**Characterization of dietary fucoxanthin from *Himanthalia elongata* brown seaweed**

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# Abstract

This study explored *Himanthalia elongata* brown seaweed as a potential source of dietary fucoxanthin which is a promising medicinal and nutritional ingredient. The seaweed was extracted with low polarity solvents (n-hexane, diethyl ether, and chloroform) and the crude extract was purified with preparative thin layer chromatography (P-TLC). Identification, quantification and structure elucidation of purified compounds was performed by LC-DAD-ESI-MS and NMR (1H and 13C). P-TLC led purification yielded 18.6 mg/g fucoxanthin with 97% of purity based on the calibration curve, in single-step purification. LC-ESI-MS (parent ion at *m/z* 641 [M+H-H2O]+) and NMR spectra confirmed that the purified band contained all-trans-fucoxanthin as the major compound. Purified fucoxanthin exhibited statistically similar (p>0.05) DPPH scavenging capacity (EC50: 12.9 µg/mL) while the FRAP value (15.2 µg trolox equivalent) was recorded lower (p<0.05) than the commercial fucoxanthin. The promising results of fucoxanthin purity, recovery and activity suggested that *H. elongata* seaweed has potential to be exploited as an alternate source for commercial fucoxanthin production.

**Keywords:** Antioxidant capacity;fucoxanthin; lipophilic compound; LC-ESI-MS; NMR; preparative TLC; seaweed

# Introduction

Oxidative stress has been associated with ageing and many chronic diseases, including cancer, cardiovascular disease, inflammation, cognitive impairment, immune dysfunction and some neurological disorders (Miyashita et al., 2011; Peng et al., 2011; D’Orazio et al., 2012; Mikami & Hosokawa, 2013). The potential cause of oxidative stress is free radicals or reactive oxidation species (ROS) which are produced during oxidation reactions, a naturally occurring process within the human body. These free radicals or ROS degrade the cellular biomolecules such as DNA, proteins and lipids which causes accelerated ageing and many degenerative diseases and conditions (Roehrs et al., 2011; Kim et al., 2013; Sangeetha, Hosokawa, & Miyashita, 2013; Pisoschi & Pop, 2015; Zampelas & Micha, 2015). To combat these oxidation by-products, natural antioxidants are becoming an increasingly important ingredient and have already received much attention in the prevention of these chronic diseases (Aldini et al., 2011; Mikami & Hosokawa, 2013; Zhang et al., 2015).

In the search for natural antioxidants, carotenoids have been considered as important dietary ingredients with many biological functions. The antioxidant activity of such molecules are based upon their ability to quench singlet oxygen and ROS (Stahl & Sies, 2012). The unique structure, conjugated double bonds and attached functional end groups make carotenoids an ideal candidate to act as antioxidants. The quenching ability of these molecules increases with increasing number of conjugated double bonds in the structural backbone as well as the nature of substituent attached groups (Sachindra et al., 2007; Miyashita et al., 2011).

Fucoxanthin is one of the most abundant carotenoids of brown seaweed and contributes almost 10% of total carotenoids found in nature (Hosokawa et al., 2009). There have been several reports which stated that fucoxanthin possess a number of therapeutic activities, including antioxidant, anticancer, antiobesity, antidiabetic, antihypertensive, antitumor, antiangiogenic and antiinflammatory effects (Sugawara et al., 2002; Maoka et al., 2007; Heo et al., 2008; D’Orazio et al., 2012; Mikami & Hosokawa, 2013; Sangeetha, Hosokawa, & Miyashita, 2013; Zhang et al., 2015). Despite multiple health related activities, particularly it has been widely investigated for its antioxidant role in both food and pharmaceutical sectors (Maeda et al., 2009; Kim, Shang, & Um, 2011; Kim et al., 2013; Zhang et al., 2015).

Many brown seaweeds as well as some microalgae are known to possess fucoxanthin as a main carotenoid and are considered a promising source for its industrial production (Kanazawa et al., 2008; Peng et al., 2011; Kim et al., 2012a). The isolation of fucoxanthin was first carried out from marine brown seaweeds *Fucus, dictyota* and Laminaria in 1914 (Willstätter & Page, 1914) while its chemical structure and chirality were primarily confirmed in 1990 (Englert, Bjørnland, & Liaaen‐Jensen, 1990). From a structural point of view, fucoxanthin is an allenic carotenoid possessing a conjugated carbonyl group with epoxide and acetyl substituent groups attached on a polyene structural backbone (Yan et al., 1999). It is an energy transferring pigment which binds to several proteins and chlorophyll a pigment, and forms fucoxanthin-chlorophyll-protein complexes in the thylakoid. This unique structure of fucoxanthin distinguishes it from other plant carotenoids, such as β-carotene and lutein. (Maoka, et al., 2007; Kim, Shang, & Um, 2011).

