promising natural source of bioactive compounds and its importance as a source of novel material is increasing (Gil-Ch´avez et al., 2013). As the traditional feedstock for fucoxanthin extraction, large-size algae, including brown algae, have some disadvantages when it comes to industrial production. In the growing stage, they need deep seawater surroundings, and require an abundance of minerals, pathogen- and pollution-free water, and low water temperature to thrive. Microalgae, on the other hand, achieve high growth rate, high reproduction rate, and high photosynthetic efficiency. They can be cultivated under highly controllable conditions and have a very short harvesting cycle. Microalgae have been reported to double their biomass within 24 h when

commercially cultured. In the production stage, brown algae need more pretreatment, such as pulverization, and they produce a lot of waste themselves after extraction. On the contrary, the high surface area-to-volume ratio of microalgae allows easy solvent penetration for high extraction efficiency.

The diatom, *Thalassiosira weissflogii* is a commercially available microalga and has been commonly used to feed shrimp and shellfish larvae in hatcheries and in aquaculture. It was selected in this work as a model to investigate fucoxanthin extraction from microalgae. The relationships between fucoxanthin yield and solvent types, feedstock conditions, effects of antioxidant, and basic extraction kinetics were examined.

3.3 Materials and Methods

3.3.1 Raw materials and chemicals

Diatom *Thalassiosira weissflogii* concentrate was purchased from Reed Mariculture Inc. (Campbell, CA, USA). The diatoms were refrigerated and shipped immediately after harvest. After received, they were stored at -20°C until use. The water content of the diatom concentrate was measured in triplicates and determined to be 89.8 ± 0.8%. Acetone, ethanol, ethyl acetate, 3-tert-butyl-4-hydroxyanisole (BHA) and Sudan I of analytical or ACS grade were purchased from VWR (Radnor, PA, USA). HPLC grade acetonitrile and ethyl acetate were also obtained from VWR. High-purity water produced with an Alpha-Q system (Millipore, Marlborough, MA, USA) was used for HPLC analysis. Fucoxanthin standard solution (purity: 99.6%) was purchased from ChromaDex Inc. (Irvine, CA, USA).

3.3.2 Fucoxanthin extraction

About 0.1 g of freeze-dried diatoms or 1 g of concentrated diatoms (water content: 89.8%) was placed in an Erlenmeyer flask. Then, 20 ml of organic solvent (acetone, ethanol, or ethyl acetate, with or without 0.3% BHA) was added, and the flask was sealed, placed in an orbital shaker and shaken at 150 rpm under room temperatures for 60 min. The flask was completely covered with aluminum foil during the extraction to prevent potential light-induced degradation of fucoxanthin. When the extraction finished, 75 ml of ethanol and 5.00 ml of 0.40 mg/ml Sudan I (internal standard for HPLC analysis) were immediately added into the flask. After a brief mixing, a 1-ml aliquot of the mixture was moved into a glass vial and further diluted with 4.0 ml of ethanol. The resulted solution was filtered through a 0.2 μm syringe filter and subjected to HPLC analysis.

The fucoxanthin content in the diatom concentrate was measured in quintuplicates by depleting extraction. About 1 g of diatom concentrate and 20 ml ethanol (with 0.3% BHA, w/v) were put in an Erlenmeyer flask and shaken in an orbital shaker at 150 rpm in dark under room temperatures for 20 min. After settling for 1 min, the supernatant was decanted and stored at -20 ºC until the subsequent extraction steps were completed. The remained diatoms were extracted 4 more times with fresh ethanol (with 0.3% BHA) until the extract was almost colorless and the diatoms became light gray. The five extracts were combined for HPLC analysis.

3.3.3 Extraction kinetics

About 1 g of wet diatom concentrate was placed in an Erlenmeyer flask. Then, 20 ml of organic solvent (ethanol or acetone, with or without 0.3% BHA) was added, and the flask

was sealed, placed in an orbital shaker and shaken at 150 rpm under room temperatures. At a series of predetermined times (2min, 4min, 6min, 8min, 10min, 15min, 30min and 60min), 0.10 ml of the extract was sampled from the flask, then 100 µl of 0.40 mg/ml Sudan I were added, and the sample was further diluted with 5.0 ml of ethanol. After mixing, the resulting solution was filtered through a 0.2   m syringe filter and subjected to HPLC analysis. The flask was completely covered with aluminum foil during the extraction to prevent potential light-induced degradation of fucoxanthin. All the experiments were conducted in triplicate.

3.3.4 HPLC analysis and quantities calculation

An HPLC system (LC-10 series, Shimadzu, Kyoto, Japan), equipped with a UV-VIS detector and a Restek Ultra C18 column (4.6 × 250 mm with a 4.6 × 10 mm guard cartridge, Restek Corporation, Bellefonte, PA, USA), were used to quantify the amount of fucoxanthin extracted. The chromatography method was adapted from an algal pigment analysis method by van Leeuwe and others (2006). Multiple-step linear gradient was used. Mobile phase A consisted of acetonitrile/water (90:10, v/v), and mobile phase B was ethyl acetate. The initial mobile phase was 100% A. After holding for 1 min, the mobile phase was linearly changed to 80% A and 20% B over 9 min, followed by changing to 63% A and 37% B over 4 min, and to 20% A and 80% B over 3 min. Then, the mobile phase composition was held at 20% A and 80% B for 13 min. The column was used under room temperatures. The chromatogram was recorded at 450 nm. The flow rate was 0.8 mL/min, and the sample injection volume was 20 microliters. The typical retention times for the internal standard,

Sudan I, and fucoxanthin were 11.7 min and 12.8 min, respectively. The yield of fucoxanthin in the fraction was calculated by the follow equation:

$$\text{Yield of Fucoxanthin } (\mu g/g) = (143.42 \times R.A. - 0.0918)/2*M$$

where, R.A equals the Area (Fucoxanthin)/Area (Sudan I), and M is the weight of sample.

In order to compare the effects of the different solvents, (i.e., acetone, ethanol, and ethyl acetate with and without BHA) on extraction, wet base yield was converted to dry base yield using the following equation (water content in the fresh diatoms: 89.8%):

$$\text{Dry base yield } (\mu g/g) = \text{Wet base yield } (\mu g/g) / (1-0.898)$$

3.3.5 Data analysis

Statistical analyses were performed by JMP (JMP® 11.0.0, Cary NC, U.S.A.). A one-way and a two-way analysis of variance were used to test for statistically significant differences. Yield of fucoxanthin was analyzed by a one-factor ANOVA with repeated measures. A Least Squared Means Difference Student's t test was performed to determine whether there were differences among the individual groups. A difference with $P \leq 0.05$ was considered significant.

3.4 Results and discussion

3.4.1 Effects of solvent types and feedstock conditions

To evaluate extraction efficiencies of different solvents, three frequently used organic solvents (i.e., acetone, ethanol and ethyl acetate) were examined. The results are illustrated in Figure 8. From both the freeze-dried diatoms and wet diatoms, acetone had the highest

extraction efficiency among these three solvents followed by ethanol, while the efficiency of ethyl acetate was the lowest. But, within the freeze-dried diatoms, after 60 min of extraction, there was no significantly differences by using acetone or ethanol as the solvent, while acetone extracted 263% more fucoxanthin than ethyl acetate (140.5 μg /g vs. 38.7 μg /g, $p<0.05$)). Within the wet diatoms, after 60 min of extraction, acetone extracted 50.3% more fucoxanthin than ethanol (297.1 μg /g vs. 194.1 μg /g, $p<0.05$)), and 531% more than ethyl acetate (297.1 μg /g vs. 47.1 μg /g, $p<0.05$)).

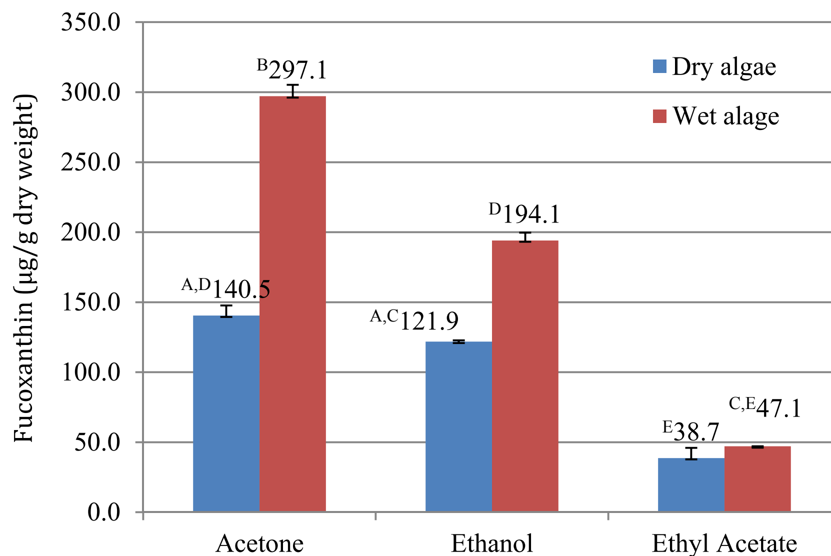

Fig. 8 Fucoxanthin extraction with acetone, ethanol and ethyl acetate from freeze-dried or wet diatoms

Note: Means with different letters are significantly different

Under similar conditions, fucoxanthin yields from wet diatoms were higher than those using freeze-dried diatoms when using acetone and ethanol as the solvent. Acetone extracted 111.5% more fucoxanthin from wet diatoms than from freeze-dried diatoms, and

the yield increased by 59.2% when using ethanol. When wet diatoms were used in the extraction, the diatoms were more evenly distributed in the acetone and ethanol during the extraction, which could help to accelerate the mass transfer. As a result, the fucoxanthin yield from acetone and ethanol extraction significantly increased when wet algae were used. However, the yield of fucoxanthin from ethyl acetate extraction was not significantly increased when wet diatoms were used as compared to freeze-dried diatoms. This is due to the poor water solubility of ethyl acetate. The wet diatom concentrate could not efficiently mix with ethyl acetate and settled at the bottom of the Erlenmeyer flask during the extraction, which essentially limited the transfer rate of fucoxanthin and reduced the extraction yield. Therefore, ethyl acetate was not a suitable extraction solvent when feedstock contains a large amount of water.

Another reason for the higher yield of fucoxanthin from wet algae than from dried algae may be fucoxanthin losses during dehydration. It is reported that thermal processing used in seaweed extraction might be a major factor responsible for the reduction in fucoxanthin, so that, freeze-dried seaweed is considered to give better fucoxanthin yields compared to hot-wind dried samples (Mise et al., 2011; Fung et al., 2013). The process of drying at high temperature is thought to cause fucoxanthin decomposition through oxidation (Mise et al., 2011). In this study, it is found that even the process of freeze-drying, which is identified as safe and effective drying method, can reduce the fucoxanthin yield significantly. So, thawed diatom concentrates were directly used as feedstock to minimize fucoxanthin losses before extraction in this study.

In previous studies, brown algae, such as *Undaria pinnatifida*, have been recognized as a potential source of fucoxanthin. However, in this work, the diatom, *Thalassiosira*

weissflogii was selected as a model microalga to investigate the extraction of fucoxanthin. Mise et al. (2011) examined the effect of different particle sizes of ground *Cladosiphon okamuranus* (brown seaweed, macro-algae) on yield of fucoxanthin. They found a 50-μm freeze-dried powder had a higher extraction efficiency compared with 200 and 1000 μm particle sizes. Because of their inherently small size, the necessity to pulverize diatoms before extraction could be avoided. The microalgae *Thalassiosira weissflogii,* for example, is a short cylindrical diatom varying in size from 4 to 32 μm in diameter.

3.4.2 Effects of BHA and time

Antioxidants are frequently used to prevent or slow down degradation of carotenoids. In preliminary experiments, we examined the effects of vitamin C and BHA on a 4-hour extraction of fucoxanthin from dried algae by using three different solvents (Table 5). After 4h of extraction in the presence of an antioxidant (vitamin C or BHA) or without an antioxidant, the yields of fucoxanthin with BHA increased by 1.42 times when acetone was the solvent, by 1.88 times when ethanol was the solvent, and by 1.90 times when ethyl acetate was the solvent as compared to the respective yields without an antioxidant. Vitamin C significantly increased the yield of fucoxanthin when ethanol was the solvent, but did not increase the yield when acetone or ethyl acetate was used as the solvent. BHA, a widely used fat-soluble antioxidant in the food industry, appeared to have better antioxidant activity than did Vitamin C, a water soluble antioxidant, during the 4-hour fucoxanthin extraction.

