Journal of Applied Phycology (2019) 31:1675–1683
https://doi.org/10.1007/s10811-018-1726-5

Phenolic compounds, carotenoids, and antioxidant capacities of a thermo-tolerant *Scenedesmus* sp. (Chlorophyta) extracted with different solvents

Onur Bulut[1,2,3] · Dilan Akın[2] · Çağla Sönmez[1] · Ayşegül Öktem[2] · Meral Yücel[2] · Hüseyin Avni Öktem[2]

Received: 19 July 2018 / Revised and accepted: 27 December 2018 / Published online: 9 January 2019

Abstract

The human body can fight against the adverse effects of chronic exposure to environmental pollutants and stress by consumption of a diet rich in antioxidants. Although fruits and vegetables are the predominant sources, alternative sources of antioxidants such as microalgae are also being explored. Here, we investigate the antioxidant capacity, total phenolic, flavonoid, and carotenoid contents of novel thermo-resistant green microalga *Scenedesmus* sp. ME02. This strain has previously been shown to have a high polyunsaturated fatty acid content. Four different solvents were used for extraction and the antioxidant capacity was determined to be 3.71 ± 0.11 and 47.01 ± 3.14 μmol Trolox eq. g^{-1} DW in ethanol/water mixture by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays, respectively. Total phenolic, flavonoid, and carotenoid contents in ethanol/water were measured as 5.40 ± 0.28 mg gallic acid eq. g^{-1} DW, 1.61 ± 0.76 mg quercetin eq. g^{-1} DW, 0.61 ± 0.05 mg g^{-1}, respectively. This is one of the few studies that reports the presence and quantification of total flavonoids in microalgae. The correlation between the total phenolic content and FRAP assay, but not the DPPH assay, was statistically significant. Finally, 12 different phenolic compounds were analyzed by reverse-phase HPLC and ethyl acetate extract showed substantial amounts of quercetin (0.84 ± 0.12 mg g^{-1} DW) and rutin (0.11 ± 0.08 mg g^{-1} DW). Quercetin amount was also high in the ethanol/water extract along with gallic acid, 4-hydroxy benzoic acid, and chlorogenic acid. To the best of our knowledge, this is the first study that reports significant amounts of quercetin and rutin in a microalgal species.

Keywords Green microalgae · Antioxidant capacity · Phenolic compounds · Flavonoids · Quercetin

Introduction

Accumulation of reactive oxygen species (ROS) at high levels in cells as a result of normal cellular activities as well as exposure to oxidative stress is attributed to many chronic degenerative disease and conditions in humans including cardiovascular disease, cancer, and aging-related conditions such as Alzheimer's disease (Christen 2000; Waris and Ahsan 2006; Li et al. 2007; Sugamura and Keaney Jr 2011). Several enzymes including superoxide dismutase, glutathione peroxidase, and catalase are involved in the cell's endogenous defense system against free radicals; however, certain conditions such as cigarette smoking, exposure to environmental pollutants and UV radiation, and unhealthy eating habits may exert additional stress. In that situation, humans rely on exogenous sources of antioxidants for additional support. Many studies have shown that a diet rich in fruits and vegetables can circumvent the negative effects of oxidative damage (Prior 2003; Holt et al. 2009). The antioxidants found in high amounts in certain plant species serve as the natural scavengers of ROS (Masella et al. 2005; Pandey and Rizvi 2009). Both natural and synthetic antioxidants are also widely used in the food industry as additives to prolong the shelf life of food products. There is an ongoing search for replacement of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT) with their natural counterparts

✉ Hüseyin Avni Öktem
haoktem@metu.edu.tr

[1] Department of Molecular Biology and Genetics, Faculty of Agriculture and Natural Sciences, Konya Food and Agriculture University, 42080 Konya, Turkey

[2] Department of Biological Sciences, Middle East Technical University, Üniversiteler Mah., Dumlupınar Blv., No. 1, 06800 Ankara, Turkey

[3] Research and Development Center for Diagnostic Kits (KIT-ARGEM), Konya Food and Agriculture University, 42080 Konya, Turkey

due to concerns about the potential toxic and carcinogenic effects of the former (Ito et al. 1986; Shebis et al. 2013).