Though research has proved that fucoxanthin is an economically valuable pigment for both food and pharmaceutical industry, its commercial production and usage has been limited due to the low extraction efficiency and purification recovery from marine sources (Kanazawa et al., 2008; Kajikawa et al., 2012). Furthermore, as the chemical synthesis of fucoxanthin is difficult and expensive, the possibility of obtaining this precious compound directly from marine sources should not be underestimated (D’Orazio et al., 2012; Kajikawa et al., 2012). Therefore, the present study explored the Irish brown seaweed *Himanthalia elongata* or ‘sea spaghetti’ as a potential source for fucoxanthin production. This edible brown seaweed is commonly harvested along the European side of the Atlantic Ocean and has traditionally been used as fertilizer or a raw material for the potash industry. Recently, *H. elongata* has been explored for its potential phenolic antioxidants, antimicrobial property and free radical scavenging capacity (de Quirós et al., 2010; Plaza et al., 2010; Rajauria et al., 2013; Rajauria, Foley & Abu-Ghannam, 2016). However, to the best of our knowledge, this is the first detailed report on purification and characterization of fucoxanthin from *H. elongata* seaweed. In this study, the tested seaweed was submitted to extraction using a mixture of low polarity solvents, and the crude extract was then purified with preparative thin layer chromatography (P-TLC). Identification and structure elucidation of purified fucoxanthin was carried out by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) and nuclear magnetic resonance (NMR), and *in vitro* investigation of its antioxidant activity was performed.

# Materials and methods

## 2.1. Seaweed material and extraction procedure

Edible brown seaweed *H. elongata* used in the present study was purchased from Quality Sea Veg., Co Donegal, Province of Ulster (Northern part), Ireland. Samples were collected in bulk in February/March (between the winter and spring) and washed thoroughly to remove epiphytes and eliminate foreign materials such as sand, shells and debris and stored at -18 °C until further analysis. Extraction of fucoxanthin was carried out from liquid nitrogen crushed seaweed powder with equal-volume mixture of low polarity solvents (n-hexane, diethyl ether, and chloroform). The samples were filtered with Whatman #1 filter paper and centrifuged at 9,168 x *g* (Sigma 2–16PK, SartoriusAG, Gottingen, Germany) for 15 min (Rajauria et al., 2013; Rajauria & Abu-Ghannam, 2013). The resulting supernatant was evaporated to dryness, and the dried extract was dissolved in LC-MS grade methanol for further analysis. The whole extraction procedure was carried out under dark conditions to minimize the possibility of oxidation/degradation by light.

## 2.2. Preparative thin layer chromatography (P-TLC) based isolation

Purification of fucoxanthin from crude seaweed extract was carried out using preparative thin layer chromatography (P-TLC) reported in our earlier publication (Rajauria & Abu-Ghannam, 2013). A streak of crude extract was applied manually on a thick TLC glass plate with an inorganic fluorescent indicator binder (Analtech, Sigma-Aldrich, Steinheim, Germany). After air drying, the plate was developed with chloroform/diethyl ether/n-hexane/acetic acid (10:3:1:1, v/v/v/v) as mobile phase in a pre-saturated glass chamber with eluting solvents for 30min at room temperature. The developed plate was visualized under visible light and the compounds of interest were scratched carefully using a scalpel. The collected samples were dissolved in methanol and centrifuged at 9,168 x *g* for 15 min in order to remove the silica. The supernatant was collected, filtered using a 0.22 µm filter and dried under reduced pressure. The dried sample was passed under nitrogen stream for 5 min and then dissolved in LC-MS grade methanol for further characterization and bioactivity analysis.

