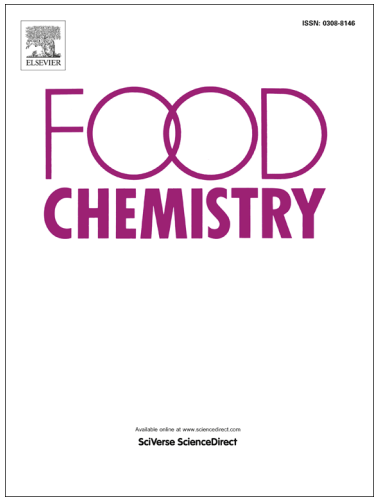

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PII: S0308-8146(17)31541-8
DOI: http://dx.doi.org/10.1016/j.foodchem.2017.09.075
Reference: FOCH 21744

To appear in: *Food Chemistry*

Received Date: 8 June 2017
Revised Date: 11 September 2017
Accepted Date: 14 September 2017

Please cite this article as: Wang, N., Manabe, Y., Sugawara, T., Paul, N.A., Zhao, J., Identification and biological activities of carotenoids from the freshwater alga *Oedogonium intermedium*, *Food Chemistry* (2017), doi: http://dx.doi.org/10.1016/j.foodchem.2017.09.075

Identification and biological activities of carotenoids from the freshwater alga *Oedogonium intermedium*

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Running title: Carotenoids from the freshwater alga *Oedogonium intermedium*

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Abstract

The chemical and biological properties of carotenoids in the freshwater alga *Oedogonium intermedium* were investigated in this study. Carotenoids were extracted from the alga by dichloromethane and purified by saponification. The carotenoid content was determined both spectrometrically and by HPLC, the carotenoids identified by HPLC-PDA-APCI-IT-TOF-MS and the extracts analysed for several health-related bioactivities. The crude and saponified extracts contained 3,411.2±20.7 and 2,929.6±5.9 µg carotenoids/g dry algal biomass, respectively. Seven major carotenoids were identified, namely neoxanthin, 9'-*cis*-neoxanthin, loroxanthin, violaxanthin, lutein, α-carotene and β-carotene, which were present in similar amounts in the alga. Both the crude and saponified carotenoid extracts exhibited significant antioxidant activities as well as potent inhibitory effects against several metabolically important enzymes including α-amylase, α-glucosidase, pancreatic lipase and hyaluronidase, but they were poor inhibitors of angiotensin converting enzyme (ACE). *Oedogonium* could be an important new source of carotenoids, specifically loroxanthin, which is lacking in terrestrial plants.

Keywords: Algae, *Oedogonium*, carotenoids, biological activities, LC-MS.

1. Introduction

Carotenoids are an important group of natural pigments that are widely distributed in nature. More than 700 different carotenoids have been identified, mostly in plants but also in animals, microorganisms and algae (Saini, Nile & Park, 2015). Carotenoids are broadly divided into carotenes and xanthophylls with the former being tetra-terpenoid hydrocarbons that are made only of C and H, while the latter being oxidised carotenoids that contain some *O*-substituent groups such as hydroxyl, keto and epoxy groups (Saini *et al*, 2015). Carotenoids are excellent scavengers of singlet oxygen in plants and, thus, protect cellular components, such as chlorophylls, lipids, proteins and DNA, from oxidative damage (Raposo, De Morais & De Morais, 2015). Carotenoids are also shown to exhibit protective effects against several human health disorders such as certain cancers and eye conditions (*e.g.*, cataracts/macular) and cardiovascular disease, and improve the function of the immune system, skin texture and gap-junction communication (Raposo *et al*., 2015). These properties have formed the basis for their wide application not only as food colorants, but increasingly also as functional ingredients in foods, nutraceuticals, cosmetics and other products. However, to date, the vast majority of research on carotenoids is concerned with those from fruit and vegetables, while other important sources of carotenoids, such as algae, have received much less attention.

Compared to terrestrial plants, algae offer a number of advantages as a source of carotenoids, including fast growth rates, year-round availability and low or potentially positive impact on the environment (Ahmed, Fanning, Netzel, Turner, Li & Schenk, 2014; Lawton, Cole, Roberts, Paul & de Nys, in press). Furthermore, many algal carotenoids are lacking in terrestrial plants (Sugawara, Ganesan, Manabe & Hirate, 2014). Commercial production and extraction of carotenoids from algae already exists,

although it is limited to a few species of microalgae. For example, β-carotene from *Dunaliella salina* and astaxanthin from *Haematococcus pluvialis* are extracted for use as food colourants (Hu, Lin, Lu, Chou &Yang, 2008), while *Muriellopsis* has been used commercially to produce lutein due to its high lutein content and high growth rate (Lin, Lee & Chang, 2015). Recently, there has been a growing interest in sourcing carotenoids from marine macroalgae (seaweed), including siphonaxanthin from the green seaweed *Codium fragile* and fucoxanthin from the brown seaweed *Undaria pinnatifida* (Sugawara *et al.*, 2014, Kotake-Nara, Asai & Nagao, 2005). While these studies have focused on marine algae, freshwater algae, such as *Oedogonium*, represent another important source of carotenoids that is yet to be explored.

Oedogonium is a genus that grows in freshwater and has recently been domesticated for intensive aquaculture (Lawton *et al.*, in press). Research on this group of freshwater macroalgae, done at commercial scales, has shown that *Oedogonium* can be used in wastewater treatment for aquaculture, as a bio-sorbent of metals and as a fertiliser and soil conditioner. It has also been analysed as a feedstock biomass for bioenergy applications since it has a high bio-crude yield compared with many other algal species (Cole *et al.*, 2016). Proximate, biochemical and ultimate analyses of the dry biomass of *Oedogonium* has revealed a profile of carbohydrate content of ~40%, lipid 10%, protein 30% and mineral content 20% (Neveux *et al.*, 2014). In addition to bioenergy applications, there is also potential for the development of this alternative protein as a higher value commodity for animal feeds. However, to date, there has been no research to investigate its bioactive components and related biological activities. The objective of this study was to investigate the carotenoid content, composition and health-related biological activities of the green alga *Oedogonium*

intermedium cultured under intensive conditions. Carotenoid content of the alga was determined using both spectrophotometric and HPLC methods; carotenoids were identified by HPLC and LC-MS and the carotenoid extracts were analysed for their antioxidant capacities as well as *in vitro* inhibitory activities against several metabolically important enzymes including α-amylase α-glucosidase, pancreatic lipase, hyaluronidase and ACE.