Table 5. The effects of BHA and Vitamin C on the yield of fucoxanthin (μg/g)

	Acetone	Ethanol	Ethyl Acetate
Without antioxidant	189.5^{A,G}	316.5^{B,C}	80.5^{F}
0.3% BHA	270^{B}	604^{D}	151.5^{G}
0.3% VITAMIN C	211^{A}	379.5^{E}	93.5^{F}

Note: Means with different letters are significantly different

To study the effect of BHA on fucoxanthin extraction, acetone and ethanol with 0.3% BHA (w/v) were used to extract fucoxanthin from wet diatom concentrate. The results showed that over the 60 min extraction time, the addition of BHA did not increase the yield of fucoxanthin (Figure 9). At 60 min, there was an approximate 5% decrease in fucoxanthin yield for both acetone and ethanol extractions, however this difference was not statistically significant. It appears that under the conditions of this study, significant oxidation of fucoxanthin does not occur within the 60-minute extraction time. Thus, BHA did not result in greater fucoxanthin yields. Consistent with this interpretation is the finding the BHA could increase fucoxanthin yield during a 4-hour extraction (see Table 5). Therefore, BHA may play a role to increase fucoxanthin yields during extraction times that are greater than 60 minutes. As fucoxanthin is sensitive to oxidation, there is a potential need to use antioxidants to stabilize the extraction process and especially prolong fucoxanthin's shelf life. The extracted fucoxanthin could be further processed into relatively stable formats such as a frozen product and/or dried product for longer periods of time by adding BHA. The variety and dosage of other antioxidants will need to be investigated in the future.

The extraction kinetics in the first 60 min using acetone and ethanol with wet algae are illustrated in Figure 9. The fucoxanthin content in the diatom concentrate was determined to be 322.3 ± 16.6 μg /g dry weight by the depletion method. From this, the corresponding fucoxanthin extraction efficiencies were calculated and summarized in Figure 10. At 10 min, the fucoxanthin extraction efficiency reached 100.7 ± 5.8%, or 324.5 ± 8.4μg fucoxanthin per g alga dry mass using acetone with BHA. This suggests that a large portion of fucoxanthin is extracted in the first several minutes from diatoms under these extraction conditions.

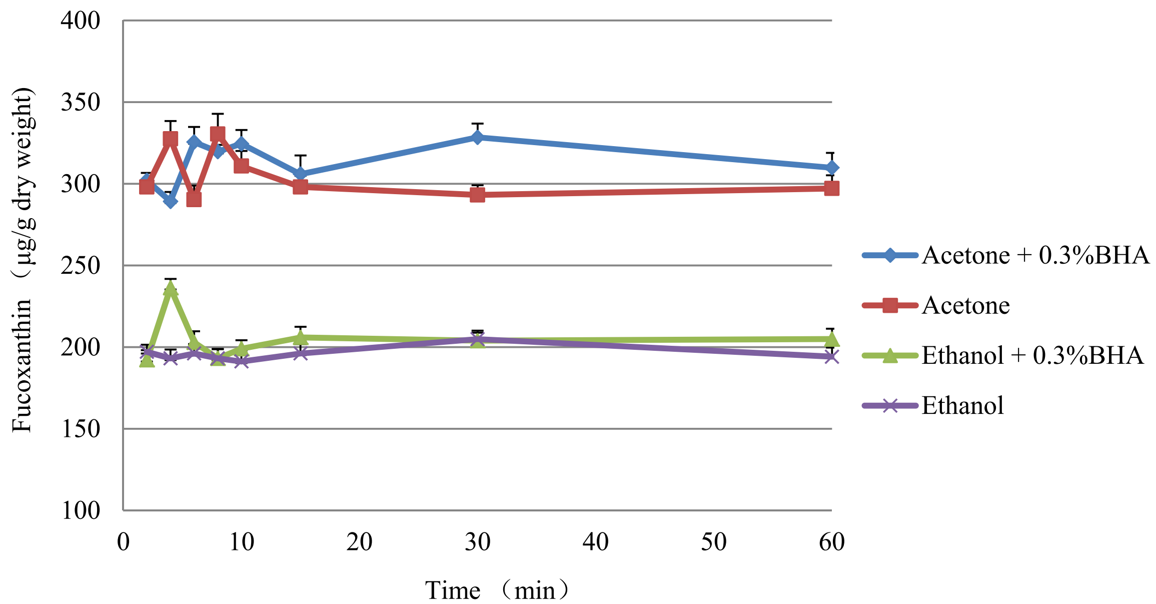

Fig. 9 Extraction yields of fucoxanthin in acetone and ethanol with or without BHA

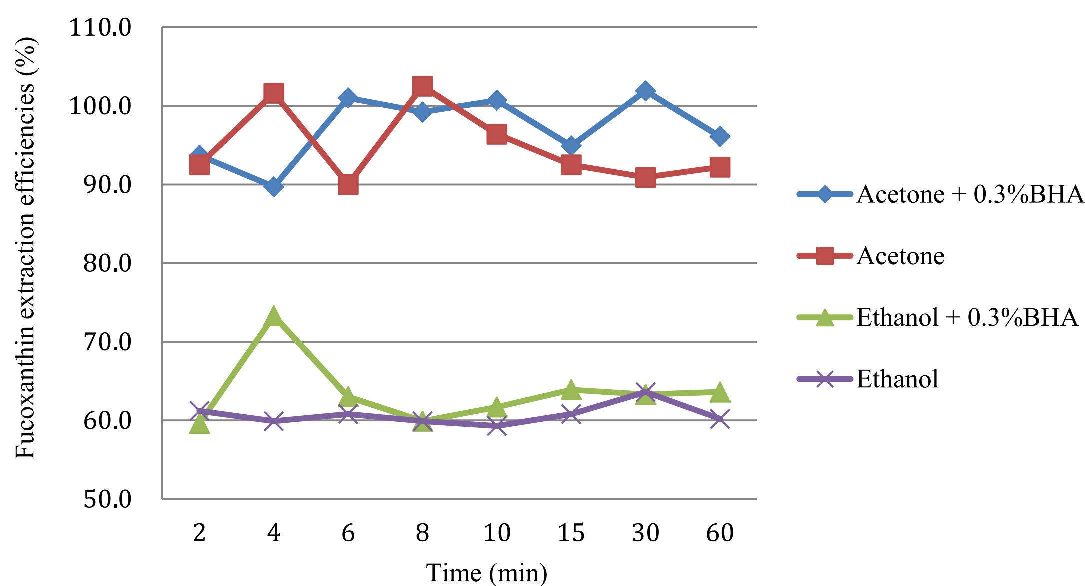

Fig. 10 Extraction efficiencies of fucoxanthin in acetone and ethanol with or without BHA

Mori et al. (2004) determined the content of fucoxanthin in *Undaria pinnatifida* and three other edible brown algae. They extracted for two days to deplete fucoxanthin from the brown algae using methanol as the solvent. In contrast, the extraction of fucoxanthin from diatoms used in this study could be depleted in only 10 min using acetone as the solvent. Difficulties have been reported with maintaining a uniformity of substrate distribution during commercial extraction processes using brown algae as a substrate. This was thought to be a result of the high concentrations of agar and carrageenan found naturally in brown algae, both of which tend to promote the formation of a colloidal suspension that hinders mixing with the extraction medium and limits the mass transfer. Microalgae should not have these issues and could, therefore, reduce the capital cost of extraction equipment.

These results provide a beneficial reference for the industrial production of fucoxanthin. Acetone seems to be the best solvent based on the extraction efficiency.

However, ethanol is also a good solvent for industry, because it is potentially less hazardous to human health and the environment, and is allowed for use in food processing. The industrial extraction time could be shortened to 10 min because of the high extraction efficiency of microalgae.

3.5 Summary

The efficiency of fucoxanthin extraction involves two aspects: the net extraction rate and the decomposition rate. The results of this study showed that acetone and ethanol could achieve excellent extraction efficiency in a relatively short period of time when using wet microalgae as the raw material. The addition of 0.3% BHA during the extraction could not increase the yields significantly in the short time, but may prevent the further potential decomposition of fucoxanthin. Compared with brown algae studied previously, fresh wet diatom might be a more cost efficient source for the extraction of fucoxanthin. The information obtained in this study will provide the scientific groundwork for the efficient extraction of fucoxanthin, which can be used to explore novel applications to the nutriceutical industry.

3.6 References

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4. Potential of Fucoxanthin as an Anti-Obesity Functional Food

4.1 Abstract

Fucoxanthin, a major carotenoid that exists in the Phaeophyta and most other heterokonts such as diatoms, has been suggested to exerts some amazing health benefits in recent metabolic and nutritional studies. Functional foods are defined as foods that have health-promoting or disease-preventing properties beyond that of the basic nutrients they supply. In this regard, fucoxanthin could be considered as a functional food that has been suggested to have antiobesity and antidiabetic properties. It is attracting more and more attention from researchers in food science and human nutrition areas.

In this study, we examined whether daily administration of fucoxanthin would have an anti-obesity effect in Wistar rats fed a high fat, high sucrose diet. Fucoxanthin administration (0.5 mg/kg/day for the first 4 weeks and up to 1.0 mg/kg/day for an additional 8 weeks) did not result in any anti-obesity or anti-diabetes activity. Rather, the rats gavaged with fucoxanthin had more fat and fat% (a percentage of carcass weight). The lipid gain of rats fed the high-fat diet and treated with fucoxanthin was 37% greater than that of rats fed the high-fat diet and treated with vehicle. The energy efficiency of rats treated with fucoxanthin was also significantly greater than in rats not treated with fucoxanthin. While food intake was not significantly different between fucoxanthin- and vehicle-treated rats fed the high fat diet, food intake was numerical greater in the fucoxanthin-treated rats between weeks 4 and 8. Data calculated from the examination of energy balance showed that high fat-fed fucoxanthin-treated rats had lower energy expenditures for a given amount of food than did high fat-fed vehicle-treated rats. The fucoxanthin-treated rats did have lower averages of ambulatory activity, but the variation

was too high to be any statistical differences between treatment groups. As opposed to our hypothesis, but consistent with an increase in body fat in fucoxanthin-treated rats, rats treated with fucoxanthin were glucose intolerant as compared to either the low fat-fed rats or the high fat-fed treated with vehicle. This was inconsistent with fucoxanthin having anti-diabetic effects. Fucoxanthin- treated Wistar rats fed a high diet seemed to store energy into fat more readily through small increases in food take over a long period time and increased energetic efficiency. This study questions the hypothesis that fucoxanthin supplementation has anti-obesity and anti-diabetic effects.

4.2 Introduction

Fucoxanthin is a major carotenoid that exists in the Phaeophyta, a division of algae that have chlorophyll masked by brown pigment, and most other heterokonts, such as diatoms. Fucoxanthin is thought to be a functional food, for which recent metabolic and nutritional studies have purported some amazing health benefits. Functional foods are defined as foods that have health-promoting or disease-preventing properties beyond that of the basic nutrients they supply. In this regard, fucoxanthin could be considered as a functional food that has been suggested to have multi-benefits on human health. Recent research suggests that fucoxanthin might anti-obesity, anti-cancer, anti-diabetic, and anti-oxidation properties. It is attracting more and more attention from researchers in the areas of food science, food engineering, and human nutrition.

The anti-obesity effect of fucoxanthin has been demonstrated in several studies using the mouse as a model (Maeda et al., 2005, 2007, 2009; Woo et al., 2009; Jeon et al., 2010). Fucoxanthin promotes the expression of uncoupling protein 1 (UCP1) to accelerate

fat burning within fat cells in white adipose tissue (Maeda et al., 2005). It also inhibits intracellular lipid accumulation during adipocyte differentiation in murine 3T3-L1 cells (Maeda et al., 2006). Furthermore, a human test suggested that fucoxanthin promotes weight loss, reduces body and liver fat content, and improves liver function tests in obese non-diabetic women (Abidov et al., 2010).

Obesity is defined as the accumulation of excess body fat. The accumulation of excess fat, especially around the internal organs, is a major risk factor increasing the risk of several chronic diseases, such as diabetes, hypertension, and hyperlipemia. Energy balance is key to maintaining a healthy weight. Energy balance describes the relationship between the metabolizable calories (energy) consumed in foods and beverages and the calories (energy) burned by the body. For most people, when calories (IN) = calories (OUT), body weight remains stable, which is a desirable condition for adults who are at a healthy weight. When intake consistently exceeds expenditure, body weight (fat) increases. When more calories are consistently burned than consumed, weight/fat loss occurs.

In this study, we examined whether daily administration of fucoxanthin would have an anti-obesity effect in Wistar rats fed a high fat, high sucrose diet. High fat and low fat diets were fed to the rats for 12 weeks, while the rats were gavaged daily with a defined dose of fucoxanthin. After 10-11 weeks of treatment, a subgroup of rats was placed in metabolic cages for 3 days (Promethion, Sable Systems, Las Vegas, NV) possessing a computer-controlled indirect calorimetry system. A body composition analysis was performed on the rats at the end study to determine the change in body energy due to fucoxanthin treatment over the course of the study.

Contrary to our hypothesis, we found that fucoxanthin treatment was associated with an increase in the gain of body fat in rats fed a high fat diet.