Vitamins (particularly vitamins C and E), carotenoids, and phenolic compounds are the three major groups of antioxidants (Freile-Pelegrín and Robledo 2013; Landete 2013). Among these, phenolic compounds or phenols comprise a broad range of structural characteristics with diverse physiological effects (Balasundram et al. 2006). Anti-carcinogenic and anti-proliferative activities of phenols on breast, colon, prostate, and human leukemia tumor cell lines as well as on animal models of various cancer types have been documented (Dai and Mumper 2010). Additionally, consumption of dietary supplements rich in polyphenols, particularly in early developmental stages, has been associated with positive cognitive outcomes in patients with Down syndrome and related disorders (Vacca et al. 2016). Other effects of phenolics that have been reported up-to-date are antimicrobial, anti-inflammatory, and antibiofilm activities (Jagani et al. 2009; Zhang et al. 2011; Borrás-Linares et al. 2015). The mechanisms of antioxidant activity include inhibition of enzymes such as glutathione S-transferase, NADH oxidase, and protein kinase C or chelation of trace metals (e.g., iron or copper) that are involved in ROS production, scavenging free radicals, and upregulating the antioxidant defense pathways (Pietta 2000). The overall structure and certain structural components of flavonoids, a distinct class of phenolic compounds, have a significant contribution to the efficiency of the antioxidant activity. Besides fruits and vegetables, flavonoids can also be found in many forms in medicinal plants, herbs and spices, nuts, tea, and cereals (Pietta 2000).

Although a large number of studies have focused on the antioxidant potential of terrestrial plants (Brewer 2011), microalgae are emerging as an alternative source of antioxidants. Several studies have reported the phenolic content and antioxidant activity of microalgae (Li et al. 2007; Hajimahmoodi et al. 2010; Guedes et al. 2011; Goiris et al. 2012; Machu et al. 2015). The vast diversity of microalgae with the ability to produce different metabolites, the ease of cultivation with minimal land requirement, the feasibility of growth under different conditions including on wastewater make microalgae attractive candidates in the quest for different antioxidant sources. Some species of microalgae, particularly *Chlorella* and *Arthrospira* (*Spirulina*) are commercially available as natural food supplements and are known as "super-foods" due to their rich phytochemical content and health benefits. Other microalgae (e.g., *Nannochloropsis* spp., *Tetraselmis* spp.) with high polyunsaturated fatty acid (PUFA) composition are used as fish feed in aquaculture (Guedes and Malcata 2012; Sørensen et al. 2017).

In this study, we investigated the antioxidant capacity of novel, thermo-resistant freshwater green microalga, *Scenedesmus* sp. ME02 in relation to its total phenolic, flavonoid, and carotenoid contents. This strain was previously isolated and characterized in our laboratory as part of a collection of thermal spring water flora from Central Anatolia (Onay et al. 2014) and can withstand a wide temperature range between 10 and 50 °C. It is composed approximately of 56% proteins and the total lipid content varies between 10 and 20% depending on different culture conditions and extraction methods (Onay et al. 2016; Sonmez et al. 2016). The fatty acid composition is also highly variable and the PUFA content can be as high as 75% of total fatty acids when grown at 16 °C. In the present study, we evaluated the total phenolic, total flavonoid, and total carotenoid contents of *Scenedesmus* sp. ME02 using different solvents with varying polarity. We measured the antioxidant capacity by two different assays, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. We determined the correlation between the antioxidant capacity and phenolic, flavonoid, and carotenoid contents of *Scenedesmus* sp. ME02. Finally, we assessed the presence and amounts of 12 different phenolic compounds in our samples.

Materials and methods

Chemicals and reagents

DPPH, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), iron(III) chloride hexahydrate, Folin and Ciocalteu's phenol reagent, sodium carbonate, aluminum chloride, trolox, gallic acid, and quercetin were from Sigma-Aldrich (USA). Sodium hydroxide, sodium acetate, and sodium nitrite were supplied by AppliChem GmbH (Germany). Solvents including methanol, ethanol, ethyl acetate, and hexane and the phenolic compound standards used in RP-HPLC were from Merck (Germany). All chemicals and reagents used in the study were analytical or HPLC grade.