2. Materials and methods

2.1 Chemicals and reagents

4-Methylumbelliferyl oleate (4-MUO), 4-nitrophenyl-a-D-glucopyranoside (*p*-NPG), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), acarbose, ACE from rabbit lung, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), DPPH (2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl), hyaluronic acid sodium salt from *Streptococcus equi*, hyaluronidase from bovine testes, pancreatic lipase from *Candida rugose,* N-Hippuryl-his-Leu (HHL), sodium citrate, sodium potassium tartrate tetrahydrate, α-amylase from *Bacillus subtilis* (380U/mg), gallic acid, α-glucosidase from *Saccharomyces cerevisiae* (100U/mg), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) and trolox were purchased from Sigma-Aldrich (Sydney, Australia). Acetic acid, acetone, acetonitrile, 3,5-dinitrosalicylic acid, ammonium acetate, boric acid, citric acid, hexane, hydrochloride acid (32%), iron (III) chloride hexahydrate, methanol, potassium carbonate, potassium persulfate, sodium chloride, sodium phosphate diabasic, sodium phosphate monobasic and potato starch were purchased from Ajax Finechem Pty Ltd (Sydney, Australia). 4-Dimethylaminobenzaldehyde (DMAB) was purchased from BDH Chemicals Ltd (Poole, UK), dichloromethane from Merck (Sydney, Australia), methyl-tert-butyl ether (MTBE) from Fisher Scientific (Sydney,

Australia), orlistat from Tokyo Chemical Industry (Tokyo, Japan) and potassium hydroxide and sodium hydroxide from Chem-Supply Pty Ltd (Sydney, Australia). All chemicals were of at least analytical grade unless stated otherwise. Solvents used in HPLC were of liquid chromatography grade and water used in all experiments was purified by reverse osmosis using the MilliQ RO system (referred to as MilliQ water).

2.2 Seaweed sample collection and storage

The freshwater alga *Oedogonium intermedium* (hereafter *Oedogonium*) was harvested in December 2012 from stock cultures grown in outdoor tanks at James Cook University (JCU), Townsville, Queensland, Australia. *Oedogonium,* a filamentous macroalga that naturally grows under eutrophic conditions in streams and drainage ditches, was cultured in two 10,000 L parabolic tanks, using de-chlorinated tap water, enriched with Guillard's f/2 nutrient medium to 1 g/L, as described in Cole, Mata, Paul and de Nys (2014). Monocultures of *Oedogonium* were maintained in open system throughout the sampling period and verified by visual inspection and microscopy. Biomass was harvested weekly, using large hand nets or pumped through nylon bags, to maintain the stocking density at 0.5 g fresh weight/L. Use of a coarse filter to harvest the *Oedogonium* ensured that any microalgae or microbes from the water column were not collected in the sample. The biomass was centrifuged and freeze-dried (Labogene ScanVac Coolsafe 110-4 Pro Freeze Dryer, Lynge, Denmark) for 48 h. The freeze-dried biomass was vacuum packed immediately in plastic bags and air freighted to the University of New South Wales, Sydney, Australia, where they were stored at -80°C until use.

2.3 Extraction of carotenoids from seaweeds

The extraction procedure was a modified Bligh-Dyer method (Rao, Baskaran, Sarada & Ravishankar, 2013). Briefly, 15 mg of freeze-dried samples was mixed with 1.2 mL of MilliQ water, 3 mL of methanol and 1.5 mL of dichloromethane, followed by vigorous mixing. After 10 min incubation at room temperature, the mixture was added with 1.5 mL each of distilled water and dichloromethane, and was mixed vigorously again. The organic layer was separated by centrifugation (10 min at 3,500 g) and collected. The extraction step was repeated twice more using 3 mL of dichloromethane each time. The solvent was dried with nitrogen (N_2) gas, and the resultant extract was dissolved in 1 mL of methanol or DMSO for further analysis. The crude extract was then saponified with 10% methanolic KOH and kept overnight at room temperature in the dark; after which, the carotenoid extract was washed with water to remove the alkali, and dried over N_2 gas. Precautions were taken to minimise potential loss of carotenoids due to light, heat and oxidation. These precautions included conducting the extraction procedures under dim lighting, use of low temperatures, and evaporation of solvents with N_2 streams where applicable and performing each operation in the shortest possible times.

2.4 Determination of total carotenoids

Determination of total carotenoids was carried out spectrophotometrically according to Lichtenthaler (1987). The dried extract (15 mg dry algal biomass weight equivalent) was re-dissolved in 5 mL of methanol, and the absorbance of the extracts was measured at 470, 665.2 and 652.4 nm by a Spectramax Plus M2 spectrophotometer (Molecular Devices, Australia). The pigment contents (chlorophyll a and chlorophyll b and total carotenoids) were calculated using the Lichtenthaler equations (Lichtenthaler, 1987):

$$C_{chlorophyll\ a} = 16.72A_{665.2} - 9.16A_{652.4}$$

$$C_{chlorophyll\ b} = 34.09A_{652.4} - 15.28A_{665.2}$$

$$C_{carotenoid} = \frac{1000A_{470} - 1.63C_{chlorophyll\ a} - 104.96C_{chlorophyll\ b}}{221}$$

2.5 Identification and quantification of carotenoids

2.5.1 HPLC-PDA-APCI-IT-TOF-MS analysis of carotenoids

The analysis was performed using a Prominence LC system (Shimadzu, Kyoto, Japan) connected to a PDA detector (SPM-M20A, Shimadzu, Japan) followed by an ion trap-time of flight mass spectrometer (LCMS-IT-TOF, Shimadzu, Japan) equipped with an atmospheric pressure chemical ionisation (APCI) source. The extract was separated on a TSK gel ODS-80TsQA column (2.0 × 250 mm, 5µm, Tosoh, Tokyo, Japan) at 40°C. The injection volume was 5 µL and flow rate was 0.2 mL/min. The mobile phase consisted of methanol/water (90/10, v/v) containing 0.1% of ammonium acetate (A) and methanol/ethyl acetate (70/30, v/v) containing 0.1% of ammonium acetate (B). The gradient elution was performed as follows: 0 to 5 min 0% B; 5 to 20 min, 0 to 100% B linear, 20 to 35 min, 100% B; 35 to 40 min, 100 to 0% B linear; 40 to 45 min, 0% B. To quantify the carotenoids, absorbance at 450 nm was monitored. Mass spectra were recorded in positive ion mode with a detection voltage of 1.7 kV, APCI temperature of 400°C, curved desolation line of 200°C and block temperature of 200°C. Carotenoids were identified by comparing their retention times and mass spectra with those of reference standards and the mass spectra published in the literature.

2.5.2 HPLC-PDA quantification of carotenoids

Quantification of the detected carotenoids was carried out by HPLC-PDA using the external standard method by constructing standard curves of reference carotenoids. Due to the high costs of neoxanthin, loroxanthin and α-carotene, these three carotenoids were quantified based on the standard curves of 9'-*cis*-neoxanthin, lutein and β-carotene, respectively, because they have similar absorption spectra.

2.6 Assays of antioxidant capacity

The ABTS radical scavenging, DPPH radical scavenging and the ferric reducing antioxidant power (FRAP) assays were conducted as previously described (Magnusson, Mata, Wang, Zhao, de Nys & Paul, 2015). The results of ABTS radical scavenging and FRAP assays are expressed in mg Trolox equivalents per 100 g dry weight (mg TE/g DW), and those of DPPH radical scavenging assay as mg gallic acid equivalents (GAE)/g DW.

