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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 (^{1}H and ^{13}C). 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 $[M + H\text{-}H2O]^{+}$) 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 (EC_{50}: 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.

1. 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, Yuan, Wu, & Wang, 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, Yeum, Niki, & Russell, 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, Okada, Mikami, Konishi, & Miyashita, 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, Baskaran, Tsuzuki, & Nagao, 2002; Maoka, Fujiwara, Hashimoto, & Akimoto, 2007; Heo et al., 2008; D'Orazio et al., 2012; Mikami & Hosokawa, 2013; Sangeetha et al., 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, Hosokawa, Sashima, Murakami-Funayama, & Miyashita, 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

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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, Chuda, Suzuki, & Nagata, 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 et al., 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, Frecha-Ferreiro, Vidal-Perez, & López-Hernández, 2010; Plaza et al., 2010; Rajauria, Jaiswal, Abu-Ghannam, & Gupta, 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.

2. 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 9168 ×*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 30 min 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 9168 ×*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 4.5 kV) and fragmentor voltage (30, 50, 70, 100, 120 and 140 V) were optimized. The final operating conditions selected were: positive ionization mode, capillary voltage 3.5 kV, fragmentor voltage 120 V and collision energy 10 eV.

2.5. Nuclear magnetic resonance (NMR) analysis

Proton (^{1}H NMR) and carbon (^{13}C 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 32 K 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 9168 ×*g* for 15 min in order to remove the silica. The supernatant was collected and filtered