Culture and growth conditions of *Scenedesmus* sp. ME02

Scenedesmus sp. ME02 strain was isolated from thermal springs of Haymana, Ankara (latitude 39.4° N, longitude 32.48° E) and characterized as previously described (Onay et al. 2014). Cultures of *Scenedesmus* sp. ME02 were maintained mixotrophically in tris-acetate-phosphate (TAP): BG-11 (1:1 *v/v*) growth media either in Petri plates containing 1.5% agar or in flasks with constant shaking at 150 rpm at 25 °C under 16:8 h of light/dark photoperiod with 54 μmol photons m^{-2} s^{-1} light intensity. The composition of TAP/BG-11 (1:1 *v/v*) growth medium was previously described in Sonmez et al. (2016). *Scenedesmus* sp. ME02 at 2–3 × 10^5 cells mL^{-1} was inoculated in 1 L TAP/BG-11 (1:1 *v/v*) medium in 2-L Erlenmeyer flasks and grown until cells reached late logarithmic growth phase and harvested at 7 days

after cultivation. This time point marked the highest biomass concentration obtained. Final biomass concentration was measured as 0.4 ± 0.02 g L^{-1} at the harvest time. The growth characteristics of this strain in TAP/BG-11 (1:1 *v/v*) growth medium were previously reported in Sonmez et al. (2016).

Preparation of microalgal extracts

Cells were grown as described above and harvested by centrifugation at 3600× *g* for 20 min at 4 °C. The pellet was frozen at − 80 °C overnight and freeze-dried. Four different solvents, ethanol/water mixture (3:1 *v/v*), ethyl acetate, hexane, and water were used for extraction. Then, 0.2 g freeze-dried microalgal biomass was suspended in 5-mL solvent in separate tubes and mixed vigorously. Tubes were sonicated in an ultrasonic water bath for 20 min and stirred on an orbital shaker for 1 h at room temperature. The extracts were centrifuged at 3800× *g* for 10 min and the supernatant was collected. The pellet was resuspended in the same solvent and extracted one more time following the same procedure. Both extracts were combined and filtered through a 0.45-μm pore size syringe filter. Extracts (10 mL) were dried using a rotary evaporator (Hei-VAP Precision, Germany) until the solvents were completely removed. The dried residues were resuspended in methanol to a concentration of 10 mg mL^{-1} and stored at − 20 °C for further analysis. All experiments were done using three biological replicates.

Determination of total phenolic content

Total phenolic content (TPC) of the microalgal biomass extracted in the above-mentioned solvents were determined by the Folin and Ciocalteu method (Singleton and Rossi 1965). Briefly, 100 μL of each sample was mixed with 400 μL of 10% Folin–Ciocalteu reagent and allowed to stand at room temperature for 5 min; then, 500 μL of 7.5% (*w/v*) sodium carbonate solution was added to the mixture. After incubating for 1.5 h in the dark at room temperature, the absorbance of each sample was measured at 760 nm. A standard curve prepared with serial gallic acid solutions ranging from 10 to 400 mg L^{-1} was used for calibration. The analyses were performed in triplicate, and TPC was expressed as milligram gallic acid equivalents per gram dry weight of sample (mg GAE g^{-1} DW).

Determination of total flavonoid content

Total flavonoid content (TFC) of the extracts were determined using the aluminum chloride colorimetric method (Zhishen et al. 1999). Briefly, 1 mL of extract was mixed with 4 mL of distilled water, and 0.3 mL of 5% (*w/v*) $NaNO_2$ was added. After 5 min, 0.3 mL of 10% (*w/v*) $AlCl_3$ was added and the mixture was incubated for 6 min. Then, 2 mL of 1 M NaOH was added and the total volume was adjusted to 10 mL by adding 2.4 mL of distilled water. The mixture was vortexed briefly, and the absorbance was measured at 510 nm. A standard curve was also prepared using serial quercetin solutions ranging from 10 to 400 mg L^{-1} and the results were expressed as milligram quercetin equivalents per gram dry weight of sample (mg QE g^{-1} DW).

Determination of total carotenoid content

Carotenoid content of the extracts was calculated by the spectrophotometric method (Lichtenthaler and Buschmann 2001). Absorbance values of the extracts in pure methanol were recorded at 470, 652, and 665 nm and carotenoid contents were calculated according to the Lichtenthaler equations.

$$c_a\left(\mu g\ mL^{-1}\right) = 16.72A_{665} - 9.16A_{652}$$
$$c_b\left(\mu g\ mL^{-1}\right) = 34.09A_{652} - 15.28A_{665}$$
$$c_{(x+c)}\left(\mu g\ mL^{-1}\right) = \left(1000A_{470} - 1.63c_a - 104.96c_b\right)/221$$

where c_a and c_b are concentrations of chlorophyll *a* and *b*, respectively, and $c_{(x+c)}$ is the concentration of total carotenoids. The results were expressed as milligrams carotenoid per gram dry weight of sample.