2.7 Assays of inhibitory activity against metabolically important enzymes

2.7.1 *In vitro* pancreatic lipase inhibition assay

The inhibition of pancreatic lipase activity was determined following the method described by Sakulnarmrat and Konczak (2012) with slight modifications. Initially, stock solutions of the sample extracts were prepared at a concentration of 15 mg dry algal biomass weight equivalent/mL (henceforth expressed as dry weight equivalent) in DMSO, and working sample solutions were prepared by diluting the stock extract solutions in DMSO to 10, 5 and 1 mg dry weight equivalent/mL. Porcine pancreatic lipase (65 mg/mL in Mcllvaine's buffer, pH 7.4) and 0.1 mM 4-MUO in DMSO was used as the enzyme and fluorogenic substrate, respectively. Prior to use, the enzyme solution was centrifuged at 3,500g for 10 min and the supernatant collected. For the

assay, a mixture of 100 µL of the substrate and 50 µL of sample solution, orlistat (positive control) or solvent blank (negative control) in DMSO was incubated at 37°C for 5 min, followed by addition of 50 µL of the enzyme solution. After reaction at 37°C for 20 min, 1 mL of 0.1N HCl was added to terminate the reaction followed by the addition of 2 mL of 0.1 M sodium citrate. The amount of 4-methylumbelliferone released by the pancreatic lipase was measured fluorometrically at an emission wavelength of 460 nm and excitation wavelength of 320 nm using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA). Orlistat was used to plot a standard calibration curve with R^2=0.957. The pancreatic lipase inhibition activity was calculated using the following formula:

$$\%\ inhibition = 1 - \frac{(F_S - F_{SB})}{(F_C - F_{CB})} \times 100$$

Where F_S and F_C were the values of sample and negative control measured fluorometrically, and F_{SB} and F_{CB} were the fluorescence readings of sample and control blanks, respectively.

2.7.2 *In vitro* α-amylase inhibition assay

This activity was determined as previously described by Lordan, Smyth, Soler-Vila, Stanton and Rose (2013). Briefly, equal volumes (100 µL) of sample extract (1, 5, 10 and 15 mg dry weight equivalent/mL) and 1% (w/v) starch solution were incubated in Eppendorf tubes at 25°C for 10 min. A volume of 100 µL of α-amylase (0.5 mg/mL) was added to each tube and the reaction mixtures were incubated at 25°C for a further 10 min. The reaction was stopped with the addition of 200 µL of 3,5-dinitrosalicyclic acid colour reagent solution and incubation at 100°C for 5 min. Once the mixtures had cooled to room temperature, 50 µL was removed from each tube and transferred to the

wells of 96-well microplates. The reaction mixture was diluted by adding 200 µL of MilliQ water to each well and absorbance was measured at 540 nm. The pharmacological inhibitor, acarbose, was included as a positive control and a standard calibration curve was plotted with R^2=0.958. Blank readings (no enzyme) were substrate from each well and results were compared to the control. The activity of amylase inhibition was calculated as follows:

$$\%\ inhibition = \frac{(A_C - A_{CB}) - (A_S - A_{SB})}{(A_C - A_{CB})} \times 100$$

Where A_C and A_S were the absorbance readings of the control and sample, while A_{CB} and A_{SB} were the readings of the sample and control blanks, respectively.

2.7.3 *In vitro* α-glucosidase inhibition assay

This activity was determined following the method described by Rengasamy, Aderogba, Amoo, Stirk and Van Staden (2013). Briefly, α-glucosidase (0.1 U/mL) was dissolved in 0.1 M potassium phosphate buffer (pH 6.8) which was used as the enzyme solution. The substrate, 0.375 mM of *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG), was prepared in the same buffer and the sample extracts were dissolved in DMSO at the concentration of 1, 5, 10 and 15 mg dry weight equivalent/mL. Aliquots of (30 µL) each sample or acarbose (positive control) were mixed with the enzyme solution (30 µL) in 96 well microplates and the reaction was initiated by adding 60 µL of the substrate. The reaction mixture was incubated at 37°C for 40 min in darkness; after that, 120 µL of 0.2 M sodium carbonate in 0.1 M potassium phosphate buffer (pH 6.8) was added to each well to stop the reaction. The amount of *p*-nitrophenol (*p*NP) released was quantified using a 96-well microplate reader at 405 nm. A

standard calibration curve of acarbose was plotted with R^2=0.941. The percentage inhibition (%) of α-glucosidase was calculated as follows:

$$\%\ inhibition = \frac{A_C - A_S}{A_C} \times 100$$

Where A_C and A_S were the absorbance readings of the control and sample, respectively.

2.7.4 Hyaluronidase inhibition assay

The colorimetric Morgan-Elson assay was used to determine the inhibitory activity of algal extracts against hyaluronidase following the procedure described by Muckenschnabel, Bernhardt, Spruss and Buschauer (1998) with some modifications. The extracts at 5, 10, 15 and 30 mg dry weight equivalent/mL or quercetin (0.1 mg/mL, positive control) dissolved in DMSO (27 µL), were mixed with a reagent mixture that contained 300 µL of citrate-phosphate buffer (pH 5.0) (0.1 M Na_2HPO_4/0.1M NaCl and 0.1M citric acid/0.1M NaCl mixed in appropriate portions to arrive at pH 5.0), 75 µL BSA solution (0.2 mg BSA/mL water), 75 µL substrate hyaluronic acid (HA) solution (5 mg/mL in water), 129 µL MilliQ water and 75 µL of the enzyme solution (100U). The mixture was incubated at 37°C for 1 h. After that, the enzymatic reaction was stopped by the addition of 160 µL of alkaline borate solution and heating for 4.5 min in a boiling water bath. The alkaline borate solution was prepared by combining 10 volumes of borate solution (2.8 M H_3BO_4/1.4 M KOH) and 1 volume of 5.8 M potassium carbonate solution. After cooling on ice for 2 min, 90 µL of the reaction mixture was transferred to a 96-well microplate and mixed with 110 µL of Ehrlich's reagent. The mixture was incubated at 37°C for 20 min, and the absorbance of the coloured product was measured at 590 nm.

Inhibition of the enzyme activity was calculated according to the equation:

$$\text{Enzyme activity } \% = \frac{(A_S - A_{SB})}{(A_C - A_{CB})} \times 100$$

Where A_S was the absorbance of the incubation mixture containing the extract, A_C was the absorbance without the extract (extract solution replaced with DMSO); A_{SB} was the absorbance of the incubation mixture containing the inhibitor without the enzyme (enzyme solution replaced with buffer) and A_{CB} was that in the absence of both the enzyme and extract (enzyme solution replaced with the buffer and extract solution replaced with DMSO).

2.7.5 ACE inhibition assay

ACE inhibitory activity of the algal carotenoid extracts was assayed according to the method of Boschin, Scigliuolo, Resta and Arnoldi (2014) using HHL as the substrate, freshly prepared daily, and HPLC-PDA to detect hippuric acid (HA), the product of the enzymatic reaction. A volume of 100 µL of 2.5 mM HHL in 100 mM Tris buffer (containing 300 nM NaCl, pH 8.3) was mixed with 30 µL of the sample extract (10 mg dry weight equivalent/mL). The mixture was pre-incubated at 37°C for 15 min, and then 15 µL of ACE solution, corresponding to 3 mU of enzyme in the same buffer, was added. The mixture was incubated for 60 min at 37°C, and the reaction was stopped with the addition of 125 µL of 0.1 M HCl. The reaction mixture was extracted twice, each with 600 µL of ethyl acetate. The solvents were combined and evaporated under nitrogen gas, and the resultant residue was dissolved in 500 µL of the same buffer and analysed by HPLC.