## 2.3. HPLC-DAD guided identification

Identification of the purified compound was done by HPLC-DAD according to the method described by Sugawara et al. (2002). The HPLC system was an Alliance e2695 separation module equipped with online degasser, a quaternary pump programmable for gradient elution, a thermostatic controlled column chamber, an auto-sampler connected to a variable-wavelength diode array detector (DAD 2998), controlled by a Waters Empower 2 software (Waters, Ireland). The column employed was Atlantis C-18 (250×4.6 mm, 5 µm particle size) fitted with a suitable C-18 (4.0×3.0 mm) guard cartridge. The mobile phase consisting of a ternary solvents of acetonitrile/methanol/water (75:15:10, v/v/v/) contained 1.0 g/L ammonium acetate, and the separations were performed by using isocratic mode. The elution was performed at a flow rate of 1.0 mL/min for 25 min with 20 µL injection volume and 25°C column temperature. All chromatographic data were recorded from 200 to 700 nm range and were extracted at 450 nm absorption wavelength specific for carotenoids.

To quantify the fucoxanthin in *H. elongata* seaweed, a 5 point calibration curve (area vs. concentration) was constructed using reference fucoxanthin compound (≥95%; Sigma-Aldrich, Ireland) and the content was expressed as mg/g dry weight of seaweed sample. Each fucoxanthin standard curve set was injected in duplicate before and after the injections of *H. elongata* extract.

## 2.4. Liquid chromatography mass spectrometry (LC–MS) analysis

LC/ESI–MS analysis was performed with an Agilent Technologies 6410 Triple Quad LC/MS, fitted with Agilent 1200 series LC and MassHunter Workstation software (Agilent Technologies Ireland Ltd). The LC conditions such as column, flow rate and column temperature were the same as described in above HPLC section [2.3], except for the injection volume, which was 10 µL. Nitrogen gas was used as the nebulizer and drying gas with 50 psi pressure, 10 L/min flow rate, 350 °C drying temperature and 35 nA capillary current. Mass spectral data were recorded on ESI interface mode in the mass range of *m/z* 100–1000. In terms of fragmentation and sensitivity, various ESI-MS parameters such as capillary voltage (2.0, 2.5, 3.0, 3.5, 4.0 and 4.5kV) and fragmentor voltage (30, 50, 70, 100, 120 and 140V) were optimized. The final operating conditions selected were: positive ionization mode, capillary voltage 3.5kV, fragmentor voltage 120V and collision energy 10eV.

## 2.5. Nuclear magnetic resonance (NMR) analysis

Proton (1H NMR) and carbon (13C NMR) NMR spectra were performed on purified compound at 400 MHz and 100 MHz frequency using Bruker 400 MHz, Ultra shield instrument (Bruker UK Limited, Coventry, UK) respectively. The spectra were measured at ambient temperature with 32K data points and 128-1024 scans. The purified band collected from preparative TLC plate was dried under nitrogen stream in order to remove traces of TLC developing solvents. The sample was dissolved in deuterated (*d*) acetone and centrifuged at 9,168 x *g* for 15 min in order to remove the silica. The supernatant was collected and filtered from 0.22 µm filter and then an aliquot of this mixture was transferred into a 5 mm NMR sample tube and analysed. The proton (1H NMR) and carbon (13C NMR) spectra of the standard fucoxanthin compound were recorded in similar fashion. The structure of the purified compound was confirmed by comparison of the spectral data with those of the authentic standard. Data were acquired using Bruker Topspin software version 2.1. Resonance assignments were based on chemical shifts (δ). The chemical shifts relative to the residual solvent signals are reported in ppm whereas coupling constant (J) are reported in Hertz (Hz).

## 2.6. Antioxidant activity analysis of purified compound

The purified sample was tested for the determination of antioxidant status by DPPH radical scavenging (1,1-diphenyl-2-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) assays. Both analyses were carried out as described in our earlier publications (Rajauria et al., 2013). Trolox (6-hydroxy-2, 5, 7, 8- tetrametmethylchroman-2-carboxyl acid) was used as standard for FRAP analysis and results were expressed as µg trolox equivalent (TE). However, the ability to scavenge the DPPH radical was calculated using equation (1) and results were interpreted as the “efficient concentration” or EC50 value which is the concentration of sample required to scavenge 50% DPPH radicals.

DPPH scavenging capacity (%) x 100 Eq. (1)

Where ‘A control’ is the absorbance of the control (DPPH solution without sample), ‘A sample’ is the absorbance of the test sample (DPPH solution plus test sample) and ‘A sample blank’ is the absorbance of the sample only (sample without any DPPH solution).