4.3 Materials and Methods

4.3.1 Preparation of Fucoxanthin

Purified fucoxanthin extracts (FE) were obtained from the Laboratory of Food Chemistry and Nutrition, Ocean University of China (Qingdao, China). The raw material was dried *Undaria pinnatifida* powder harvested and made by Jisyi Aquatic Foods Company (Rongcheng, China). Fucoxanthin was prepared as described previously by the author (Wang et al., 2009). Briefly, fucoxanthin was extracted from dried *Undaria pinnatifida* powder with acetone, and then recovered from the acetone extract by partitioning with ethyl acetate and water. The extracted liquid was isolated through silica gel column chromatography. HPLC, MS, and NMR verified the authenticity of the extracted fucoxanthin. The HPLC system used was an Agilent liquid chromatography system (1100 series, Agilent, Waldbronn, Germany) with a diode-array detector (DAD). The reversed phase column was an Eclipse XDB-C18 column (4.6 × 250 mm with a 4.6 × 10 mm guard cartridge, Agilent, Waldbronn, Germany). The column was maintained at a constant temperature of 25°C using a column thermostat. All solvents were HPLC grade agents. The eluent used was methanol/acetone/water (75:15:10, v/v/v) containing 1 g/L ammonium acetate. The flow rate was kept at 1.0 ml/ min. Fucoxanthin was detected at 450 nm. After fraction collection of the HPLC effluents, the fucoxanthin-containing fraction was collected and affirmed finally through continued MS and NMR detection. MS of fucoxanthin from drying Undaria pinnatifida showed the Quasi-molecular ion peak: 659.6[M+H]+. The molecular weight was

obtained at 658, which is consentaneous with the documented molecular weight of fucoxanthin (Sugawara et al., 2002). The MS of the peak detected at 10.564 min in drying Undaria pinnatifida gave positive ions at m/z 581.6, which corresponded to [M +H-36]+ of fucoxanthinol. According to spectrum unscrambling, the Delta Values corresponds to the carbon's sequence number, which is consistent with the NMR data of fucoxanthin reported previously (Hosokawa et al., 1999).

The prepared fucoxanthin extract (FE) was dissolved in 100 ml ethanol. The 100 ml aliquot was separated into 100 microcentrifuge tubes, with 1 ml per tube, and then were vacuum concentrated to remove the organic reagent (Eppendorf Vacufuge®, Hauppauge, NY). All the tubes with concentrated FE were wrapped with aluminum foil to protect from light degradation and stored frozen at -4℃.

4.3.2 Quantitative Analysis

To quantify the amount of fucoxanthin contained in the tubes, concentrated FE in one tube was dissolved in 1 ml ethanol and brought up to 100 ml with ethanol in the volumetric flask. After a brief mixing, a 0.3ml aliquot of the FE solution and 0.1 ml Sudan I (0.4mg/ml) were transferred into a glass vial and further diluted with 4.6 ml of ethanol. The resulting solution was filtered through a 0.22μm syringe filter and injected into a high performance liquid chromatography (HPLC) system. High performance liquid chromatography was performed using an HPLC system (LC-10 series, Shimadzu, Kyoto, Japan), equipped with a UV-VIS detector and a Restek Ultra C18 column (4.6 × 250 mm with a 4.6 × 10 mm guard cartridge, Restek Corporation, Bellefonte, PA, USA). The chromatography method was adapted from an algal pigment analysis method by van Leeuwe and others (2006). Multiple-step linear gradient

was used. Mobile phase A consisted of acetonitrile/water (90:10, v/v), and mobile phase B was ethyl acetate. The initial mobile phase was 100% A. After holding for 1 min, the mobile phase was linearly changed to 80% A and 20% B over 9 min, followed by changing to 63% A and 37% B over 4 min, and to 20% A and 80% B over 3 min. Then, the mobile phase composition was held at 20% A and 80% B for 13 min. The column was used under room temperatures. The chromatogram was recorded at 450 nm. The flow rate was 0.8 mL/min, and the sample injection volume was 20 μL. The typical retention times for the internal standard Sudan I and for fucoxanthin were 11.7 min and 12.8 min, respectively. The content of fucoxanthin/0.3ml FE solution fraction was calculated by the follow equation:

$$\text{Fucoxanthin } (\mu g) = (1.4342 \times \text{R.A.} - 0.0009) \times 10$$

where, R.A equals to Area (Fucoxanthin)/Area (Sudan I).

Although FE was a mixture of several constituents, fucoxanthin was detected as the main peak on HPLC chromatogram using the UV detector. The content of fucoxanthin in one tube was determined to be 1.1mg.

4.3.3 Animals and Diets

The animal care and use protocol was approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Forty male Wistar rats (~175g) were obtained from Harlan Laboratories (Indianapolis, IN). Each rat was housed individually in a wire mesh cage in an environmentally controlled room with a specific temperature (23 ± 2 °C), humidity (50 ± 5%), and light schedule (lights on from 06:00 am to 6:00 pm). Rats were given free access to diet and water. Body weight and food intake (corrected for spillage) of each rat were recorded daily throughout the experiment. After

the rats were adapted to the environment for several days, we euthanized a subgroup of rats (n=8) at the beginning of the study to calculate the relationship between body weight and body composition (i.e., percent of body water, body fat, body ash, and body protein) using a body composition analysis (see later). From this information, we were able to estimate the initial amount of body water, body fat, body ash, and body protein of the experimental animals at the beginning of the study. The other rats were randomly divided into three groups. One group (LFD-V, 10 rats) was fed a semi-purified low-fat diet (containing 10% of calories from fat). The two other groups were fed the high-fat, high sucrose diet (containing 50% of calories from fat). The high fat diet was also relatively high in sucrose (roughly 15% by calories) as compared to the low fat diet (roughly 6.5% by calories). See Table 6 for specific ingredients and amounts in each diet. One of the groups (HFD-F, 11 rats) fed the high-fat diet was gavaged daily with purified extracts of fucoxanthin. The fucoxanthin extract (FE) was dissolved in 2% ethanol, and the mixture was administrated via gastric gavage to the animals at a dosage of 0.5 mg/kg/day for the first 4 weeks and up to 1.0 mg/kg/day for an additional 8 weeks till the end of the study. The other group (HFD-V, 11 rats) fed the high-fat diet was gavaged daily with vehicle (2% ethanol). The group of rats fed the low-fat diet was also gavaged with vehicle daily. The volume of the gastric administration of fucoxanthin or vehicle was approximately 1 ml per 100 g of weight.

Table 6. Composition of Low-fat Diet (g) and High-fat Diet (g) varying in fat and sucrose

Diet Ingredients	Low fat (gm/kg)	Low fat (% of kcal)	High Fat (gm/kg)	High Fat (% of kcal)
Casein	197	20.28 [1]	264	20.1 [1]
Anhydrous milkfat	27	10.12 [2]	252.2	50.02 [2]
Soybean Oil	12.8		12.8	
Cornstarch	600.2	69.60 [3]	184	29.88 [3]
Sucrose	60		184	
Cystine	3		3	
Cellulose	50		50	
Minerals (AIN-93)	36		36	
Vitamins (AIN-93)	11		11	
Choline bitartrate	3		3	
Total	1000	3505.7 kcal/kg	1000	4663.1 kcal/kg

Note: 1 - sum of calories from casein and cysteine. Assuming the digestibility of casein is 88.7% (Calories derived from protein). 2 - sum of calories from milkfat and soybean oil. Assuming the digestibility of fat is 99% (Calories derived from fat). 3 - sum of calories from cornstarch and sucrose. Assuming the calories derived from cornstarch is 3.6656 kcal/gm. (Calories derived from carbohydrate).

4.3.4 Measurement of body weight, food intake, and epididymal and retroperitoneal fat pad weights

Food intakes and body weights of all the rats were determined daily for 12 weeks. To determine daily food intake, the difference in food cup weights between consecutive days was determined. Spill papers were put below each cage to collect and account for the

amount of food that was spilled daily. At the end of the experiment, rats were given a euthanatizing dose of pentobarbital (250mg/kg). All rats were then decapitated. The GI tract was removed and discarded. The epididymal and retroperitoneal fat pads were isolated and weighed. Fat pads were returned to the carcass and the carcasses (minus the GI tracts) weight determined. Carcasses were frozen for subsequent body composition analyses.

4.3.5 Real time monitoring metabolic cages

Between approximately 10-11 weeks of fucoxanthin treatment, a subgroup of rats (8 rats per session, 3 sessions of 3 days each, for a total of 24 rats) were monitored by using a computer-controlled indirect calorimetry system (Promethion, Sable Systems, Las Vegas, NV). The calorimetry system consisted of 8 metabolic cages with bedding, each equipped with water bottles and food hoppers connected to load cells for food and water intake monitoring. All animals had ad libitum access to their specific diet (depending on their grouping) and water throughout the study. The air within the cages was sampled through microperforated stainless steel sampling tubes located in the inner bottom rim of the cages. Ambulatory activity and position were detected with XYZ beam arrays (BXYZ-R, Sable Systems, Las Vegas, NV) with a beam spacing of 0.25 cm. Respiratory gases were measured with an integrated fuel cell oxygen analyzer, spectrophotometric CO_2 analyzer and capacitive water vapor partial pressure analyzer (GA3, Sable Systems. Las Vegas, NV). Gas sensors were calibrated daily with 100% N_2 as zero reference and with a span gas containing known concentration of CO_2 with balance N_2 (PraxAir, Tacoma WA). Promethion utilized a pull-mode, negative pressure system. Two multi-channel mass flow generators measured and controlled airflow (FR8, Sable Systems, Las Vegas, NV). The incurrent flow rate was set at 3000 mL/min. Water vapor was continuously measured and its dilution

effect on O_2 and CO_2 was mathematically compensated for in the analysis stream. Oxygen consumption and carbon dioxide (CO_2) production were measured for each rat at 8-min intervals. Respiratory quotient (RQ) was calculated as the ratio of CO_2 production over O_2 consumption. Energy expenditure was calculated using the Weir equation:

$$Kcal/hr=60*(0.003941*VO2+0.001106*VCO2).$$

Data acquisition and instrument control were coordinated by MetaScreen v. 1.6.2 and the obtained raw data was processed using ExpeData v. 1.4.3 (Sable Systems, Las Vegas, NV) using an analysis script detailing all aspects of data transformation.

4.3.6 The oral glucose tolerance test (OGTT)

At the end of the study, an OGTT was performed on the rats from all three groups, which was performed according to the standard method (Du Vigneaud and Karr, 1925). In short, all 32 rats were given an OGTT test after an overnight fast for 16 hours. Before the glucose solution was administered, the baseline glucose concentrations were measured by a handheld glucometer (Walgreens, TRUEtrack blood glucose test meter). The tip of the tail (a couple of millimeters) was cut off with scissors. From this wound, a blood sample was obtained. The wound quickly formed a scab. Successive samples were obtained from the same site by opening the scab. Only about 25 ul of blood was required to determine the glucose concentration by a handheld glucometer. A glucose solution was gavaged at the dose of 2 g/ml. The resulting blood glucose concentrations were obtained from sampling the tail vein at time 15, 30, 60 and 120 minutes after gavage of the glucose solution.

4.3.7 Body composition analysis

Carcasses were placed in glass containers covered with foil and were autoclaved for 1-1.5 hour (depending on the weight of rats) at 120℃. Three volumes of distilled water was added to each carcass and blended. The mixture was then homogenized with a tissue homogenator (PowerGen 700, Fisher Scientific, Norcross, GA). During this time, two sets of samples were taken in triplicate for the determination of fat, water, and ash percentages. The percentage of body fat was determined by chloroform–methanol extraction. The percentage of body water was determined by drying the samples for 48 h at 85℃. The samples were subsequently heated at 600℃ overnight to determine the percentage of ash. The percentage of protein was determined by the difference. Total body fat, protein, water and ash were determined by multiplying the ratio of each component by the carcass weight. To calculate carcass energy and energy intake, we assumed the energy content of protein and carbohydrate to be 4 kcal/gm and fat to be 9 kcal/gm. From the baseline control rats, the relationships between carcass components and body weight were determined. Using these relationships and the experimental animal's body weight before the introduction of test diets, the initial carcass composition of each rat was estimated. From the difference between the final carcass composition and the estimated initial carcass composition, the lipid gain, protein gain, energy gain, energy efficiency (the ratio of energy gain to energy intake), and energy expenditure (the difference between energy intake and energy gain) were estimated.

4.3.8 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of fucoxanthin

Antioxidant activity is one of the important characteristics of fucoxanthin. Earlier studies had indicated that fucoxanthin was an effective free radical scavenger (Nomura et al., 1997; Yan et al, 1999). Chemically generated free radicals can be used to evaluate the antioxidant activity of fucoxanthin, thus providing an independent bioassay for the presence of authentic fucoxanthin.

Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of functional components. A rapid, simple, and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant capacity helps understand the functional properties of foods (Miller et al., 2000). To evaluate the antioxidant capacity of the fucoxanthin extracted from dried *Undaria pinnatifida* powder used in this study, a DPPH radical scavenging assay was carried out.