Hippuric acid (HA) was detected on a HPLC system with a Luna C18 column (250 x 4.6 mm i.d., 5µ) column (Phenomenex, Sydney, Australia). MilliQ water and acetonitrile were used as eluents with the following gradient program: 0 min 5% acetonitrile, 10 min 60% acetonitrile, 12 min 60% acetonitrile, 15 min 5% acetonitrile. The flow rate was fixed at 0.5 mL/min. The injection volume was 10 µL and peaks were monitored at 228 nm. The retention time of hippuric acid (HA) was 4.2 min. The detector response for standard HA was linear in the range from 1-100 µg/mL with R^2 = 0.999.

2.8 Statistical analysis

The experiments were repeated twice and each analysis was performed usually in triplicate. The results were presented as mean ± standard deviation (SD). One way analysis of variance (ANOVA) was carried out using SPSS version 22 (IBM, USA) to determine whether significant differences exist among treatments in each of the bioactive assays and Duncan triplicates range test was used to separate significant differences between the means at the 5% level. A p-value <0.05 was considered as statistically significant.

Results and discussion

3.1 Total carotenoid content

The crude and saponified extracts of *Oedogonium* were analysed for total carotenoids and chlorophylls (**Table 1**). The amounts of chlorophyll a and b in the saponified extract were significantly lower ($p<0.05$) than in the crude extract; however, the total carotenoids in both extracts were similar, only slightly more in the crude extract (2.9 vs 3.4% w/w). This demonstrated that saponification was effective for the removal of chlorophylls from the carotenoid extracts. This is not surprising as it is well

established that chlorophylls can easily be hydrolysed under the alkaline conditions of saponification (Bijttebier, D'hondt, Noten, Hermans, Apers & Voorspoels, 2014). Compared to the remarkable loss of chlorophylls with saponification, the majority of carotenoids were stable with this treatment as over 85% of the yield in the crude extract was retained. Loss of some carotenoids during saponification is typical; for example, Divya, Puthusseri and Neelwarne (2012) observed a loss of 20-30% of β-carotene and over 50% of other carotenes during saponification of coriander extracts.

3.2 Identification and quantification of carotenoids

3.2.1 HPLC-PDA-APCI-IT-TOF-MS identification of carotenoids

Figure 1 shows the HPLC-PDA chromatogram of carotenoids in the saponified extract of *Oedogonium*. The peaks were first tentatively identified by comparing their UV-Vis absorption spectra with those of the respective reference standards. Confirmation of the chemical structures of the peaks was made by comparison of their mass spectra with those of reference standards as well mass spectrometric data published in the literature. For example, peak 4 was initially identified as violaxanthin, based on comparison of its retention time and UV absorption spectra with the reference standard. The UV-Vis absorption spectral characteristics of peak 4, i.e., λ_{max} at 326, 410, 434 and 465 nm, were virtually identical to those of the reference standard violaxanthin and also agreed to those reported by Inbaraj, Chien and Chen (2006) for the same compound. The mass spectra of peak 4 and violaxanthin reference standard were identical with characteristic molecular and fragment ions $[M+H]^+$ m/z 601, $[M+H-18]^+$ m/z 583, $[M+H-18-18]^+$ m/z 565 and $[M+H-18-18-56]^+$ m/z 509, which were the same as those reported by Inbaraj *et al*. (2006) and Kao, Seo and Kim (2011) for violaxanthin.

Table 2 presents the comparison of UV-Vis absorption spectral λ_{max} and MS spectral data of the peaks with those of reference standards and those reported in literature. Using these techniques, detectable carotenoids were identified and confirmed to be comprised of neoxanthin (peak 1), 9'-*cis*-neoxanthin (peak 2), loroxanthin (peak 3), violaxanthin (peak 4), lutein (peak 5), α-carotene (peak 6) and β-carotene (peak 7).

There is no published information on the carotenoids of *Oedogonium* and, thus, this is the first report on the carotenoids of this alga and, indeed, any freshwater green macroalga from Australia. Analysis of carotenoids has been done on a few other green algal species including *Chlorococcum humicola* (Bhagavathy and Sumathi, 2012), *Botryococcus braunni* (Rao *et al.*, 2013) and *Dunaliella salina* (Hu *et al.*, 2008), using similar analytical techniques. The carotenoids found in *Oedogonium* were similar to those in the literature with the major compounds being violaxanthin, lutein, α-carotene, β-carotene, astaxanthin and zeaxanthin. However, loroxanthin was found in *Oedogonium*, which was not reported for those other algae. Another major difference appears to be the relative levels of the various carotenoids present, which could be related to taxonomic variation and environmental factors.

3.2.2 Quantification of carotenoids in *Oedogonium*

The identified carotenoids were quantified using the external standard method and the results are shown in **Table 3**. The total carotenoid content obtained by HPLC (2,257.4 µg/g) was lower than that by the spectrophotometric method (2,929.6 µg/g), equating to a ~25% reduction. It has been reported that spectrophotometric methods tend to overestimate the carotenoid content when compared to HPLC quantification. This is mainly due to the former measuring not just carotenoids, but also certain impurities such as carotenoid degradation products and also chlorophyll degradation products,

e.g., chlorophyllides, which also absorb UV-Vis light at similar wavelengths (Kimura, Kobori, Rodriguez & Nestel, 2007). The extraction method used in the study would extract other lipophilic substances and this is the reason why a saponification step was included. Saponification removed most of the non-carotenoids although some unsaponifiable products such as sterols and terpenoids may remain. However, the amount of sterols and terpenoids in the extract would be very small because, as shown in the HPLC chromatogram of the saponified extract (Fig. 1), all the major peaks have been identified as being either carotenoids or chlorophylls. As both sterols and terpenoids had significant UV absorption, they would appear as peaks in the chromatogram if they were present in appreciable amounts. The fact that they were not demonstrated that their presence in the extract, if at all, would be in very low concentrations.

Oedogonium contained seven major carotenoids, with several of them present in similar amounts. The most abundant compound was lutein (18.4% of the sum of all seven carotenoids), followed closely by violaxanthin (18.1%) and 9'-*cis*-neoxanthin (17.2%). Neoxanthin (15.5%), β-carotene (13.7%) and loroxanthin (13.5%) were also present in appreciable amounts, while α-carotene (3.7%) was the least abundant carotenoid. *Oedogonium* contained an appreciable amount of loroxanthin (298.2 µg/g), which, to date, has only been found in a couple of algal species, such as *Scenedesmus obliquus* and *Chlorella vulgaris* (Aitzetmuller, Strain, Svec, Grandolfo & Katz, 1969).