# Results and discussion

## 3.1. Isolation and identification of fucoxanthin by HPLC-DAD

Identification and quantification of fucoxanthin from purified P-TLC band and from the crude extract of *H. elongata* was assessed by HPLC-DAD by comparing its UV-visible spectra and retention time (RT) to that of the authentic standard. Initially, the crude extract was loaded on preparative TLC (P-TLC) and most active band was isolated and collected (Rajauria & Abu-Ghannam, 2013). The P-TLC purified band, the crude lipophilic extract and commercial fucoxanthin standard were loaded on HPLC and the characteristic chromatograms of each were recorded from 200-700 nm wavelengths. Fig. 1 shows the HPLC chromatograms of commercial fucoxanthin, purified P-TLC band and the crude extract from *H. elongata* seaweed. A common peak was detected at 12.33 min (RT) in all the chromatograms. The UV-visible spectra of each common peak was recorded and projected besides the peaks (Fig. 1a-c). The characteristic UV-visible spectra extracted from the same peaks exhibited the absorption maxima (λmax) within the same region (λmax: 265, 332 and 448 nm), indicating that the purified compound is tentatively fucoxanthin (Fig. 1a-c). The recorded value of λmax is in agreement with Sangeetha et al. (2010) and Kim, Shang, & Um (2010). The purity of this sample, based on the calibration curve, was about 97%, indicating that the collected TLC band contained fucoxanthin as the major component. The purity of fucoxanthin recovered from P-TLC based purification was higher than the values reported by Noviendri et al. (2011) using silica column chromatography. Furthermore, the quantity of preparative TLC purified fucoxanthin, as determined by the calibration curve, was 18.6 mg/g dry weight sample. This value was much higher than the amount reported by Plaza et al. (2010) in the same seaweed species as well as Fung, Hamid & Lu, (2013) in *Undaria pinnatifida* brown seaweed. Moreover, compared to microalgae, the fucoxanthin content of *H. elongata* macroalgae is much higher than the content reported in *Phaeodactylum tricornutum*, *Chaetoceros gracilis*, *Nitzschia* *sp*. and *Isochrysis galbana* microalgal species (Kim et al., 2012a; 2012b). The fucoxanthin content varies throughout the growing season of seaweeds, harvesting location as well as different parts of the plant, which may explain the variations in the results among other studies (Mori et al., 2004; Fung, Hamid & Lu, 2013). For instance, *U. pinnatifida* species from New Zealand exhibited higher fucoxanthin content in the blade followed by sporophyll part compared to the same Japanese and Korean species (Fung, Hamid & Lu, 2013). Additionally, solvent type, extraction time, temperature, extraction and purification methods are known to significantly affect the content of fucoxanthin in algae (Kim et al., 2012a). The higher amount of fucoxanthin observed in the present study may have been due to the climatic and regional effects, which notably have an effect on the content of fucoxanthin in brown seaweed (Mori et al., 2004). In addition, the seaweed from which fucoxanthin was extracted, was harvested between the winter and spring which is reportedly favourable season for high fucoxanthin production in seaweed (Fung, Hamid & Lu, 2013).

## 3.2. Characterization of the purified fucoxanthin by LC-ESI-MS

Mass spectrometry with ESI gives a great deal of structural information of the bioactive compounds present in plant extracts. LC-ESI-MS ionization parameters have great effects on the component separation and its performance (in terms of fragmentation and sensitivity), in the analysis of the compounds of interest (Choi & Song, 2008). HPLC-DAD data indicated that RT and λmax of the purified compound were completely similar to that of the fucoxanthin standard. Therefore, for ESI-MS analysis, fucoxanthin reference compound was used to investigate the ionization behaviours and fragmentation pattern by varying the most important parameters such as fragmentor and capillary voltage using positive ion mode.

In the present study, by varying the fragmentor voltage (30-140V) and capillary (2.0-4.5kV) voltage, the most appropriate mass spectrum (*m/z*) and ion counts (abundance) useful for the detection of fucoxanthin were recorded (data not shown). Results revealed that among all the tested fragmentor voltages, the best mass spectrum was observed at 120V. However, in terms of ion counts, the highest intensity of ions was recorded at 30 and 140V, but a high signal to noise ratio was also observed at these fragmentor voltages. High fragmentor voltage increases the rate of fragmentation due to the initiation of collision in the region between the capillary and the first skimmer cone. However, high fragmentor voltage in some instances can dissociate the compounds and produce smaller ions of no significance (Choi & Song, 2008). Thus, in this study 120V was considered the most appropriate fragmentor voltage which not only produced the correct precursor ion [*m/z*, 659.3] but also generated enough ion counts, therefore was used for further optimization process. However, variations in the ion counts at tested capillary voltage were not as much as those recorded in the fragmentor voltage, yet, the most appropriate mass spectrum pattern and the highest ion intensity appeared at a capillary voltage of 3.5kV, therefore, it was chosen for the detection of fucoxanthin. Capillary voltage influences the transmission of the ions through the capillary sampling orifice and affects the fragmentation pattern of sample ions (Choi & Song, 2008). The optimization has been carried out by studying “one-variable-at-a-time” wherein the conditions of one parameter were changed while the others remained constant (Choi & Song, 2008).