The capacity to scavenge the stable free radical DPPH was performed according to the modified method of Brand-Williams et al. (1995). Briefly, when DPPH reacts with an antioxidant compound that can donate hydrogen, it is reduced. The samples were reacted with the stable DPPH radical in an ethanol solution. The reaction mixture consisted of 4.5 ml of DPPH radical

ethanol solution (100 μ mol/L) and 1 mL ethanolic sample solution. The mixture was shaken vigorously and incubated for 30 min at room temperature in the dark. The changes in color were read [absorbance (abs)] at 517 nm using a UV- VIS spectrophotometer (Helios Omega; Thermo Scientific, Leicestershire, England). The reading on the spectrophotometer was zeroed using ethanol as the blank. Trolox (Enzo Life Sciences, New York) was used as a positive control for comparison. The radical scavenging activity percentage (RSA%) was determined according to Afolayan et al. (2007):

$$RSA\% = [1 - (A_{sample} - A_{blank\ sample})/(A_{control} - A_{blank\ control})] \times 100$$

where A_{sample} is the absorbance of the solution when the sample extract is added at a particular level mixed with DPPH solution, $A_{blank\ sample}$ is the absorbance of the solution when the sample extract is added with ethanol solution, $A_{control}$ is the absorbance of the DPPH solution, and $A_{blank\ sample}$ is the absorbance of the ethanol. The antioxidant capacity was expressed as the EC50 (the concentrations of fucoxanthin in the extract that is necessary to induced 50% of the maximal radical scavenging activity). The experiments were repeated three times.

4.3.9 Data analysis

All results are expressed as mean ± SEM. Statistical analyses were performed by JMP (JMP® 11.0.0, Cary NC, U.S.A.). A one-way analysis of variance was used to test for statistically significant differences. Daily food intake, body weight, and energy balance data were analyzed by a one-factor ANOVA with repeated measures. A Least Squared Means Difference Student's t test was performed to determine whether there were differences among the individual groups. Specific comparisons between groups were made using

Orthogonal constrast. For some studies, an analysis of covariance was performed. A difference with $P \leqslant 0.05$ was considered significant.

4.4 Results and discussion

4.4.1 Effects of Fucoxanthin on high-fat diet-induced obesity

After 12 weeks, the body weight (Figure 11) and body weight gain (Figure 12) in the HFD groups were greater than the corresponding weights in the LFD group ($p<0.0001$ for both body weight and body weight gain), suggesting that the high fat diet induced obesity. Fucoxanthin administired orally (0.5 mg/kg/day for the first 4 weeks and up to 1.0 mg/kg/day for the remainder of the study) didn't decrease the body weight or body weight gain in the HFD-F group relative to the non-fucoxanthin-treated HFD group, but rather it tended to further increase body weight. From week 4 through week 7, the weekly body weight gain of the high fat-fed fucoxanthin-treated rats was greater than in the high fat-fed vehicle-treated rats ($p=0.032$). This was at the time the body weights began to separate between the fucoxanthin-treated and vehicle-treated rats fed the high fat diet (Figure 12). This was also around the time that the amount of fucoxanthin that was gavaged daily was increased from 0.5 mg/kg/day to 1 mg/kg/day. By 8 weeks of age, this increase in body weight gain of the fucoxanthin-treated rats had returned to the level of the high fat fed-controls. At the end of the study, there was a trend ($p=0.14$), but not a statistical difference between body weight or body weight gain of the fucoxanthin- and vehicle-treated high fat-fed rats.

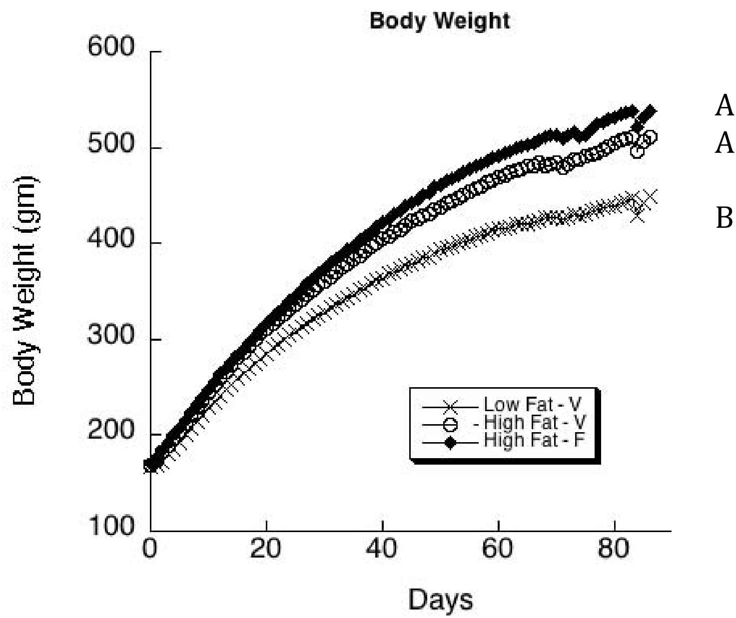


Fig. 11 Body weight changes in rats fed LFD-V, HFD-V, or HFD-F. Different letters indicate significant differences ($p<0.05$).

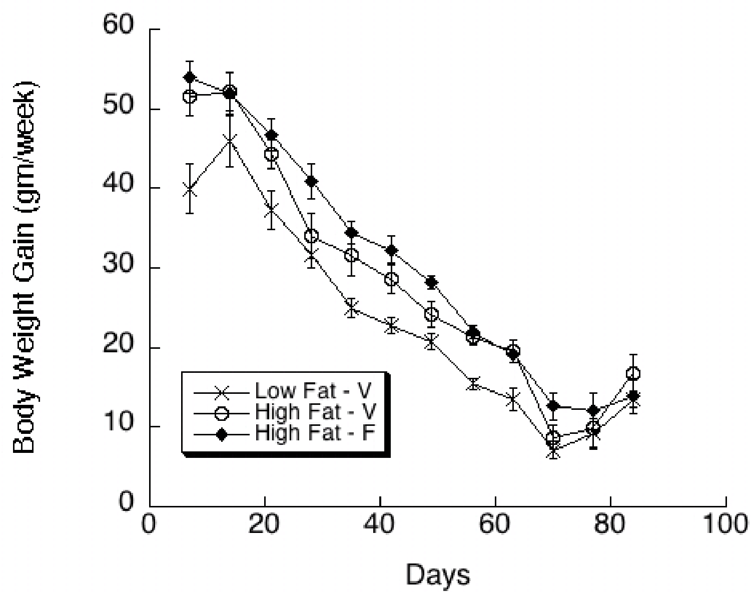


Fig. 12 Body weight gain by week in rats fed LFD-V, HFD-V, or HFD-F.

Cumulative food intake (kcal) and weekly food intake (kcal/week) are shown in Figure 13 and Figure 14, respectively. The food intake (kcal) of the high fat-fed rats was greater than that of the rats on the low fat diet ($p<0.0001$) across all 12 weeks of the study. This undoubtedly contributed to the greater body weight of the high fat-fed rats as compared to the low fat-fed rats (Figures 11 and 12). Interestingly, between week 4 and week 7 and during week 10 the food intake of the rats gavaged with fucoxanthing appeared increased relative to the high fat-fed rats gavaged with vehicle. However, like body weight, this was only a trend ($p=0.15$) and not statistical difference. By the end of the study, there was a 230 kcal difference in the average caloric intake of the fucoxanthin-treated rats (7484 ± 204) and the high fat-fed vehicle-treated controls (7254 ± 206, see Table 9). The results with body weight gain and food intake were unexpected since other investigators have observed that the gastric gavage of fucoxanthin of mice resulted in a decrease in body weight, but food intake did not differ significantly (Kang et al., 2012).

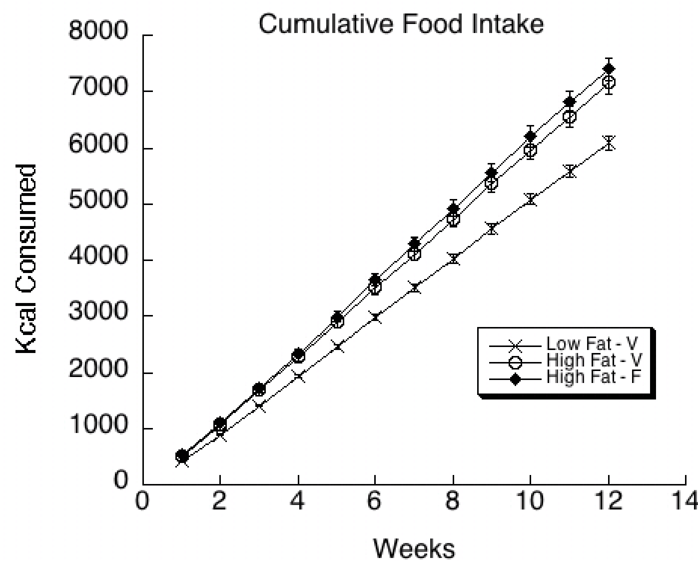


Fig. 13 Cumulative food intake (kcal) of rats

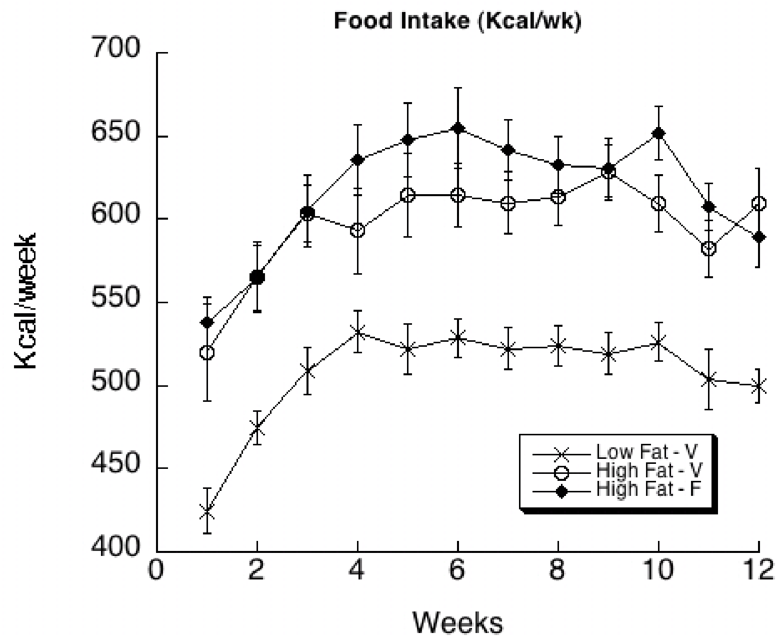


Fig. 14 Food intake in week (kcal/wk) of rats

As a preliminary indicator of potential changes in body fat, two visceral fat pads, the retroperitoneal (RETRO) and epididymal (EPI) fat pads, were isolated and weighed at the time the animals were euthanized (Table 7). As expected, both the RETRO and EPI fat pads of the rats fed the high fat diet were larger than the fat pads in the rats fed the low fat diet ($P<0.05$). While the EPI fat pad weights were similar in the high fat-fed fucoxanthin-treated rats as compared to the high fat-fed vehicle-treated rats, there was a tendency ($p=0.064$) for an increase in the weight of the RETRO fat pad in the fucoxanthin-treated rats. This was also true for total fat pad weights (i.e. sum of both the EPI and RETRO fat pads) ($p=0.092$). This suggests that fucoxanthin treatment may actually have increased the amount of body fat, which was also unexpected and different from previous studies. Kang et al. (2012)

found epididymal and perirenal fat pad weights of high fat fed fucoxanthin-treated mice to be significantly smaller than in high fat fed control mice.

Table 7. Effects of FE on RETRO and EPI fat pad weight in HFD-induced obese experimental groups after 13 weeks

	LFD-V	HFD-V	HFD-F
EPI Fat pad Weight (gm)	8.23 ± 0.56 ^{A}	13.36 ± 1.14 ^{B}	14.92 ± 0.89 ^{B}
RETRO Fat pad Weight (gm)	10.12 ± 0.61 ^{A}	17.10 ± 1.65 ^{B}	21.48 ± 2.12 ^{B*}
Total Fat pad weight # (gm)	18.35 ± 0.97 ^{A}	30.45 ± 2.72 ^{B}	36.40 ±2.95 ^{B**}

Note: Different letters indicate significant differences (p<0.05)

Sum of EPI and RETRO fatpads weights.

* High Fat – Vehicle vs High Fat – Fucoxanthin (p=0.064)

** High Fat – Vehicle vs High Fat – Fucoxanthin (p=0.092)

At the beginning of the study, a subgroup of rats (n=8) was used to calculate the relationship between body weight and body composition (i.e., percent of body water, body fat, body ash, and body protein). From this, the initial amount of body fat, body protein, body ash, and body water of the experimental animals was estimated just prior to the introduction of the high fat diet and fucoxanthin treatment (Table 8). There were no differences in the amount of body fat, body protein, body ash, or body water between the three groups at the beginning of the study.