Carotenoids have been shown to have a preventive effect against a number of diseases, including arteriosclerosis, cardiovascular disease, cancer (liver, medulla and prostate tumours), macular degeneration and neurodegenerative disorders like Alzheimer's disease (Raposo *et al.*, 2015). For example, lutein and its isomer zeaxanthin play a

vital role in the maintenance of normal visual function of the human macula, (Koushan, Rusovici, Li, Ferguson & Chalam, 2013). Violaxanthin exhibits a strong anti-proliferative activity on a human mammary cancer cell line (Pasquet *et al.*, 2011) and β-carotene is a well-known antioxidant that is believed to mitigate the harmful effects of free radicals implicated in various disorders such as many forms of gastrointestinal cancer and lung cancer (Goralczyk, 2009) and may also help prevent the symptoms of Alzheimer's disease through the inhibition of amyloid beta formation, deposition and fibril formation either by reducing the levels of p35 or inhibiting corresponding enzymes (Obulesu, Dowlathabad & Bramhachari, 2011). 9'-*cis*-neoxanthin can induce apoptosis through caspase-3 activation in PC-3 human prostate cancer cells (Kotake-Nara *et al.*, 2005). The presence of these carotenoids in *Oedogonium* in appreciable quantities implies that the alga has the potential to be developed into bioactive ingredients for functional food, nutraceuticals and pharmaceuticals.

3.3 Antioxidant activities of carotenoid extracts of Oedogonium

Antioxidants have a positive effect on human health as they can protect the human body from damage by reactive oxygen species, which attack macromolecules such as membrane lipids, proteins and DNA, leading to many health disorders such as cancer, diabetes mellitus, aging and neurodegenerative diseases (Le Tutour, Benslimance, Gouleau, Gouygou, Saadan & Quemeneur, 1998). Although carotenoids are widely used as colorants, they also play an important role as antioxidants. In this study, the antioxidant activities of crude and saponified extracts of *Oedogonium* were assessed using three different *in vitro* assays, ABTS scavenging capacity, DPPH scavenging capacity and FRAP (ferric reducing capacity) assays.

Figure 2 shows the ABTS, DPPH radical scavenging and FRAP capacities of the crude and saponified extracts of *Oedogonium*. The crude extracts produced higher antioxidant values than the saponified extracts for all three assays. This result is expected as the saponification step removed considerable amount of chlorophylls (**Table 1**), which are known to exhibit antioxidant activities (Le Tutour *et al*., 1998). Saponification also caused some loss of carotenoids and probably some other components such as lipids, which would otherwise contribute to the antioxidant activities (Bijttebier *et al.,* 2014). There are no previous reports on the antioxidant activities of carotenoids in saponified extracts of algae; therefore direct comparison with the literature is not possible. The antioxidant activities of algae published in the literature are usually reported on extracts obtained by aqueous alcohols or acetone, and the results vary considerably among different algal species. For example, Ahmed *et al*. (2014) screened 12 algal species for their carotenoid profiles and *in vitro* antioxidant capacity (ORAC) and showed that the ORAC values varied between 45 and 577 μmol/g (DW). The carotenoids in the algae, including violaxanthin, astaxanthin, lutein, zeaxanthin, neoxanthin, α-carotene and β-carotene, were believed to be major contributors to the antioxidant activity. The strong antioxidant activities of many carotenoids have been well documented and are believed to be responsible for the antioxidant activities of a number of algal species (Rao *et al.*, 2013). It is likely that the presence of these compounds in *Oedogonium* contributed significantly to the strong antioxidant activities of its extracts, especially the saponified fraction where carotenoids were the predominant antioxidants.

3.4 Inhibitory effects of Oedogonium carotenoid extracts against metabolically significant enzymes

3.4.1 *In vitro* pancreatic lipase inhibition

Obesity is one of the major health issues worldwide, with the World Health Organization estimating that there are more than 1.9 billion overweight adults worldwide in 2014, of which 13% being clinically obese (WHO, 2016). Obesity can lead to adverse chronic conditions such as cardiovascular disease, diabetes mellitus, certain cancers, hypertension and sleep-breathing disorders. An imbalance between calorie intake and expenditure is a central factor in many cases of obesity and reducing the intake and absorption of energy-dense fats is one of the main strategies for managing body weight. Pancreatic lipase is the key enzyme in the digestion and utilisation of dietary fats. It hydrolyses triglycerides into monoglycerides and fatty acids which can then be absorbed by the body. Orlistat, a pancreatic lipase inhibitor, is one of the standard prescription drugs used to treat obesity and it is reported that intestinal fat absorption can be reduced by up to 30% by oral administration of 120 mg Orlistat with main meals (Guerciolini, 1997).

The pancreatic lipase inhibitory effect of crude and saponified carotenoid extracts of *Oedogonium* was assessed and a dose-dependent inhibition effect was observed (**Figure 3**). With increases in the concentration of both crude and saponified extracts, the inhibition of pancreatic lipase increased. The activities were higher for the saponified extract than the crude extract. From a dosage perspective, the results mean that the pancreatic lipase inhibition effect from the consumption of 1 mg of orlistat is equivalent to that of about 3 mg of the saponified extract of *Oedogonium*.

There are no reports on the pancreatic lipase inhibition by carotenoid extracts of algae, and the current study is the first on this aspect of biological activities of algae. However, a number of studies have investigated the inhibitory effect of individual carotenoids on pancreatic lipase and their preventive effect on obesity. For example, fucoxanthin, which has a similar structure to neoxanthin, was found to have a potent

inhibitory effect, comparable to that of Orlistat, on rat pancreatic lipase *in vitro*, and reduce lymphatic triglyceride absorption in laboratory rats *in vivo* (Matsumoto, et al, 2014). *Oedogonium* contains an appreciable level of neoxanthin (361.8µg/g DW) which, coupled with the high pancreatic lipase inhibitory activities of the algal extracts, indicates that the alga could be developed into functional food ingredients with the potential to play a part in the management of body weight. However, the mechanism for the enzymatic inhibitory actions of carotenoids has not yet been elucidated and remains a worth topic for future investigations.

3.4.2 *In vitro* antidiabetic effects

Diabetes mellitus has become an epidemic in adults and, increasingly, in children throughout the world and is a leading cause of kidney failure, heart attack, blindness and lower limb amputation. WHO estimated that 8.5% the world's adult population are diabetic and 1.5 million deaths were directly caused by diabetes in 2012 (WHO, 2016). It is the fourth main cause of death in most developed countries. Effective management of diabetes mellitus, especially the non-insulin-dependent Type II, involves preventing the excessive rise of the blood glucose level through the inhibition of starch digestive enzymes, i.e., pancreatic α-amylase and intestinal α-glucosidase, in the digestive system. α-Amylase catalyses the hydrolysis of α-1,4-glucosidic linkages of starch to oligosaccharides, which, in turn, are further broken down by α-glucosidase to glucose, ready for absorption by the body. The inhibition of these two enzymes are two of the most commonly used *in vitro* assays for determining the anti-type II diabetic potential of a substance. In recent years, natural sources of α-amylase and α-glucosidase inhibitors have received a lot of interest due to the side effects associated with synthetic enzyme inhibitors such as acarbose, sulfonylureas, biguanides, glinides, metformin and orlistat (Kunyanga, Imungi, Okoth, Biesalski &

Vadivel, 2012). These synthetic drugs provide a benchmark with which to compare the efficacy of natural inhibitors such as the carotenoid extracts from *Oedogonium*.