The optimized parameters such as 120V fragmentor voltage and 3.5kV capillary voltage were selected for LC-ESI-MS analysis of purified compounds. The identification of the purified compound was carried out by comparing its retention time, characteristic UV-visible spectra and ESI-MS fragmentation data with that of the authentic standard. Results from Fig. 2a indicate that TIC chromatogram of the purified compound showed a strong signal at 14.892 min (RT) with a base peak at *m/z* 659 [M+H]+. Upon ESI-MS fragmentation using 10eV collision energy, the major fragments were produced at *m/z* 641 [M+H-18]+ and 581 [M+H-78]+ due to the loss of water (18 amu) and acetic acid (AcOH) along with water (78 amu) from the base peak ion (*m/z* 659) (Fig. 2b). These assignments are consistent with the ESI-MS fragmentation pattern of fucoxanthin standard and they are in agreement with the mass fragmentation data reported by de Quirós et al. (2010), Kim, Shang, & Um (2010) and Kim et al. (2011) wherein similar fragmented ions (*m*/*z* 659, 641, 581) were recorded for fucoxanthin. Based on the response of HPLC-DAD and ESI-MS fragmentation, the purified compound was confirmed as fucoxanthin.

## 3.3. Structure elucidation of the purified fucoxanthin using NMR

Because of an extended system of conjugated double bonds, fucoxanthin is structurally very unstable and it can easily deteriorate by heat, co-extractants, aerial exposure, oxygen, enzymes, light and illumination, therefore, the handling of fucoxanthin during extraction and purification is very important (Rivera & Canela, 2012; Piovan et al., 2013; Zhao et al., 2014). The instability of fucoxanthin can lead to isomerisation(*cis-trans*), oxidative cleavage and/or epoxidation of the backbone which may further limit its biological properties (Davey, Mellidou, & Keulemans, 2009; Piovan et al., 2013). Additionally, algal material often contains carotenoids or mixtures of carotenoids with similar structures that can interfere with the analytes of interest. Therefore, the isolation, identification, and quantification of these pigments are challenging. Thus, in order to confirm the structure of the purified compound, NMR spectroscopy was utilized as it is considered a useful tool for the investigation of the molecular composition of compounds. The results show that the 1H and 13C NMR spectra of the active compound revealed signals assignable to two quaternary geminal dimethyls and methyls of oxygen, four olefinic methyls, conjugated ketone and polyene having acetyl functionalities. The complete assignments of 1H and 13C NMR spectra are listed in Table 1. The NMR structural elucidation data identified that the purified compound is all-*trans*-fucoxanthin. These data matched well with previously published results (Yan et al., 1999; Mori et al., 2004; Heo et al., 2008; Kim, Shang, & Um, 2010). The physicochemical features outlined above suggested that the active compound was a carotenoid in which one of the hydroxyl groups was acetylated. The 13C and 1H NMR spectral data of the purified compound were identical with those of an authentic fucoxanthin standard, the purified active compound was confirmed as fucoxanthin.