At the completion of the study, a body composition analysis was performed on each of the rats. The percentage of body fat, protein, ash and water were determined. From these values and the carcass weights, the amount of body fat, protein, ash, and water were

determined. From the difference in the final amount of body fat, protein, ash, water and the estimated initial amount of body fat, protein, ash, and water, the gain in body fat, protein, ash, and water were determined. These values are shown in Table 8. The high fat diet (HFD-V) was able to increase lipid gain as compared the rats on the low fat diet (LFD-V) (58.1 ± 4.8 gm vs. 36.9 ± 2.5gm, respectively, $p<0.05$). Most interesting, fucoxanthin treatment further increased lipid gain in high fat-fed rats (HFD-F) compared to high fat-fed rats treated with vehicle (HFD-V) (79.4 ± 8.7 gm vs. 58.1± 4.8 gm, respectively, ($p<0.05$). On average, the effect of the high fat diet increased lipid gain by 57%, while the increase in lipid gain was doubled to a 115% with addition of fucoxanthin treatment. The increase in lipid was also reflected by increases in the percent of body fat. These findings were contrary to the hypothesis that fucoxanthin treatment has anti-obesity effects. It in fact suggests that fucoxanthin treatment could under certain conditions promote lipid gain. These results indicate that fucoxanthin treatment could increase body fat gain without affecting body weight. This find appears inconsistent with some previous studies, Kang et al. (2012) reported that fucoxanthin gavage (0.5mg/kg/day) for 70 days in C57BL/6 mice could decrease HFD-induced body weight gain and adipose tissue weight by activating AMP-activated protein kinase. Meada et al. (2005) reported reductions in abdominal WAT weights in rats and mice by feeding lipids from *Undaria pinnatifida* (~10% fucoxanthin).

No diet or fucoxanthin-related differences were found in gain of body protein or body ash. The gain in body water was greater in rats fed the high fat diet (HFD-V, HFD-F) as compared to rats fed the low fat diet ($p<0.05$).

Table 8. Carcass composition of LFD-V, HFD-V, and HFD-F rats

Treatment Groups	% Lipid	% Protein	% Ash	% Water
Low Fat - V	12.4 ± 0.5 ^{A}	23.2 ± 0.5 ^{A}	2.6 ± 0.1 ^{A}	61.8 ± 0.6 ^{A}
High Fat - V	15.4 ± 0.7 ^{B}	22.8 ± 0.8 ^{A,B}	2.5 ± 0.1 ^{A}	59.2 ± 1.1 ^{A,B}
High Fat - F	18.8 ± 1.3 ^{C}	20.8 ± 0.9 ^{B}	2.7 ± 0.2 ^{A}	57.7 ± 1.4 ^{B}
	Grams Lipid	Grams Protein	Gram Ash	Gram Water
Low Fat - V	51.5 ± 2.5 ^{A}	95.9 ± 2.1 ^{A}	10.8 ± 0.5 ^{A}	256.0 ± 6.3 ^{A}
High Fat - V	72.9 ± 4.9 ^{B}	107.3 ± 4.5 ^{A}	11.8 ± 0.7 ^{A}	278.1 ± 7.8 ^{B}
High Fat - F	94.4 ± 8.8 ^{C}	103.0 ± 5.1 ^{A}	13.3 ± 1.2 ^{A}	283.9 ± 4.6 ^{B}
	Est. Initial Lipid (gm)	Est. Initial Protein (gm)	Est. Initial Ash (gm)	Est. Initial Water (gm)
Low Fat - V	14.5 ± 0.4 ^{A}	26.2 ± 0.2 ^{A}	3.60 ± 0.02 ^{A}	92.5 ± 0.8 ^{A}
High Fat - V	14.8 ± 0.4 ^{A}	26.3 ± 0.2 ^{A}	3.60 ± 0.02 ^{A}	93.0 ± 0.8 ^{A}
High Fat - F	15.0 ± 0.4 ^{A}	26.5 ± 0.2 ^{A}	3.60 ± 0.02 ^{A}	93.5 ± 0.7 ^{A}
	Gain of Lipid (gm)	Gain of Protein (gm)	Gain of Ash (gm)	Gain of Water (gm)
Low Fat - V	36.9 ± 2.5 ^{A}	69.7 ± 2.1 ^{A}	7.2 ± 0.5 ^{A}	163.5 ± 6.5 ^{A}
High Fat - V	58.1 ± 4.8 ^{B}	80.9 ± 4.4 ^{A}	8.2 ± 0.6 ^{A}	185.1 ± 7.7 ^{B}
High Fat - F	79.4 ± 8.7 ^{C}	76.5 ± 5.0 ^{A}	9.7 ± 1.1 ^{A}	190.4 ± 4.4 ^{B}

Note: Different letters indicate significant differences ($p<0.05$)

From the body composition analysis and the food intake, the energy balance of the rats was determined (Table 9). The difference between the total energy intake and total energy gain in the body was calculated to be the total energy expenditure. Total energy gain (lipid energy gain plus protein gain) was greater in the HFD-V rats than in the LFD-V rats (847 ± 56 kcal vs. 611 ± 24 kcal, respectively) ($p<0.05$). While technically not statistically different, total energy gain of HFD-F rats was very close to being statistically greater than in HFD-V rats (1021± 83 kcal vs. 847 ± 56 kcal, respectively $p=0.0501$). The calculated energy

expenditure was greater in the high fat-fed rats (HFD-V & HFD-F) as compared to the low fat-fed rats ($p<0.05$). However, there was no difference in the calculated total energy expenditure between the HFD-V rats (6407 ± 163 kcal) and the HFD-F rats (6463 ± 128 kcal). The energy efficiency was greater in both the high fat-fed rats (HFD-V) as compared the low fat-fed rats (LFD-V) (11.59 ± 0.48% vs. 9.92 ± 0.38%, respectively, $p<0.05$), and in the fucoxanthin-treated rats (HFD-F) as compared to the vehicle-treated high fat-fed rats (HFD-V) (13.47 ± 0.73% vs. 11.59 ± 0.48, respectively, $p<0.05$).

Table 9. Energy Balance of rats

	Low Fat - Vehicle	High Fat -Vehicle	High Fat - Fucoxanthin
Est. Baseline energy (kcal)	236 ± 4 ^{A}	238 ± 4 ^{A}	241 ± 4 ^{A}
Final carcass energy (kcal)	847 ± 24 ^{A}	1085 ± 56 ^{B}	1262 ± 85 ^{B*}
Total Energy Gain (kcal)	611 ± 24 ^{A}	847 ± 56 ^{B}	1021 ± 83 ^{B**}
Gain from Lipid (kcal)	332 ± 22 ^{A}	523 ± 44 ^{B}	715 ± 78 ^{C}
Gain from Protein (kcal)	279 ± 8 ^{A}	324 ± 18 ^{A}	306 ± 20 ^{A}
Total Energy Intake (kcal)	6164 ± 123 ^{A}	7254 ± 206 ^{B}	7484 ± 204 ^{B}
Calculated Energy Expenditure (kcal)#	5553 ± 118 ^{A}	6407 ± 163 ^{B}	6463 ± 128 ^{B}
Energy Efficiency ##	9.92 ± 0.38 ^{A}	11.59 ± 0.48 ^{B}	13.47 ± 0.73 ^{C}

Note: Different letters indicate significant differences ($p<0.05$)

* High Fat – Vehicle vs. High Fat – Fucoxanthin ($p = 0.0507$)

** High Fat – Vehicle vs. High Fat – Fucoxanthin ($p = 0.0501$)

Energy expenditure was calculated by the balance method

(energy expenditure = energy intake – energy gain)

Energy efficiency was calculated as (energy gain/energy intake) x 100

Theoretically, changes in body energy should be due to either a change in metabolizable energy intake (energy in) and/or a change in energy expenditure (energy out). Total energy expenditure is determined mainly by the basal metabolic rate, the energy used for physical activity, and the thermic effect of food. The equation for energy balance is energy gain = $Energy_{in}$ - $Energy_{out}$. If the energy in equals energy out then balance is achieved and there is no change in the energy gain. It is the desirable condition for adults who are at a healthy weight. Understanding the relationship between energy requirements and desirable body weights should take into account not only the total weight, but also the composition of the weight. This is important because muscle mass and body fat make different demands on daily energy requirements and can have different long-term health consequences. Body composition is a much better predictor of one's level of health and risk of disease than is weight.

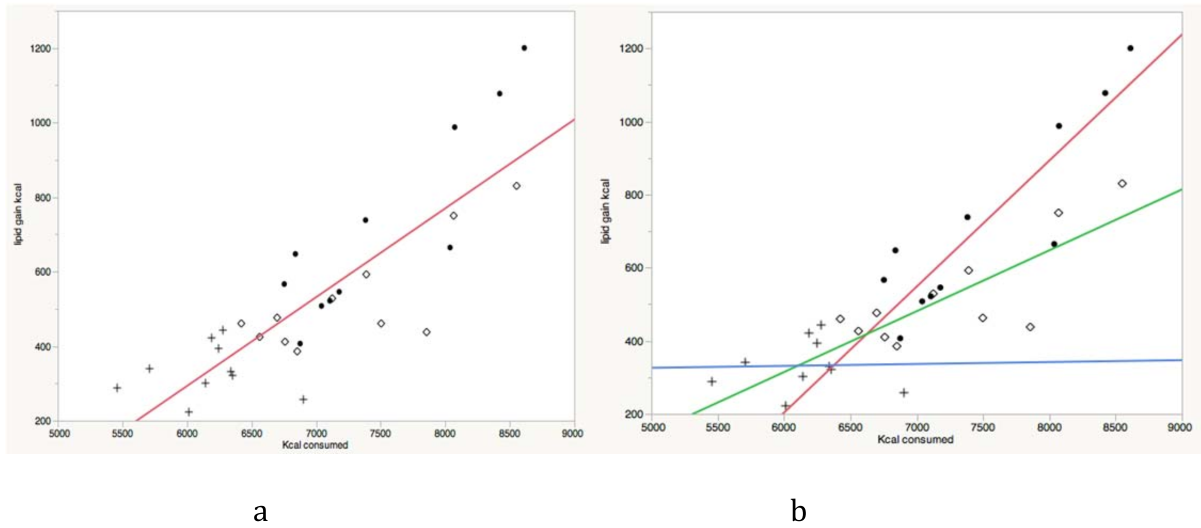
To further look at the relationship between food intake and lipid gain, food intake was plotted against lipid gain for all the rats in the study (Figure 15a). A linear regression was fit through the points (R^2= 0.69, p<0.0001, y = 0.238x - 1137). The positive correlation between these factors appears to suggest the more calories that were consumed, the more lipid was gained in the body. However, when a linear regression was fit for the rats within the individual treatment groups, differences in the relationship between food intake and lipid gain between the groups were revealed (Figure 15b). The regression line for LFD-V rats was basically flat with a slope of 0.005. This indicates that within the low fat fed-rats, lipid gain was basically independent of food intake. However, for the two groups of rats fed the high fat diet (HFD-V and HFD-F), there was a significant correlation between food intake and lipid gain. (HFD-V rats - R^2 = 0.58, p=0.0009, y = 0.166x - 685; HFD-F rats - R^2=

0.78, p=0.0002, y = 0.345x -1864, respectively). This suggests that the increased lipid gain in the high fat-fed rats was due at least in part to an increase in food intake. An analysis of covariance showed that the slope relating food intake and lipid gain was statistically steeper (p=0.022) in HFD-F rats (0.345) than in HFD-V rats (0.166). The intersection of the two lines was at a food intake of 6587 kcal, which corresponded to a lipid gain of 408 kcal. Thus, for any food intake more the 6587 kcal, high fat-fed fucoxanthin-treated rats gained more fat than high fat-fed rats treated with vehicle. When the difference in food intake was corrected for in the analysis of covariance, the LS means of the lipid gain of HFD-F rats (675 kcal) was still greater (p=0.011) than that of the HFD-V rats (542 kcal). This suggests that in addition to food intake, some other fucoxanthin-inducible factor was also responsible for the increased lipid gain. This finding is consistent with the increased energy efficiency of high fat-fed fucoxanthin-treated rats as compared to the high fat-fed vehicle-treated rats (Table 9).

The relationship between food intake and energy expenditure was also examined (Figure 16a). Total food intake was plotted against total energy expenditure for all the rats in the study. A linear regression was fit through the points (R^2= 0.96, p<0.0001, y = 0.722x + 1107). Thus, a very tight correlation was found between food intake and energy expenditure. When a linear regression was fit through the points of the rats within the individual treatment groups, subtle differences in the relationship between food intake and energy expenditure were revealed between the groups (Figure 16b). There was a significant correlation between food intake and energy expenditure for each of the groups (LFD-V rats – R^2=0.95, p<0.0001, y= 0.946x – 279) (HFD-V rats – R^2=0.96, p<0.0001, y = 0.775x + 788) (HFD-F rats – R^2=0.95, p<0.0001, y = 0.612x +1879). The low fat-fed rats had

a slope of 0.946, indicating that nearly 95% of the ingested calories were accounted for by a change in energy expenditure. This was most likely the reason why there was no correlation between food intake and lipid gain in the low fat-fed rats (Table 15b) and why the lipid gain in this group was independent of food intake. An analysis of covariance showed that the slope relating food intake and energy expenditure was statistically steeper (p=0.025) in HFD-V rats (0.775) than in HFD-F rats (0.612). The intersection of the two lines was at a food intake of 6700 kcal, which corresponded to an energy expenditure of 5980 kcal. Therefore, for any food intake above 6700 kcal, there is a relative decrease in the energy expenditure in high fat-fed fucoxanthin-treated as compared to high fat-fed rats treated with vehicle. When the difference in food intake was corrected for in the analysis of covariance, the LS means for energy expenditure of HFD-F rats (6393 kcal) was less (p=0.029) than that of the HFD-V rats (6497 kcal). This suggests that fucoxanthin treatment actually decreased energy expenditure by about 100 kcal on average as compared to vehicle treatment.