The inhibition of α-amylase was observed over four different concentrations of both crude and saponified extracts of *Oedogonium* (**Figure 3**). With increases in the concentration of the crude extract, the inhibition of α-amylase increased. For the saponified extract, the inhibition of α-amylase also increased with increases in its concentration, although the trend was not as clear as that of the crude extract. Surprisingly, the α-amylase inhibition activities of the saponified extract were lower than their crude counterpart in most cases, especially at the higher concentration range. This indicates that some of the impurities in the crude extracts that were removed by saponification also contributed to the inhibitory activity against α-amylase. Significantly, the α-amylase inhibitory activities of the crude and saponified extracts were comparable to that of acarbose, one of the standard prescription drugs for the management of diabetes.

α-Glucosidase was inhibited by the carotenoid extracts of *Oedogonium* at four different concentrations, with the inhibition activities also comparable to that of acarbose (**Figure 3**). At concentrations higher than 10 mg/mL, the crude extract showed higher inhibition than acarbose at 1 mg/mL, demonstrating their potency as an inhibitor of this enzyme. With regard to the saponified carotenoid extract, its inhibition on α-glucosidase was also examined at four concentrations; however, the saponified extracts showed very low activities compared with the corresponding concentration of the crude extract. This appeared to indicate that it is primarily the non-carotenoid components in the crude extract that were responsible for the α-glucosidase inhibitory activity. These non-carotenoid components may include chlorophylls, sterols and terpenoids and fat soluble vitamins.

It has been recently reported that the fucoxanthin-rich fraction of two brown seaweeds (*Sargassum siliquosum and Sargassum polycystum*) had significant α-amylase and α-glucosidase inhibitory activities comparable to those of voglibose, a common drug for managing diabetes (Nagappan et al., in press). The present study found that the carotenoid extracts of *Oedogonium* were potent inhibitors of the two enzymes, comparable in potency to that of acarbose, another standard prescription drugs for diabetic care. This implies that consumption of green algae from the genus *Oedogonium* could be beneficial to diabetic patients or, if that is unpalatable, that extracts of the carotenoids have the potential to be developed into nutraceutical supplements as natural alternatives to acarbose and similar drugs for the treatment of diabetes. In this regard, crude carotenoid extracts of the alga appear to be a better candidate for development into bioactive ingredient as they had significant inhibitory effects on both α-amylase and α-glucosidase whereas the saponified extract only exhibited substantial inhibition on the former.

The antidiabetic effects of several carotenoids have been well documented. For example, several studies have shown that dietary β-carotene intake has an inverse association with type II diabetes (Montonen, Knekt, Jarvinen & Reunanen, 2004). Montonen *et al.* (2004) reported that β-cryptoxanthin intake is significantly associated with a reduced risk of type II diabetes. The presence of these carotenoids in *Oedogonium* further demonstrates its potential role in diabetic prevention as well as management.

3.4.4 Hyaluronidase inhibitory activity

Hyaluronidase is an enzyme which predominantly catalyses the hydrolysis of hyaluronic acid by splitting glucosaminidic bonds to yield oligosaccharides, and it is

found both in human organs and body fluids. This enzyme plays an important role in angiogenesis, carcinogenesis, type I allergic reactions, inflammatory diseases and inhibition of its activity is regarded as an effective anti-allergic and anti-inflammatory therapy (Muckenschnabel *et al*., 1998).

To evaluate the potential anti-allergic and anti-inflammatory activities of carotenoid extracts of *Oedogonium*, their inhibitory effect on hyaluronidase was investigated (**Figure 3**). Hyaluronidase inhibition increased significantly with increases in the concentrations of both crude and saponified extracts ($p<0.05$). For the crude extract, the inhibitory effect reached a plateau when the concentration reached 15 mg/mL. For the saponified extract, the inhibitory effect increased continuously with increasing concentrations within the range studied. At the highest concentration, the inhibitory effect of the saponified extract was the same as that of the crude extract. These results indicate that the anti-hyaluronidase effect of the extracts was mostly due to the carotenoids present. However, the hyaluronidase inhibition effects of these extracts were considerably lower than quercetin at 0.1 mg/mL, the positive control, which is a flavonoid with well reported anti-inflammatory activity.

There are no reports on the hyaluronidase inhibition effects of carotenoid extracts of algae, and therefore direct comparisons with the literature is not possible. However, several carotenoids have been reported to exhibit anti-allergic and anti-inflammatory activities. For example, astaxanthin, β-carotene, fucoxanthin and zeaxanthin have been found to exhibit significant inhibitory activities against antigen-induced degranulation of rat basophilic leukemia RBL-2H3 cells and bone marrow-derived mast cells (Sakai, Sugawara, Matsubara & Hirata, 2009). β-Carotene and lutein have also been reported to have a beneficial effect for the treatment of oxidative stress-mediated gastric inflammation, because they showed inhibitory effects on H_2O_2-

induced increase in intracellular levels of reactive oxygen species, activation of NF-$_K$B and IL-8 expression in AGS cells (Kim, Seo & Kim, 2011). The presence of these carotenoids in the algal extracts are expected to contribute to their inhibitory effect on hyaluronidase activity.

3.4.5 ACE inhibitory activity

Hypertension is a major risk factor for a number of disorders including cardiovascular disease, kidney failure and stroke. Over-expression of ACE plays a key role in the elevation of blood pressure, and identification and incorporation of natural ACE-inhibitors into functional food could constitute a critical part in the overall strategy in combating the hypertension epidemic (Boschin et al., 2014).

The inhibitory effect of the carotenoid extracts of *Oedogonium* on ACE was examined. At the concentration of 10 mg/mL, the inhibition of ACE activity was less than 8% for both crude and saponified extracts with the crude extract at 4.48±0.02% and saponified extract at 7.37±0.02%, which were comparable with the effect of 200µg/mL of captopril (6.15±0.01%). When the concentrations of the extracts were increased, no significant increases in the activity were observed. It is therefore concluded that the carotenoid extracts of *Oedogonium* were poor inhibitors of ACE. There is some evidence that carotenoids exhibit anti-hypertensive activities; for example, the administration of astaxanthin at a dose of 50 mg/kg for 5 weeks was found to cause a significant reduction in the arterial blood pressure in spontaneously hypertensive rats (SHR), and also delay the incidence of stroke in the stroke-prone SHR (Hussein *et al.*, 2005). However, astaxanthin was not found in *Oedogonium*, which might explain the low ACE-inhibitory effects of the extracts. Furthermore, ACE-inhibition is only one of several routes through which blood pressure can be

lowered. It is possible that carotenoids produce anti-hypertensive effects through other mechanisms. Recently, Nagappan et al. (in press) reported that the fucoxanthin-rich fraction of the brown seaweeds *Sargassum siliquosum* and *Sargassum polycystum* also had low ACE-inhibitory activities.

Overall, it can be concluded that the inhibitory effects of the *Oedogonium* extracts were mostly due to the carotenoids, especially for the saponified extract, as HPLC-MS analysis showed the saponified extract essentially consisted of carotenoids and some chlorophylls. However, small amounts of unsaponifiable compounds such as sterols, terpenoids and non-carotene fat soluble vitamins could be present in the extract and have a minor contribution to the effects.