## 3.4. Antioxidant activity determination of purified fucoxanthin

In order to test the biological activity, the purified fucoxanthin was screened for its antioxidant capacity using DPPH and FRAP assays. The commercial fucoxanthin was used as a reference standard to compare the results. Results from Table 2 show that the purified fucoxanthin exhibited statistically similar (p>0.05) antioxidant capacity against DPPH radicals compared to the commercial fucoxanthin. Both commercial fucoxanthin and purified fucoxanthin scavenged the DPPH radical in a dose-dependent manner, with an EC50 value of 13.4 µg/mL and 12.9 µg/mL, respectively (Table 2). However, in terms of reducing power, the purified compound showed statistically significant lower antioxidant activity (p<0.05) than the commercial standard. The purified fucoxanthin gave a FRAP value equivalent to 15.2 µg trolox whereas the commercial compounds exhibited 16.5 µg trolox equivalent (Table 2). It has been determined that the major active compounds in brown seaweed extracts analysed with FRAP and DPPH scavenging assays have been reported to be polyphenol and carotenoid pigments (de Quirós et al., 2010; Plaza et al., 2010; Airanthi et al., 2011). In solvent extraction approach, the extraction of targeted compounds is associated with the type of solvents. The low polarity solvents especially helps to extract lipophilic compounds (for instance, fucoxanthin in this case) which contributes majorly in its antioxidant properties. The higher FRAP value indicates that the antioxidant compounds are electron donors and are capable of reducing the oxidized intermediate during the lipid peroxidation process, thereby acting as primary and secondary antioxidants. Previous studies also confirmed that fucoxanthin efficiently quenches chemically-generated DPPH free radicals and shows strong radical scavenging capacity against them, however, the exact mechanism of action is still not clear (Nomura et al., 1997; Yan et al., 1999; Sachindra et al., 2007; Kim, Shang, & Um, 2010; Fung, Hamid & Lu, 2013).

Several studies have established that fucoxanthin is an important dietary nutrient with antioxidant property which is based on its ability to quench singlet oxygen as well as to trap free radicals (Sachindra et al., 2007; Stahl & Sies, 2012; Fung, Hamid & Lu, 2013; Zhang et al., 2015). Fucoxanthin is the most efficient singlet oxygen quencher and acts as an antioxidant under anoxic conditions whereas other carotenoids such as β-carotene and lutein have practically no quenching abilities (Nomura et al., 1997; D’Orazio et al., 2012). Furthermore, it has been reported that the antioxidant activity or pro-oxidant effect of fucoxanthin is one of the main mechanisms behind its anti-carcinogenic effect (Kim, Shang, & Um, 2010; Peng et al., 2011).

Unlike the other antioxidants which usually are proton donors, fucoxanthin donates electron to mitigate the free radicals. The unique structural back bone with high number of double bonds make fucoxanthin more suitable for quenching singlet oxygen as well as free radicals (D’Orazio et al., 2012). The high number of conjugated double bonds in the backbone increases its ability to scavenge free radicals or quench singlet oxygen (Sachindra et al., 2007). During singlet oxygen quenching, the excess energy of singlet oxygen transfers onto the conjugated double bond back bone of the fucoxanthin molecule. Due to which, fucoxanthin molecule reaches into excited state (triplet state), and upon losing the energy, it relaxes back to the ground state (singlet state). Furthermore, to act as a free-radical scavenger, these excited fucoxanthin molecules donate electrons or form an adduct with free radicals and mitigate them (Sachindra et al., 2007; Roehrs et al., 2011; D’Orazio et al., 2012).

A unique combination of such properties, which is rarely available among natural antioxidants, make fucoxanthin a potential candidate to prevent or manage cancer, lifestyle-related (such as obesity, diabetes mellitus) and other chronic diseases including Parkinson’s disease, atherosclerosis, acute myocardial infarction, Alzheimer’s disease, and chronic fatigue syndrome (Yan et al., 1999; Maeda et al., 2009; Kim, Shang, & Um, 2010; D’Orazio et al., 2012; Kim et al., 2013; Mikami & Hosokawa, 2013; Zhang et al., 2015). Although these diseases are very different, oxidative stress is a common cause amongst them, thus versatile effects of fucoxanthin could be investigated in prevention approaches (D’Orazio et al., 2012; Mikami & Hosokawa, 2013; Sangeetha, Hosokawa & Miyashita, 2013; Zhang et al., 2015).

# Conclusion

The present study explored the *H. elongata* brown seaweed as a potential source for fucoxanthin. The active compound was extracted from an equal-volume mixture of n-hexane, diethyl ether and chloroform, and the crude extract was purified from preparative TLC. The purified compound was identified and characterized by LC-ESI-MS and NMR spectroscopy and the data was compared with an authentic fucoxanthin standard. The amount of purified fucoxanthin extracted using solvent extraction approach is much higher than the amount previously reported in the same or other brown seaweed species. Conventional purification methods for carotenoids have included silica gel chromatography but preparative TLC as applied in the present study gave significantly higher yield which provides a new insight for the purification procedures of fucoxanthin. The results suggest that the identified fucoxanthin might be a major contributor to the antioxidant activities of *H. elongata* seaweed. Results of the present research may contribute to a rational basis for the use of marine seaweed extract or functional compounds in possible prevention of disease related to oxidative stress. Additionally, due to the high demand of fucoxanthin in food and pharmaceutical industry, present results may aid the commercial development of *H. elongata* brown seaweed for large-scale fucoxanthin production.