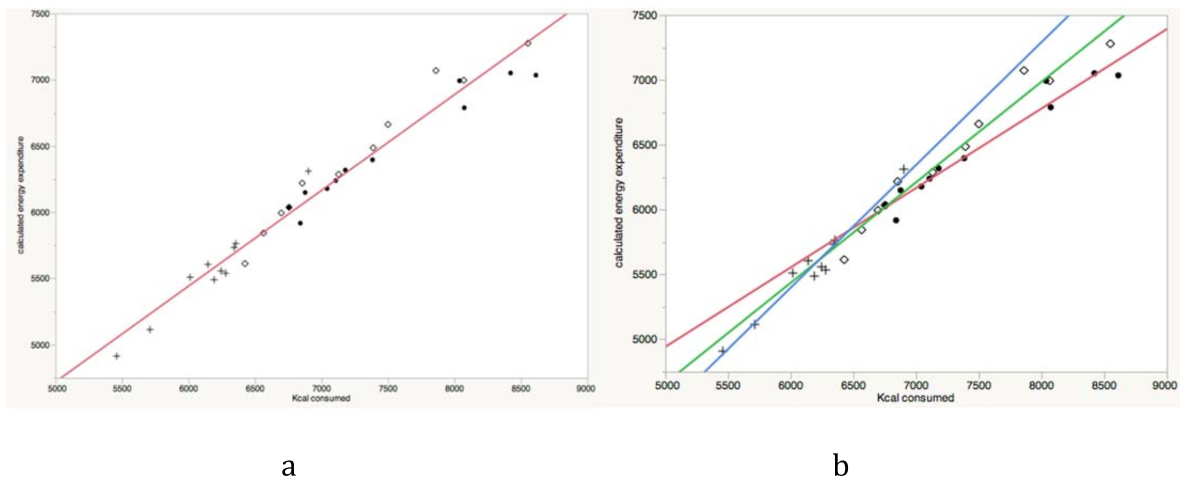
Together these data suggest that when the total food intake was less than 6600-6700 kcal, there was corresponding change in energy expenditure, so that lipid gain was independent of food intake. The high fat diet increased food intake to levels greater than 6600-6700 kcal for most of the rats. This contributed to an increase in lipid gain for both HFD-V and HFD-F rats. The increase in food intake also contributed to further increases in energy expenditure. However, the increase in total energy expenditure for a given increase in total food intake was attenuated in fucoxanthin-treated rats. This allowed more calories to be stored as fat and accounted for the increased lipid gain for a given amount food intake of high fat-fed fucoxanthin-treated rats as compared to high fat-fed vehicle-treated rats.



a b

Fig. 15 The correlation between kcal consumed and lipid gain (kcal) in all rats (a) and in three groups separately (b)

Note: + LFD-V (Blue); ◇ HFD-V (Green); ● HFD-F (Red).



a b

Fig. 16 Correlation between kcal consumed and calculated energy expenditure in all rats (a) and in three groups separately (b)

Note: + LFD-V (Blue); ◇ HFD-V (Green); ● HFD-F (Red).

The suggestion of an anti-obesity effect of fuxoxanthin was supported by up-regulation of the protein and gene expressions of UCP1 in WAT (Meada et al., 2005). UCP1 is known as a mitochondrial protein that partially uncouples the electron transport system from oxidation phosphorylation. This induces fat oxidation and the conversion of the energy to heat in BAT, which would be expected to increase energy expenditure. What role UCP1 has in WAT and its quantitative role in energy balance remain to the determined, but it may indicate the conversion of the WAT into BAT. There was no information on the dietary induction of UCP1 expression in WAT until the reports of fucoxanthin. However, contrary to these findings, the results of the present study showed that fucoxanthin treatment decreased energy expenditure under a higher food intake as compared to vehicle treatment and, thus, promoted more fat storage.

4.4.2 Effects of Fucoxanthin on high-fat diet-induced high blood glucose levels

Prior to the gastric gavage of glucose, blood glucose concentrations did not differ between the LFD-V, HFD-V, and HFD-F rats after an overnight fast (Figure 17). In comparison with the baseline concentrations, the blood glucose concentration of each group of rats increased after 15 minutes and returned to the baseline value after 120 min, except the HFD-F rats, whose blood glucose concentrations remained elevated as compared to baseline levels. Post-OGTT blood glucose values did not differ between the HFD-V group and the LFD-V group at any of the times examined. However, blood glucose concentrations of HFD-F rats were greater than LFD-V rats at all the observed times, except at 60 min. At 60 min, the blood glucose concentrations of the LFD-V and HFD-V rats showed an increase over their respective concentrations at 30 min. This increase may be caused by continued

absorption of glucose from the GI tract between 30 and 60 minutes. The area under the curve (AUC) was calculated for the three groups of rats (Figure 18). The high fat-fed fucoxanthin-treated rats had a larger ($p<0.05$) AUC than either of the other two groups. This indicates that the high fat-fed fucoxanthin-treated rats were relatively glucose intolerant as compared the either high fat-fed vehicle-treated rats or low fat-fed rats. This is contrary to the hypothesis that fucoxanthin-treated rats would have anti-diabetic properties and several previous studies (Maeda et al., 2007; Woo et al., 2007; 2010). Maeda et al. (2007) suggested that dietary fucoxanthin (0.2% in diet) decreases the blood glucose and plasma insulin concentrations of KK-Ay mice, and the combination of fucoxanthin (0.1%) and fish oil shows similar improvements and is more effective at attenuating the weight gain of WAT than feeding fucoxanthin alone. Animals were fed with AIN-93G. Woo et al. (2010) investigated the effects of fucoxanthin on lipid metabolism and blood glucose concentrations in C57BL/6N mice fed a high fat diet (20% fat in diet). The blood glucose concentrations of the two fucoxanthin groups were significantly lower during the entire experimental period, which is consistent with the findings of Maeda et al. (2007) regarding the administration of the dietary combination of fucoxanthin and fish oil in obese/diabetic KK-Ay mice. Both of these two studies used mice, and the fat content in the diet used were 13.5% and 40% respectively, which were different with our study. In this study, the development of glucose intolerance in the HFD-F rats may be related to the increased lipid gain that was observed in this group (Table 8). Somewhat surprisingly, there was no indication of glucose intolerance in high fat-fed vehicle-treated rat as compared to the low fat-fed vehicle-rats. Perhaps the difference in body fat between these groups was not large enough to affect glucose tolerance or perhaps there were differences in the distribution of

fat that accounted for the difference in glucose tolerance in the high fat-fed rats. It has been hypothesized that glucose intolerance is related to the accumulation of fat in non-adipose tissues, like skeletal muscle or liver (Unger, 2003).

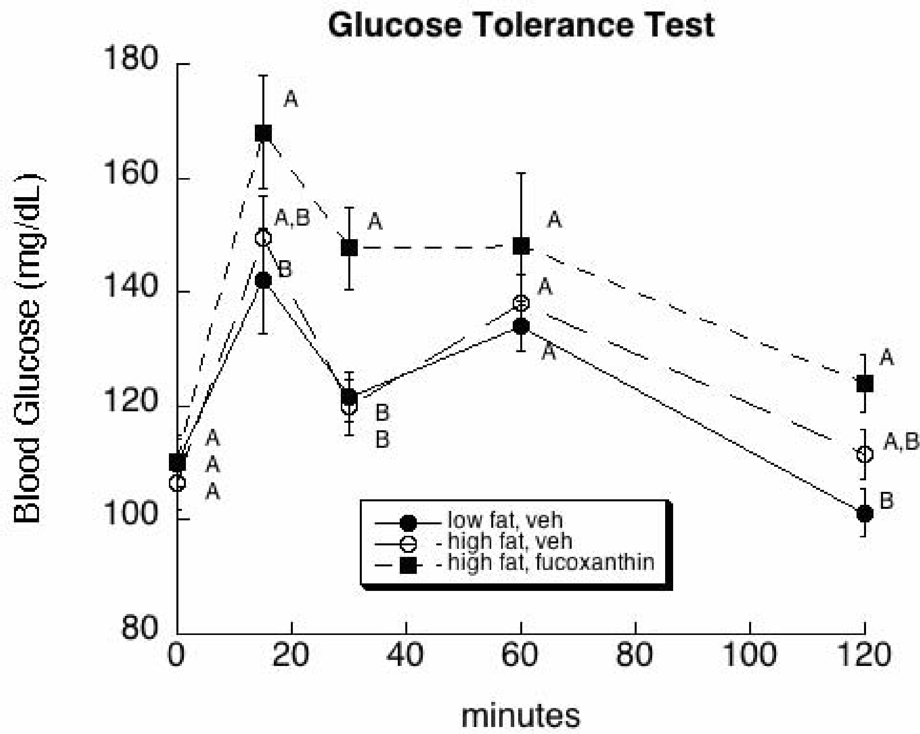


Fig. 17 Effects of fucoxanthin on OGTT values

Note: Means are shown ± SEM. Different letters indicate significant differences ($p<0.05$)

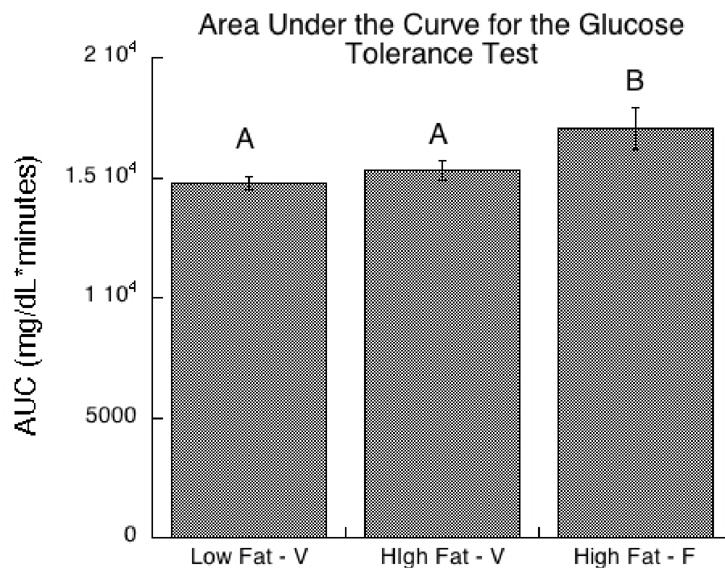


Fig. 18 Effects of fucoxanthin on AUC for the OGTT

Note: Different letters indicate significant differences ($p<0.05$)

4.4.3 DPPH scavenging activity of fucoxanthin extract

Because the results of this study were contrary with previous studies, hypothesizing anti-obesity and anti-diabetes activities of fucoxanthin, it was important to ensure that authentic fucoxanthin was actually used in this study. Although HPLC, MS, and NMR had verified the authenticity of the resulting fucoxanthin from the extraction procedures used in this study, the biological activity of the extract used in this study still needed to be confirmed. Therefore, the antioxidant activity of the fucoxanthin extract used in this study was determined.

Trolox, a standard antioxidant, was used as positive control (Figure 19). The antioxidant activity of the sample was expressed in micromoles of Trolox Equivalent (TE)

per 100 gm of sample (the sample here was the fucoxanthin extract). TE is defined as μmol of Trolox necessary to provide the same antioxidant capacity as a gram of the sample.

As the fucoxanthin concentration increased from 0.02 mg/mL to 0.8mg/mL, the percent radical scavenging activity (RSA%) increased in a concentration-dependent manner (from 4.4% to 99%) (Figure 20). The effective concentration the caused 50% scavenging (EC50) was 0.14 mg/mL. Figure 21 showed various corresponding concentrations of Trolox and fucoxanthin that achieved the same RSA%. The TE of the fucoxanthin extract at the EC50 was 1025, so its antioxidant activity (TE/100 mg) was 102500. Therefore, in addition to HPLC, MS, and NMR conformation of the presence of fucoxanthin using the extraction procedures of this study, the fucoxanthin extract used in this study showed significant antioxidant activity, which was characteristic of fucoxanthin.