DPPH radical scavenging assay

DPPH assay was performed according to the method described by Cheng et al. (2006). A 100-μL aliquot of extracts at concentrations ranging from 50 to 2000 μg mL^{-1} in methanol was mixed with 100 μL of 0.2 mM DPPH solution (prepared with methanol) in a 96-well microplate and incubated for 30 min in the dark at room temperature. The absorbance of each sample was recorded at 515 nm using a microplate reader (Synergy H1, Biotek, USA). The percentage of scavenged DPPH radical was calculated according to the following equation:

$$\text{DPPH scavenging activity}(\%) = \left[1 - \left(A_s - A_{sc}\right)/A_c\right] \times 100$$

where A_s is the absorbance of the sample, A_{sc} is the absorbance of the sample control (100 μL of sample with 100 μL of methanol), and A_c is the absorbance of the control (100 μL of DPPH solution with 100 μL of methanol). Results were expressed as micromoles Trolox equivalents per gram dry weight of sample (μmol TE g^{-1} DW) and (%) DPPH radical scavenging effect of the extract at 1 mg mL^{-1} concentration.

Ferric reducing antioxidant power assay

The antioxidant capacity of the extracts was also evaluated by FRAP assay through monitoring the reduction of Fe^{3+}-TPTZ to blue-colored Fe^{2+}-TPTZ (Firuzi et al. 2005). The working FRAP solution was prepared by mixing 10 volumes of

300 mM acetate buffer (pH 3.6), 1 volume of 10 mM TPTZ (dissolved in 40 mM HCl), and 1 volume of 20 mM ferric chloride hexahydrate. A 25-μL aliquot of extracts at different concentrations in the range of 50 to 2000 μg mL^{-1} was mixed with 175 μL of freshly prepared and pre-warmed FRAP solution in a microplate. The absorbance of each sample was recorded at 593 nm using a microplate reader (Synergy H1, Biotek, USA) after incubation for 30 min in the dark. FRAP values were expressed as micromoles Trolox equivalents per gram dry weight of sample (μmol TE g^{-1} DW).

Reverse-phase high performance liquid chromatography analysis

Phenolic compounds were identified by reverse phase HPLC as described in Farvin and Jacobsen (2013). The extract at a concentration of 1000 ppm was passed through a 0.45 μm polytetrafluoroethylene filter prior to injection. Reverse phase HPLC was performed with Waters Alliance 2695 series HPLC (Waters Corporation, USA), equipped with Waters 2489 UV–Vis detector. The column used was an ACE 5 C18 analytical column (250 × 4.6 mm) (Advanced Chromatography Technologies Ltd., Scotland) with 5-μm packing material. Elution was performed with a gradient pump mode (mobile phase A: 2% acetic acid, mobile phase B: acetonitrile and 0.5% acetic acid (1:1 *v/v*), and mobile phase C: acetonitrile) at a flow of 1.2 mL min^{-1}. Detection was done using a UV–Vis detector with reference wavelength of 280 nm. Retention times and peak areas were monitored and computed automatically by Empower 3 software (Waters Corporation). Individual phenolic compounds were identified by the retention time of sample chromatographic peaks being compared with those of authentic standards using the same HPLC operating conditions.

Statistical analysis

All analyses were performed in triplicate and results are expressed as mean ± standard error (SE). The mean values of data were analyzed by analysis of variance (ANOVA) followed by Tukey's post hoc comparison test to test for differences between data. Statistical significance was determined at the 5% level ($p < 0.05$). Correlations among different assays were calculated using Pearson's correlation coefficient (r). The statistical analysis was carried out by R version 3.4.2 (R Core Team 2013).

Results

Extraction yield

In this study, four different solvents; ethanol/water mixture (3:1 *v/v*), ethyl acetate, hexane, and water were used for extraction of antioxidants from *Scenedesmus* sp. ME02. The highest extraction yield of 23.98 ± 2.47% was obtained with ethanol/water used to extract both polar and nonpolar compounds followed by 15.32 ± 0.18% and 8.47 ± 0.55% extraction yields for water and hexane, respectively. The lowest extraction yield of 5.42 ± 1.06% was obtained for ethyl acetate, which has medium polarity.