Conclusion

This study showed that the freshwater alga *Oedogonium* contained substantial amounts of carotenoids, ranging from 2,929.6 to 3,411.2 µg/g algal dry mass, depending on the quantification method used, with the spectrophotometric method estimating a 25% higher carotenoid content than the HPLC method. Seven major major carotenoids were identified in the alga, namely neoxanthin, 9'-*cis*-neoxanthin, loroxanthin, violaxanthin, lutein and β-carotene which were present in similar amounts, and a smaller amount of α-carotene. Both the crude and saponified carotenoid extracts exhibited significant antioxidant capacities, as well as potent inhibitory effects against several metabolically important enzymes including α-amylase, α-glucosidase, pancreatic lipase and hyaluronidase. However, the algal extracts were found to be a poor inhibitor of ACE. Overall, carotenoid extracts of *Oedogonium* were found to have the potential to be developed into bioactive ingredients for application in functional food or nutraceutical products to supplement

drugs such as acarbose and orlistat for the management of diabetes, control of weight gain and mitigation of inflammation. This presents an alternative avenue for the utilisation of *Oedogonium* which has, to date, been domesticated only for use in the treatment of wastewater. However, animal model studies or clinical trials will be required to verify the health benefits of carotenoid extracts identified in the present work.

Acknowledgements

This research was conducted as part of the MBD Energy Research and Development program for the commercialization of algae. Thank S Lord from MBD for comments and commercial insights on a previous version of the manuscript.

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Figure captions

Figure 1. HPLC-PDA chromatogram of carotenoid extract of *Oedogonium* at 450 nm. Peak denotation: 1, neoxanthin; 2, 9'-*cis*-Neoxanthin; 3, loroxanthin; 4, violaxanthin; 5, lutein; 6, α-carotene; 7, β-carotene and. *, chlorophylls.

Figure 2. Antioxidant capacity of crude and saponified carotenoid extracts of *Oedogonium*. Results for ABTS scavenging capacity and FRAP values were measured as mg Trolox equivalent per gram dry weight (mg TE/g DW), and DPPH scavenging capacity as mg Gallic acid equivalent per gram dry weight (mg GAE/g DW). Data are mean ± SD of at least three independent experiments. Different lower case letters indicate the values being significantly different for the same extraction condition with $p<0.05$ (n=3).

Figure 3. Dose-dependent inhibition effects of crude (a) and saponified (b) carotenoid extracts of *Oedogonium* on pancreatic lipase, α-glucosidase, α-amylase and hyaluronidase activities. Data represent the mean ± SD of at least three independent experiments. Different lower case letters indicate values being significantly different for the same enzymatic inhibition but different concentrations with $p<0.05$ (n=3).