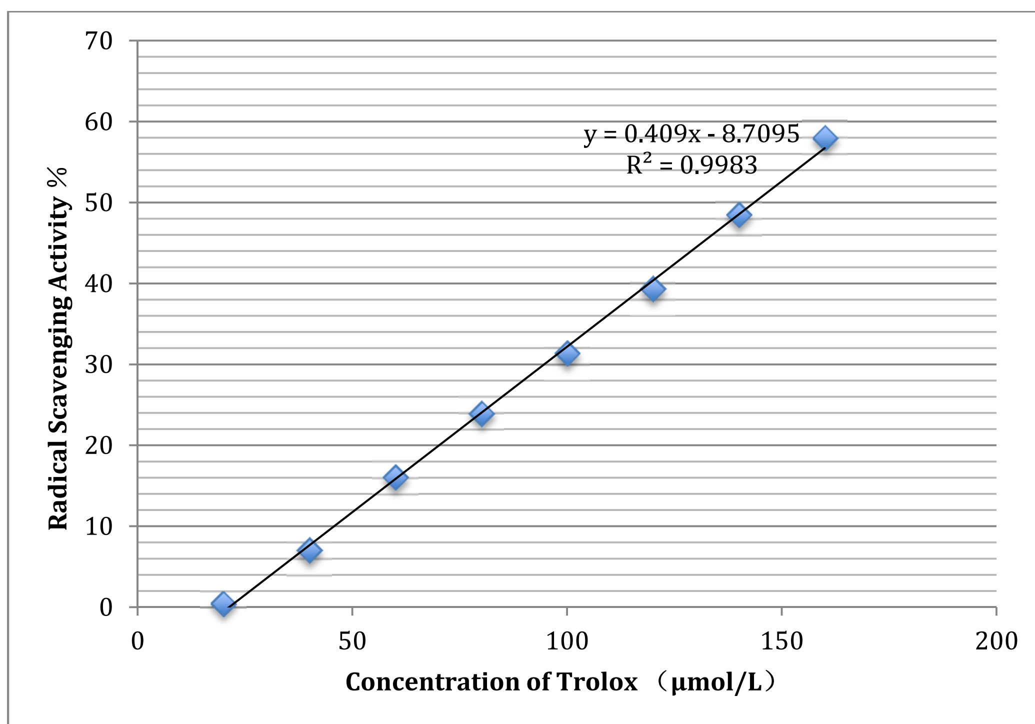


Fig. 19 The reducing power of the Trolox on DPPH

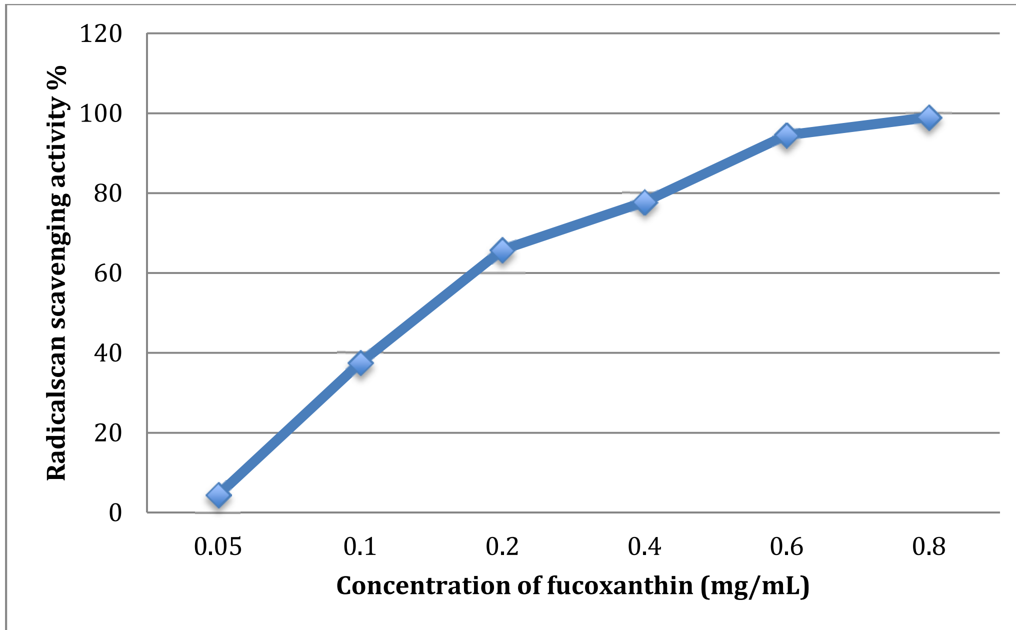


Fig. 20 The DPPH scavenging activity of fucoxanthin in different concentration

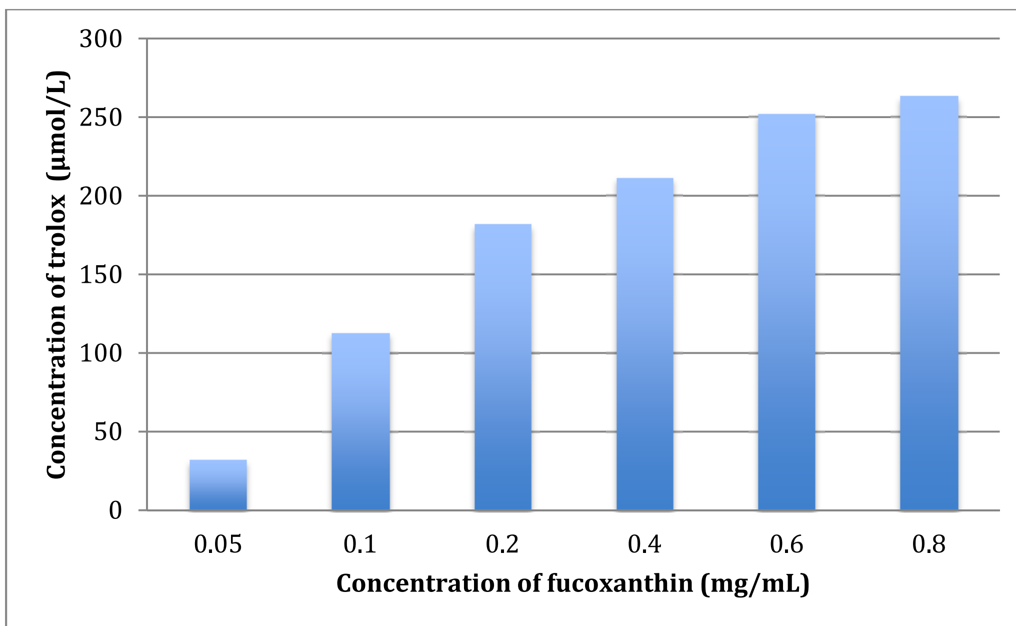


Fig. 21 The corresponding concentration of trolox and fucoxanthin under the same RSA%

4.4.4 Real time monitoring metabolic cages

Energy expenditure was determined in a subset of rats using real time monitoring metabolic cages (Figure 22). A well-defined circadian rhythm was noted where energy expenditure was greater in the dark hours (6:00pm-6:00am) that in the light hours (6:00am-6:00pm). No differences in energy expenditure were found between any of the groups at any of the light/dark phases, except during the last light phase. During this time, the energy expenditure of the two high fat-fed rats was greater (p=0.028) than the low fat-fed rats. This finding is somewhat consistent with the results of the energy balance study (Table 9) that found total energy expenditure increased in the high fat-fed rats as compared to the low fat-fed rats.

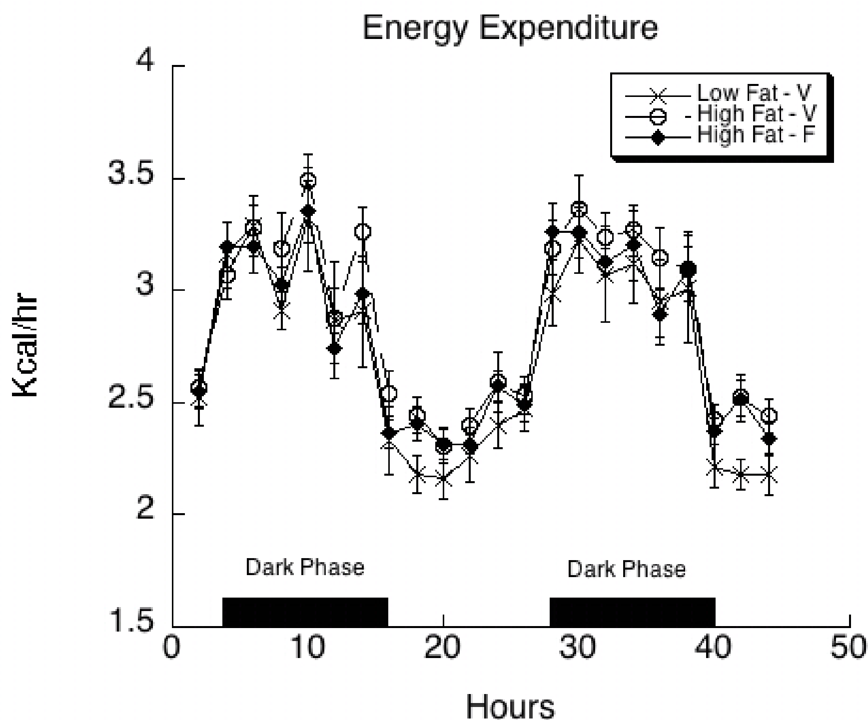


Fig. 22 Effects of high fat diet with or without fucoxanthin on total energy expenditure

The respiratory quotient (or RQ or respiratory coefficient) is a dimensionless number used in calculations of basal metabolic rate (BMR) when estimated from carbon dioxide production. BMR and the closely related resting metabolic rate (RMR), is the rate of energy expenditure by humans and other animals at rest, and is measured in kJ per hour per kg body mass. Rest is defined as existing in a neutrally temperate environment, while in the post-absorptive state. The RQ value corresponds to a caloric value for each liter (L) of CO_2 produced. If O_2 consumption numbers are available, they are usually used directly, since they are more direct and reliable estimates of energy production. The range of RQ for organisms in metabolic balance usually ranges from 1.0 (representing the value expected for pure carbohydrate oxidation) to ~0.7 (the value expected for pure fat oxidation). A mixed diet of fat and carbohydrate results in an average value between these numbers. An RQ may rise above 1.0 for an organism burning carbohydrate to produce or "lay down" fat (for example, a bear preparing for hibernation).

Figure 23 showed that LFD-V rats used carbohydrates as the main energy source in the dark phase, but used fats as the main energy source in the light phase. The dark phase is the rat’s active phase, while during the light phase rats are more sedentary. Fucoxanthin treatment did not appear to alter the RQ of high fat fed rats. The average RQ values of the two groups of high fat-fed rats were not different from each other in either the light or dark phase, and they both used fats as the main energy source during both dark and light phases.

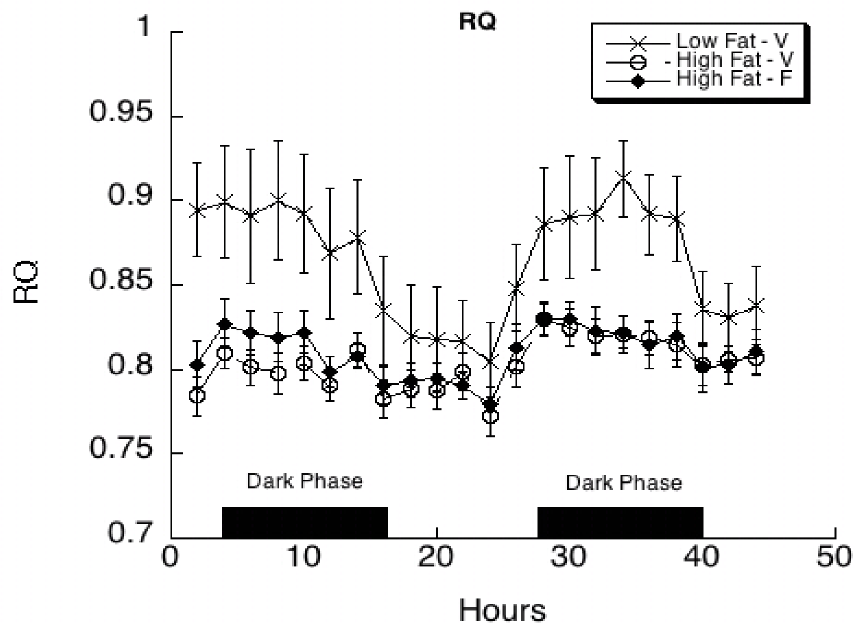


Fig. 23 Effects of high fat diet with or without fucoxanthin on RQ values

Figure 24 shows data concerning the ambulatory activity (actually the number of beam breaks) of the rats. There was a difference between the activity at night and the activity during day. As expected, rats were more active at night than during the day. However, no statistical differences in activity were found between the three groups of rats. The fucoxanthin-treated rats did have lower averages, but the variation was too large for any statistical differences between treatment groups to be observed.