Antioxidant capacity of *Scenedesmus* sp. ME02

The antioxidant capacity of *Scenedesmus* sp. ME02 extracts was measured by two different assays, namely; the DPPH and FRAP assays. The results of the antioxidant capacity of *Scenedesmus* sp. ME02 are given in Table 1 as % DPPH radical scavenging activity as well as micromoles TE per gram DW of microalgae. According to the data obtained by the DPPH and FRAP assays, the highest antioxidant capacity of the cell extracts was measured in ethanol/water mixture as 3.71 ± 0.11 and 47.01 ± 3.14 μmol TE g^{-1} DW, respectively. The extracts in other solvents displayed lower antioxidant capacity. The difference between ethanol/water > ethyl acetate > hexane and water were statistically significant ($p < 0.001$); whereas the difference between hexane and water was not ($p > 0.05$) in both DPPH and FRAP assays. The coefficient of determination (R^2) between the DPPH and FRAP assays was calculated as 0.96 and was highly significant ($p < 0.001$) for all solvents used (Fig. 1).

Total phenolic content of the extracts

The highest total phenolic content (TPC) of 5.40 ± 0.28 mg GAE g^{-1} DW was recorded in ethanol/water, followed by 3.73 ± 0.65 mg GAE g^{-1} DW in ethyl acetate, 1.97 ± 0.03 mg GAE g^{-1} DW in water, and 1.13 ± 0.11 mg GAE g^{-1} DW in hexane (Table 2).

Total flavonoid content of the extracts

We determined the total flavonoid content of *Scenedesmus* sp. ME02 as 1.61 ± 0.76 and 0.93 ± 0.30 mg QE g^{-1} DW in ethanol/water and ethyl acetate, respectively (Table 2). Hexane and water extracts did not contain any detectable levels of flavonoids.

Total carotenoid content of *Scenedesmus* sp. ME02 extracts

We measured the total carotenoid content of *Scenedesmus* sp. ME02 extracts as 0.61 ± 0.05, 0.80 ± 0.32, and 0.15 ± 0.02 mg g^{-1} DW for ethanol/water, ethyl acetate, and hexane extracts, respectively. No carotenoids were detected in the water extract (Table 2). Although the highest carotenoid

Table 1 Antioxidant capacity of *Scenedesmus* sp. ME02 in different solvent extracts determined by DPPH and FRAP assays

Solvent	(%) DPPH radical scavenging effect[a]	DPPH (μmol TE g^{-1} DW)	FRAP (μmol TE g^{-1} DW)
Ethanol/water (3:1 *v/v*)	25.65 ± 2.58	3.71 ± 0.11	47.01 ± 3.14
Ethyl acetate	52.02 ± 2.61	1.80 ± 0.25	14.49 ± 1.14
Hexane	12.99 ± 1.30	0.60 ± 0.08	2.85 ± 0.76
Water	8.40 ± 0.40	0.61 ± 0.03	4.30 ± 0.18

Results are mean ± standard error of three measurements ($n = 3$)

[a] Radical scavenging effects of algal extracts at 1 mg mL^{-1} concentration

content was obtained in ethyl acetate, the difference was not statistically significant among the three solvents.

Correlation of antioxidant potential with phenolic, flavonoid, and carotenoid content

We assessed the coefficients of determination (R^2) between the antioxidant potential and the total phenolic, flavonoid, and carotenoid contents of *Scenedesmus* sp. ME02 extracted in different solvents (Table 3). The coefficient of determination between the FRAP assay and total phenolics extracted in ethanol/water was significant ($p < 0.05$). This was the only statistically significant correlation determined based on our data. Although the coefficients of determination between total phenolic content in ethyl acetate and both DPPH ($R^2 = 0.99$) and FRAP ($R^2 = 0.97$) assays were high, results were not statistically significant ($p > 0.05$).

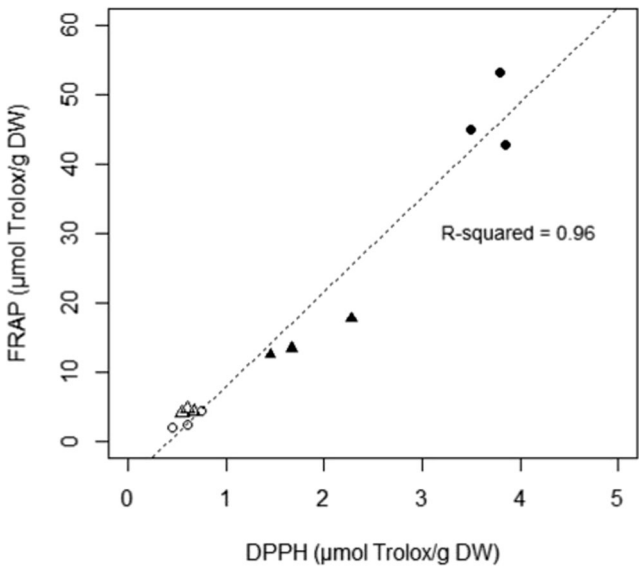

Fig. 1 Relationship between DPPH and FRAP assays. Results of both assays were expressed as micromoles of Trolox equivalent per gram DW. The dashed line represents the regression line. Filled circles, filled triangles, empty circles, and empty triangles symbolize ethanol/water, ethyl acetate, hexane and water extracts, respectively