# Conflict of Interest

# The authors declare no conflicts of interest.

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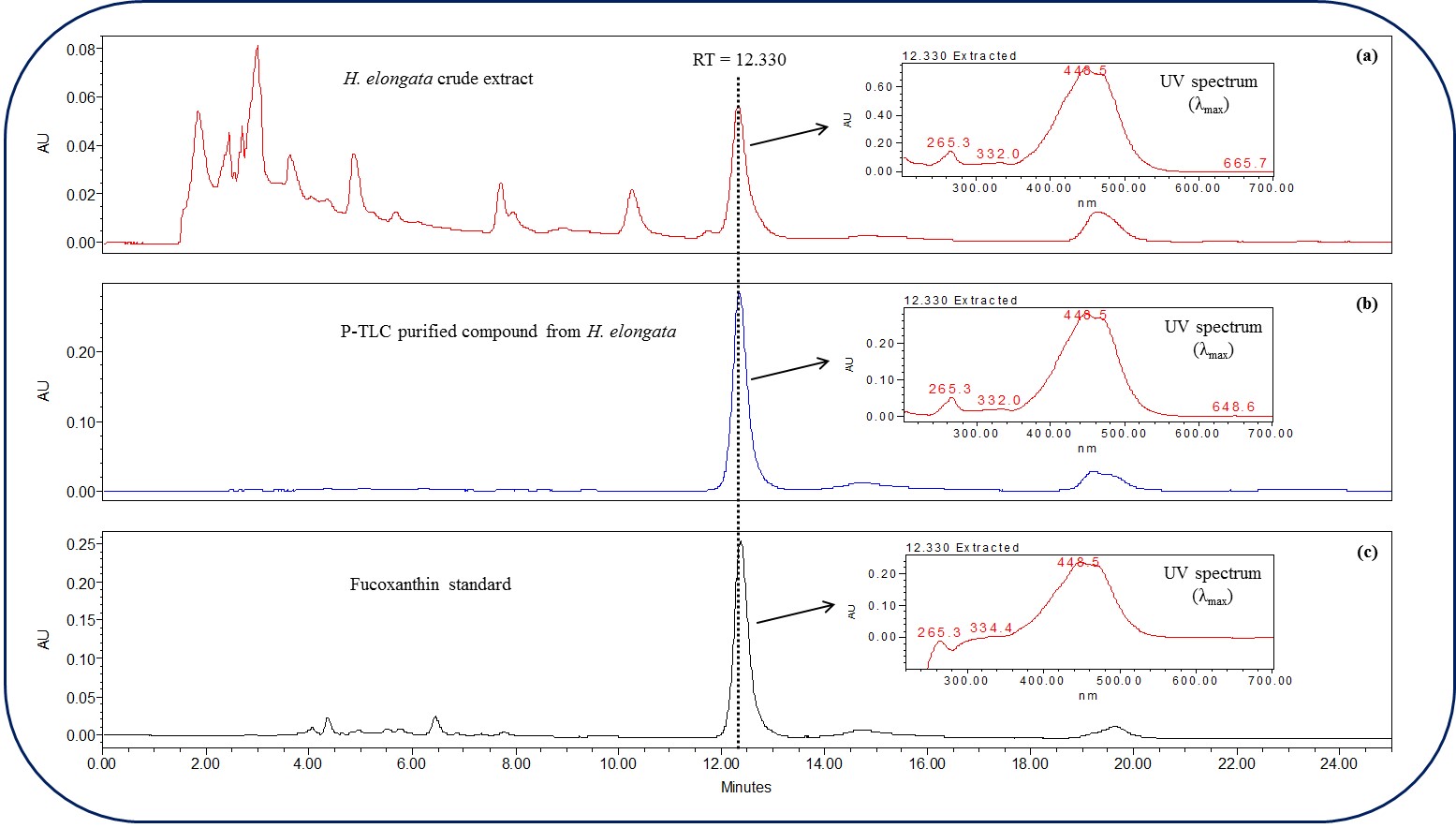
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**Figure captions**