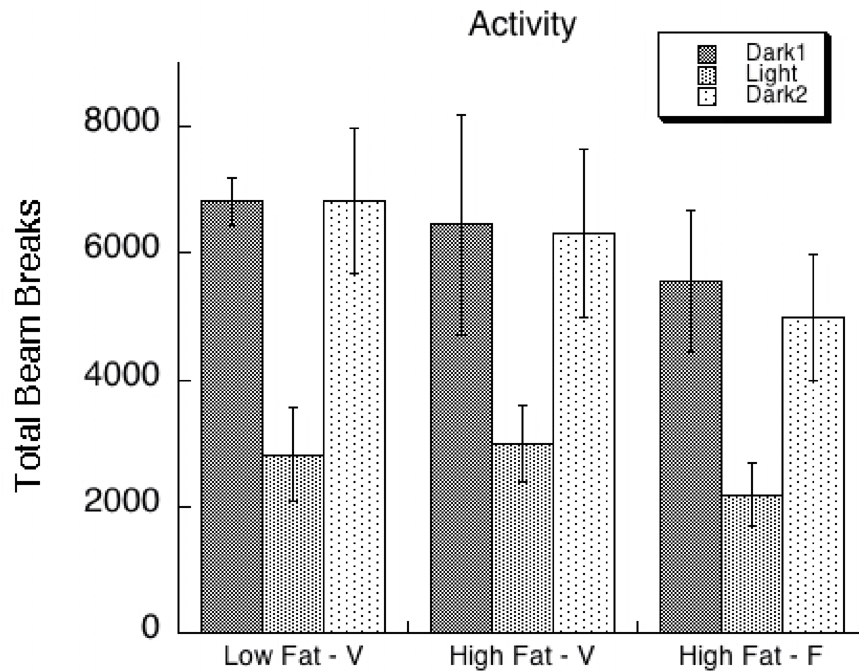


Fig. 24 Effects of high fat diet with or without fucoxanthin on ambulatory activity

4.5 Summary

Fucoxanthin has been purported to have anti-obesity and anti-diabetic activities (Maeda et al., 2005, 2006, 2007, 2008; Woo et al., 2010). Most of the previous studies examining these activities have used mice as a model. We hypothesized that gavaging fucoxanthin daily in Wistar rats fed a high fat diet would decrease the gain in body fat and improve glucose tolerance. To determine the gain in body fat, a body composition analysis was performed. To determine the cause of a potential difference in body fat gain, an energy balance analysis was performed. This is the first study to use a body composition analysis and energy balance analysis to examine the effects of fucoxanthin. Results obtained from this study were contrary with the previous studies and contrary with our hypotheses. Daily

gavage of fucoxanthin (0.5 mg/kg/day for the first 4 weeks and up to 1.0 mg/kg/day for continued 8 weeks) increased body fat gain and energy efficiency in high fat-fed rats (HFD-F) as compared to high fat-fed rats gavaged with vehicle (HFD-V). Though the average total food intake was not statistically different between these groups (p=0.15), the average of the fucoxanthin-treated rats was 230 kcal more than the average of the vehicle-treated rats. A regression analysis showed that the gain in body fat was independent of food intake in low fat-fed rats. However, for both HFD-V and HFD-F rats, body fat gain was significantly correlated with an increase in food intake. Most interesting, for a given amount of total food intake (above roughly 6600-6700 kcal), fucoxathin-treated rats gain more than twice (108% increase) as much fat as vehicle-treated rats. This is consistent with the increased energy efficiency that was observed in the HFD-F rats as compared to the HFD-V rats. A second regression analysis showed that total energy expenditure was significantly and tightly correlated with total food intake for all three groups of rats (LFD-V, HFD-V, HFD-F). However there were significant differences between the groups in how much the energy expenditure increased for a given increase in food intake. The fucoxanthin-treated rats (HFD-F) had a significantly attenuated increase in total energy expenditure for a given increase in total food intake (above roughly 6600-6700 kcal). Thus, it appears that the fucoxanthin-induced increase in body fat gain was due to a slight, though not statistically significant, increase in food intake and to an attenuation of the food intake-induced increase in energy expenditure.

Fuxoxanthin-treated rats (HFD-F) were glucose intolerant as compared to HFD-V and LFD-V rats. Again, this is counter to previously published studies (Maeda et al., 2007; Woo et al., 2010) and our hypothesis. However, it is consistent with the increased gain in body

fat that was observed in the fucoxanthin-treated rats in this study. It was a somewhat surprising that glucose intolerance was not found in HFD-V rats as compared to LFD-V, as these rats had greater gains in body fat than did the low fat–fed rats. Perhaps the amount of fat gain did not reach a threshold that would cause glucose intolerance or perhaps the distribution of the fat was different than the high fat-fed fucoxanthin-treated rats.

A major question is then, why did fucoxanthin increase body fat in this study, whereas it decreased body fat in previous studies (Meada et al., 2005; Jeon et al. 2008; Hosokawa et al., 2010). We examined the effects of fucoxanthin in rats, whereas most of the previously studies with fucoxanthin used mice as a model. Could the differences in response to fucoxanthin be due to the difference between rats and mice? There are common biological processes in different species of mammals, especially some of the most basic life processes. This is why rodents can be used in medical experiments as a model for humans. On the other hand, different species of animals have their own physiological characteristics, and responses to various factors. Because of this, different species of animals may have different effects to the same treatment. Mice are the most cited animal model used in research (Johnson, 2012). This is not surprising, 99% of mouse genes have human counterparts. Mice are relatively inexpensive to raise and to maintain. Compared to mice, rats are bigger and more resistant against various ailments. Because rats are larger and eat more food per day, they are easier to work with for feeding studies. For this anti-obesity study, we took Wistar rats as our experiment animal to examine the effects of fucoxanthin on body weight, WAT weight, food intake, and body composition of rats, which could be more accurately measured than it could in mice. The use of animals with diet-induced obesity (DIO), like in the present study, with their polygenetic basis and intact leptin

system, may provide a more robust approach for the screening of potential anti-obesity drugs or other compounds. The DIO rat model appears to have excellent predictive validity, even in regard to the modern trend towards the development of drug combinations for obesity. Vickers et al. (2011) found that the percent weight loss induced by a number of drugs in a clinical setting is comparable (R^2= 0.82) to the percent weight loss by those drugs in a DIO rat model (Vickers et al., 2011).

In the previous studies examining the effects of fucoxanthin, using KK-Ay mice and C57BL/6J mice was common (Sagawara et al., 2002; Meada et al., 2005; Tsukui et al., 2008; Jeon et al. 2008; Hosokawa et al., 2010). So it is possible that some of the differences from some previous studies and the present study may be due to species differences. However, other previous studies have used the rat as an animal model, and still observed an anti-obesity effect of fucoxanthin. Maeda et al. (2005) examined the anti-obesity effects of fucoxanthin both on Wistar rats and KK-Ay mice, and the results showed that the weight of the body and WAT decreased significantly only on mice with 2% Undaria lipid (contained 9.6%fucoxanthin), but no significantly differences were observed on rats for either the mean body weight or the food intake. Although, the weight of the perirenal and epididymal fat pads was significantly less in 2% Undaria lipid-treated rats and mice, an up-regulation of UCP1 expression in WAT, which the authors said accounted for the anti-obesity mechanism of fucoxanthin, was only found in mice. Sagawara et al. (2002) reported that dietary fucoxanthin was converted into fucoxanthinol, the deacetylated form, as it moved from the digestive tract into the blood circulation system in human and in mice, and that fucoxanthinol was the active form of the carotenoid in biological systems. Perhaps there was a difference in the metabolism of fucoxanthin to more active compounds in rats as

compared to mice. Meada et al. (2006) suggested that the anti-obesity effects of fucoxanthin and fucoxanthinol were caused by suppression of adipocyte differentiation. They suggested that fucoxanthin metabolites, including fucoxanthinol, accumulate in WAT of mice to prevent obesity.

One study used Wistar rats to investigate the effects of fucoxanthin and fucoxanthinol on the duodenal absorption of triglycerides in conscious rats. They found that fucoxanthin and fucoxanthinol (2mg/ml) could inhibit lipase activity in the gastrointestinal lumen and suppress triglyceride absorption to lower the increases in lymphatic and blood triglyceride levels in 24h (Matsumoto et al., 2010). The anti-obesity effect of fucoxanthin or fucoxanthinol was not examined in this study.

Secondly, in the present study, gastric gavage was used to administer the fucoxanthin extract as compared with other studies that added the fucoxanthin extract to the diet (Meada et al., 2005; Tsukui et al., 2008; Jeon et al. 2008; Hosokawa et al., 2010). Gastric gavage is used to dose an animal with a specified volume of material directly into the stomach. Thus, the fucoxanthin sample can be protected from light or other factors until it is administered. When added to food, fucoxathin is subjected to potential degradation/metabolism by various factors. This may lower the effective concentration of the active ingredient or even change the chemical composition of the compounds being administered. In addition, if fucoxanthin alters food intake, it would also alter the amount of the fucoxanthin that is being administered. Moreover, if fucoxanthin alters the taste or smell of the food, this could alter amount of food intake in fucoxanthin-treated animals. Gastric gavage guarantees that a specific dose of fucoxanthin extract will be delivered into

the stomach, and avoid a decrease in food intake caused by alterations in the sensory qualities of the diet. .

Thirdly, the dose of fucoxanthin used in this study (0.5 mg/kg/day for the first 4 weeks and up to 1.0 mg/kg/day for the remaining 8 weeks) was different from the dose used in the previous studies. Many studies used both a low and a high dose of fucoxanthin in the diet to examine its anti-obesity and anti-diabetic activity (Meada et al., 2005; Tsukui et al., 2008; Jeon et al. 2008; Hosokawa et al., 2010). The dose range of 0.025 – 0.05% fucoxanthin in the diet is typical. Assuming the daily food intake of a mouse is ~ 5 g and the total body weight is ~ 30 g, then this dose range represents an administration of approximately 41.6 - 83.3 mg/kg/day of fucoxanthin. A higher dose of fucoxanthin may produce a stronger biological effects or it may produce some unexpected side effects. However, Kang et al. (2012) gavaged fucoxanthin contained in a *Petalonia binghamiae* extract (PBE) at a dose of 0.5 mg/kg/day. The results showed that the PBE decreased mean body weight relative to the non-PBE-treated high-fat-fed C57BL/6 mice by 11.6%.

The fucoxanthin extract used in this study were obtained by chemical extraction and column chromatographic separation. Although fucoxanthin extract was the mixture of several constituents, fucoxanthin was detected as the main peak on HPLC chromatogram using UV detector. Apart from fucoxanthin, there were very little amounts of the other fat-soluble carotenoids and lipids, mainly consisting of glycolipids and phospholipids, which have not been reported to increase the body fat.

The present study shows that caution should be used before fucoxanthin is accepted as an anti-obesity, anti-diabetic functional food. More research is needed to explore the effects of fucoxanthin on food intake, energy expenditure, and the regulation of the body fat.

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5. Conclusion

Fucoxanthin, an important kind of carotenoid derived from algae, has been reported to have several biological activities such as anti-obesity, anti-diabetic, anti-tumor, radical scavenging, and anti-oxidant activities. It has the potential to be a functional food supplement, and an agent for obesity prevention. It plays an important role as a feed for the poultry and fisheries industries, and has the potential to affect human health. The industrial raw materials for fucoxanthin are plentiful.

In this study, a type of microalgae (diatom) *Thalassiosira weissflogii* was used as the feedstock for fucoxanthin extraction. Effects of solvent types, feedstock conditions, presence of antioxidants, and extraction time on fucoxanthin yield were investigated. In addition, an HPLC method to determine fucoxanthin content in the algae was established. Results suggested diatoms were a more cost and time effective source for fucoxanthin extraction than brown algae. Wet diatoms can achieve high extraction yields over a much shorter period. Yield was 100.7 ± 5.8% of the average total available fucoxanthin in the diatoms after 10 min of extraction with acetone. Adding 0.3% of the antioxidant (BHA) during the extraction did not increase the yields significantly over the 1-hour extraction time, but it could prevent potential decomposition of fucoxanthin during storage. The information obtained in this study will provide the scientific groundwork for the efficient extraction of fucoxanthin, which can be used to explore novel applications to the nutriceutical industry.

In addition, we sought to determine whether fucoxanthin, gavaged daily, would demonstrate anti-obesity and anti-diabetic properties in rats fed a high-fat/high-sucrose diet. To accomplish this, we performed an energy balance study on Wistar rats. Food

intakes and body weights were determined daily for approximately 12 weeks. After 10-11 weeks on the respective diets, a subset of rats from each group was placed in metabolic cages for 3 days to determine energy expenditure, RQ, and physical activity. Lastly, all rats were given an oral glucose tolerance test to determine how the diets and fucoxanthin treatment affected glucose tolerance. We found that fucoxanthin administration (0.5 mg/kg/day for the first 4 weeks and up to 1.0 mg/kg/day for an additional 8 weeks) did not result in any anti-obesity or anti-diabetes activity. Rather, fucoxanthin-treated rats fed a high-fat/high-sucrose diet gained significantly more body fat than vehicle-treated rats fed the same diet. The increase in body fat appeared to be due to a non-statistical increase in food intake and an increase in the energetic efficiency of the calories that were consumed. In addition, fucoxanthin-treated rats fed the high-fat diet also showed greater glucose intolerance as compared to the vehicle-treated rats fed the high fat diet. This study questions the hypothesis that fucoxanthin supplementation has anti-obesity and anti-diabetic effects.

These studies systemically examined the extraction, HPLC determination, anti-obesity, and anti-diabetic bioactivities of fucoxanthin. This research adds to the academic and scientific groundwork for the exploration of novel applications of fucoxanthin in food and drug fields.