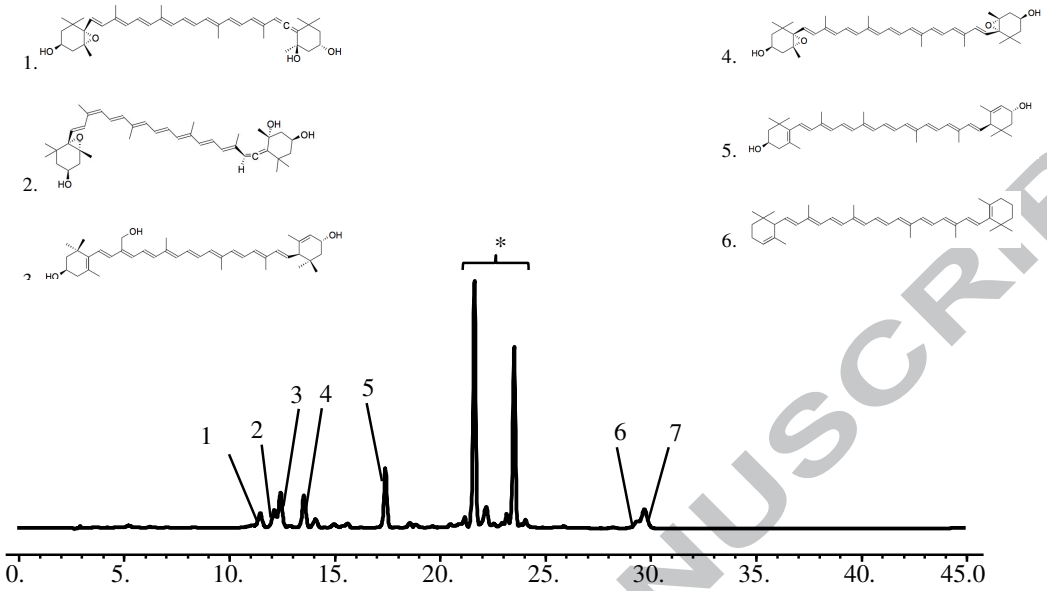

Figure 1

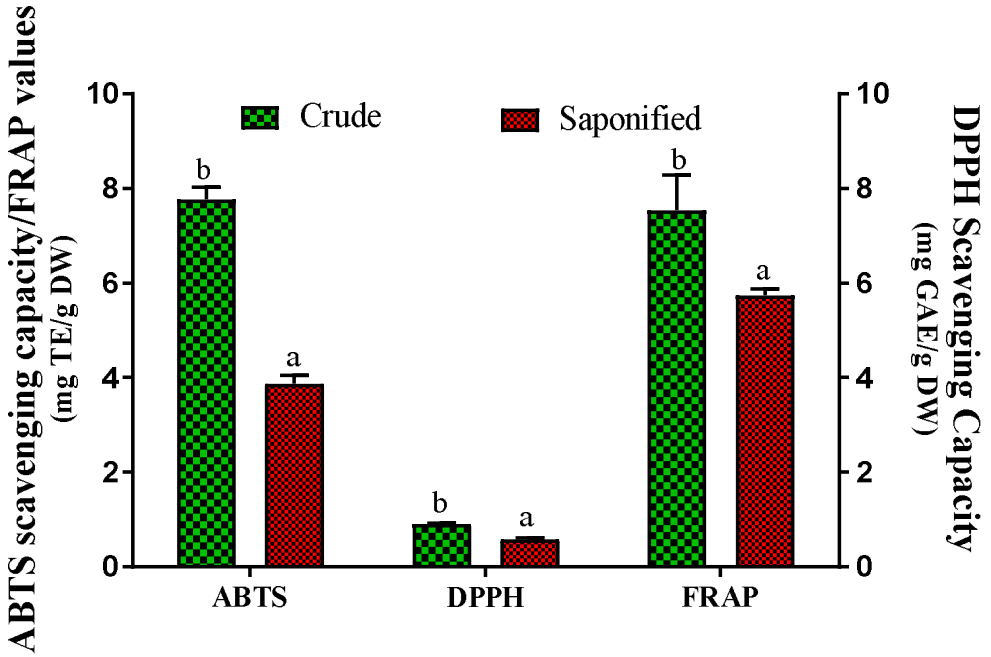

Figure 2

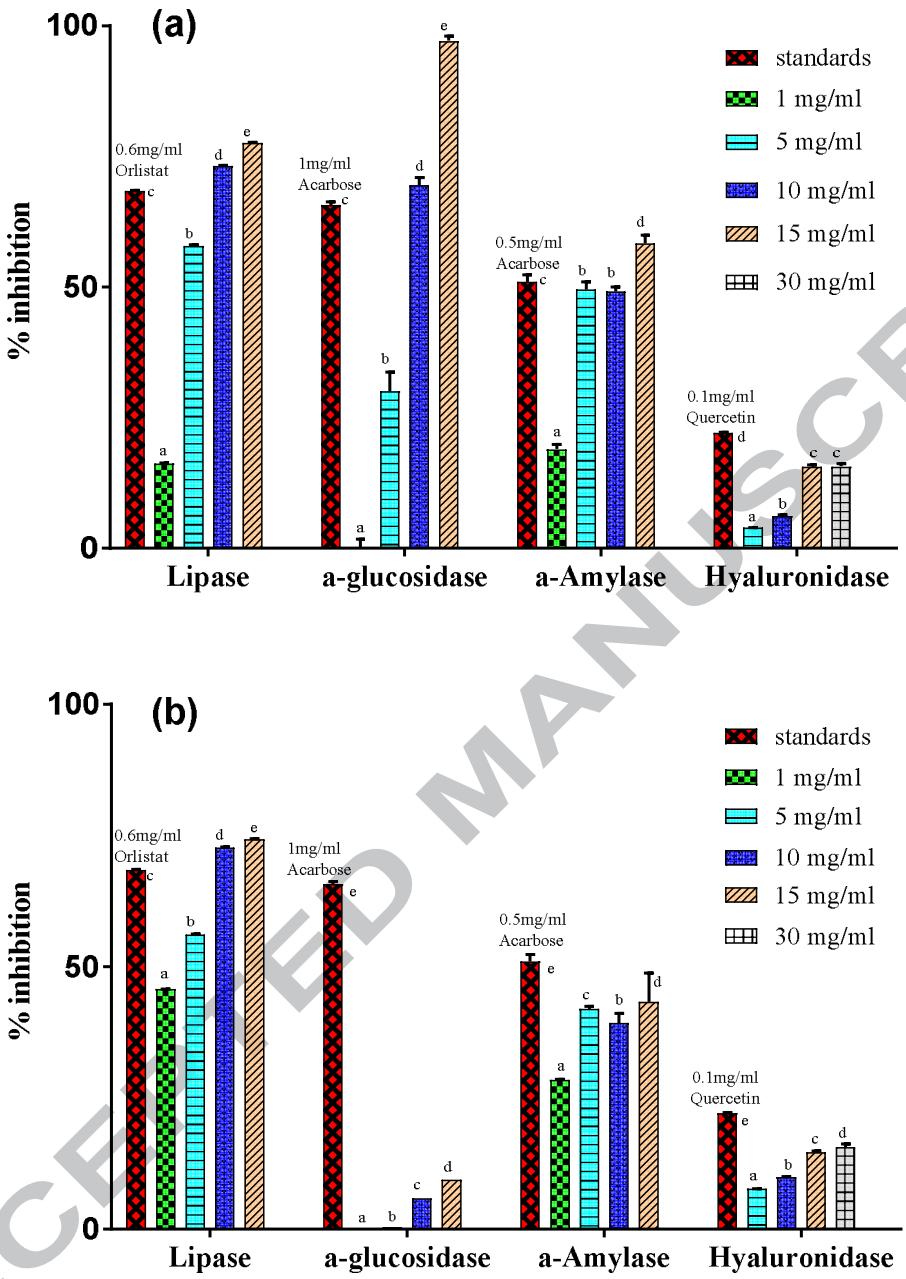

Figure 3

Table 1. Chlorophyll a, chlorophyll b and carotenoid content of *Oedogonium*

	Content (µg/g dry algal biomass)	
Component	**Crude**	**Saponified**
Chlorophyll a	21,877.0 ±16.8^{B}	688.7 ±19.2^{A}
Chlorophyll b	10,258.1 ±10.2^{B}	1,871.5 ±9.0^{A}
Total carotenoids	3,411.2 ±20.7^{B}	2,929.6 ±5.9^{A}

All values are mean ± SD for triplicate experiments (n=3). Different capital letters within the same row indicate significant difference between the values ($P<0.05$).

Table 2. UV-Vis and MS spectral data for carotenoids identified in *Oedogonium*

Peak	Compound	λ_{max} (on-line)/nm				λ_{max} (standard)/nm				λ_{max} (reported)/nm[a]			
1	Neoxanthin	-	-	439	467	n.a	n.a	n.a	n.a	-	418	440	468
2	9'-*cis*-Neoxanthin	327	412	435	464	-	412	435	464	328	414	436	464
3	Loroxanthin	-	-	444	471	n.a	n.a	n.a	n.a	-	423	447	475
4	Violaxanthin	326	415	438	468	327	415	438	468	-	416	440	468
5	Lutein	-	-	444	472	330	420	444	472	332	423	446	470
6	α-Carotene	-	-	444	472	n.a	n.a	n.a	n.a	344	426	449	476
7	β-Carotene	-	-	450	475	-	-	450	474	350	430	458	482

Peak	Compound	$[M+H]^+$ (m/z) found	$M+H]^+$ (m/z) standard	$[M+H]^+$ (m/z) reported[b]	Fragment ions (m/z) found	Fragment ions (m/z) standard	Fragment ions (m/z) reported[b]
1	Neoxanthin	601	601	601	583, 565	n.a	583, 565, 547, 509, 491, 221
2	9'-*cis*-Neoxanthin	601	601	601	583,565	583,565	583, 565, 547, 509, 393, 221
3	Loroxanthin	567	567	567	549,531	n.a	549, 531
4	Violaxanthin	601	601	601	583,565	583,565	583, 565, 509, 491, 221
5	Lutein	569	569	569	551,533	551,533	551, 533, 495, 477, 463, 459
6	α-Carotene	537	537	537	481	n.a	481, 444
7	β-Carotene	537	537	537	444	444	444

n.a: standards not available.

[a] UV-Vis and [b]MS data from Kao *et al.* (2011), Garrido, Rodriguez and Zapata (2009) and Inbaraj *et al.* (2006).

Table 3. Content of individual carotenoids in *Oedogonium*

Carotenoid	Content	
	µg /g dry algal weight	% of total carotenoids
Neoxanthin (peak 1)	361.8 ±4.5	15.5 ±0.1
9'-*cis*-Neoxanthin (peak 2)	403.3 ±2.6	17.2 ±0.2
Loroxanthin (peak 3)	298.2 ±7.5	13.5 ±0.2
Violaxanthin (peak 4)	423.7 ±2.6	18.1 ±0.1
Lutein (peak 5)	406.8 ±2.1	18.4 ±0.2
α-Carotene (peak 6)	76.8 ±1.4	3.7 ±0.1
β-Carotene (peak 7)	286.8 ±6.3	13.7 ±0.2
Total	2257.4 ±18.1	

Neoxanthin, loroxanthin, and α-carotene were quantified based on standard curves of 9'-*cis*-neoxanthin, lutein, and β-carotene, respectively. All values are mean ± SD for triplicate experiments (n=3).

Highlights

- We investigated carotenoids in the freshwater alga *Oedogonium intermedium*
- Seven carotenoids were identified including loroxanthin which is lacking in territorial plants
- The carotenoid extracts exhibited significant antioxidant capacities
- They show potent inhibition on α-amylase, α-glucosidase, pancreatic lipase and hyaluronidase