**Fig. 1.** HPLC-DAD based identification of fucoxanthin in the crude extract and P-TLC (preparative thin layer chromatography) purified fraction of *H. elongata* seaweed

**Fig. 2.** LC-ESI-MS spectra (positive-ion mode) of purified fucoxanthin from *H. elongata* seaweed: (a) Structure of fucoxanthin and its TIC chromatogram in full scan mode from *m/z* 100 to 1000 amu; (b) ESI-MS spectrum of selected ion and its major fragments due to the loss of 18 and 78 amu



**Fig. 1.**



**Fig. 2.**

**Table 1.** Proton (1H) and carbon (13C) NMR spectral data of purified fucoxanthin recorded at room temperature at 400 MHz and 100 MHz respectively, in *d*-acetone

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Position | 13C | 1H | Position | 13C | 1H |
|  | δ (ppm) | |  | δ (ppm) | |
| 1 | 35.6 |  | 1´ | 34.9 |  |
| 2 | 47.8 | 1.34 (1H, dd, J = 8.7, 14.2)  1.49 (1H, dd, J = 14.2) | 2´ | 45.8 | 1.42 (1H, dd, J = 10.4, 14.9)  2.04 (1H, dd, J = 2.9, 14.9) |
| 3 | 63.7 | 3.84 (1H, m) | 3´ | 67.9 | 5.41 (1H, tt, J = 8.8, 12.0) |
| 4 | 41.8 | 1.78 (1H, dd, J = 8.7, 14.2)  2.31 (1H, dd, J = 2.9, 17.8) | 4´ | 45.4 | 1.55 (1H, dd, J = 10.4, 14.9)  2.31 (1H, dd, J = 2.9, 17.8) |
| 5 | 66.1 |  | 5´ | 72.1 |  |
| 6 | 65.8 |  | 6´ | 117.1 |  |
| 7 | 41.1 | 2.60, 3.69 (2H, d, 20.4) | 7´ | 202.3 |  |
| 8 | 197.7 |  | 8´ | 102.5 | 6.01 (1H, s) |
| 9 | 134.8 |  | 9´ | 132.7 |  |
| 10 | 139.1 | 7.18 (1H, d, J = 12.8) | 10´ | 128.8 | 6.14 (1H, d, J = 11.6) |
| 11 | 124.2 | 6.61 (1H, m) | 11´ | 125.9 | 6.69 (1H, t, J =12.0) |
| 12 | 144.8 | 6.69 (1H, t, J = 12.8) | 12´ | 137.2 | 6.31 (1H, d, J = 11.6) |
| 13 | 136.0 |  | 13´ | 137.9 |  |
| 14 | 136.5 | 6.45 (1H, d, J = 11.6) | 14´ | 132.5 | 6.21 (1H, d, J = 11.6) |
| 15 | 129.3 | 6.69 (1H, m) | 15´ | 132.9 | 6.78 (1H, t, J = 12.0, 14.2) |
| 16 | 26.4 | 1.09 (3H, s) | 16´ | 28.7 | 1.41 (3H, s) |
| 17 | 27.2 | 0.99 (3H, s) | 17´ | 31.1 | 1.10 (3H, s) |
| 18 | 20.5 | 1.24 (3H, s) | 18´ | 32.2 | 1.38 (3H, s) |
| 19 | 11.5 | 1.95 (3H, s) | 19´ | 14.2 | 1.80 (3H, s) |
| 20 | 12.5 | 1.99 (3H, s) | 20´ | 12.7 | 2.01 (3H, s) |
|  |  |  | 21´OAc, C=O | 170.4 |  |
|  |  |  | 22´OAc, CH3 | 21.2 | 2.08 (3H, s) |

*J: calculated in Hz*

Table 2. Antioxidant capacity of commercial and purified fucoxanthin from *H. elongata* seaweed

|  |  |  |
| --- | --- | --- |
|  | Antioxidant capacity | |
|  | DPPH [EC50;µg/mL] | FRAP [µg trolox equivalent] |
| Purified fucoxanthin | 12.9 ± 1.04a | 15.2 ± 1.21a |
| Fucoxanthin standard | 13.4 ± 1.08a | 16.5 ± 1.35b |

*Values are expressed as average of three replicates.*

*Values with different letters (a–b) in each column are significantly different (p<0.05).*

*Antioxidant capacity was determined by DPPH****˙*** *scavenging and FRAP (Ferric Reducing Antioxidant Power) assays*