RP-HPLC analysis of selected phenolic compounds

In this study, the amounts of 12 different phenolic compounds, namely, benzoic acid derivatives gallic acid, benzoic acid, 4-hydroxy benzoic acid, vanillic acid, and syringic acid; cinnamic acid derivatives cinnamic acid, coumaric acid, caffeic acid, chlorogenic acid, and rosmarinic acid; and flavonols quercetin and rutin extracted in ethanol/water (3:1 *v/v*) mixture and ethyl acetate were analyzed by RP-HPLC (Table 4).

The flavonol quercetin comprised the highest amount of phenolic compound among the 12 analyzed in the ethyl acetate extract (844.5 ± 125.0 μg g^{-1} DW). Quercetin concentration in ethanol/water extract was also high (551.9 ± 90.9 μg g^{-1} DW). Gallic acid concentration was measured as 653.6 ± 54.3 μg g^{-1} DW in ethanol/water extract and detected to be much lower in the ethyl acetate extract (2.3 ± 0.1 μg g^{-1} DW). Other phenolics such as 4-hydroxy benzoic acid, vanillic acid, caffeic acid, and chlorogenic acid were also noticeably higher in the ethanol/water mixture compared to the ethyl acetate extracts (Table 4). On the other hand, rosmarinic acid and rutin concentrations were measured as 35.5 ± 2.5 and 105.8 ± 8.6 μg g^{-1} DW, respectively, in the ethyl acetate extract and not detected in ethanol/water mixture.

Discussion

The solubility of antioxidants, which depends mainly on the polarity of the solvent used is a major determinant in accurate assessment of antioxidant capacity. In this study, we used four different solvents with varying polarities; namely, ethanol/water mixture (3:1 *v/v*), ethyl acetate, hexane and water for extraction. Our results are consistent with previous studies that suggest that the choice of solvent significantly effects the yield of extractable substances (Wang et al. 2009; López et al. 2011; Jerez-Martel et al. 2017) and the measured antioxidant capacity (Goiris et al. 2012; Machu et al. 2015).

In order to estimate the antioxidant capacity of our extracts accurately, we used two different methods: the DPPH and FRAP assays. Both assays were successfully employed to

Table 2 Total phenolic, flavonoid and carotenoid contents of *Scenedesmus* sp. ME02 extracts

Solvent	Total phenolic content (mg GAE g^{-1} DW)	Total flavonoid content (mg QE g^{-1} DW)	Carotenoid content (mg g^{-1} DW)
Ethanol/water (3:1 *v/v*)	5.40 ± 0.28	1.61 ± 0.76	0.61 ± 0.05
Ethyl acetate	3.73 ± 0.65	0.93 ± 0.30	0.80 ± 0.32
Hexane	1.13 ± 0.11	ND	0.15 ± 0.02
Water	1.97 ± 0.03	ND	ND

Results are mean ± standard error of three measurements ($n = 3$)

ND not detected

measure the antioxidant activities of many fruits as well as macro and microalgae (Prior et al. 2005). In principle, DPPH assay measures the reducing capacity of tested antioxidants toward the $DPPH^{\cdot}$ radical either by direct reduction via electron transfer or by radical quenching via H atom transfer (HAT). FRAP assay, on the other hand, detects the ability of antioxidants to transfer an electron to reduce Fe(III) to Fe(II), which is known as the single electron transfer (SET) mechanism but may be limited by the reactivity of the antioxidants exhibited at different time points (Prior et al. 2005). To assess the reactivity of a broad range of antioxidants including caffeic acid, ascorbic acid, and quercetin in the extracts, the measurements of the FRAP assay were followed by a 30-min dark incubation in this study. The difference between ethanol/water > ethyl acetate > hexane and water were statistically significant ($p < 0.001$); whereas the difference between hexane and water was not ($p > 0.05$) in both DPPH and FRAP assays. In a previous study, Goiris et al. (2012) determined the antioxidant potential of 32 microalgal biomass samples by FRAP and reported a wide range of results from 3.5 to 89.7 μmol Trolox g^{-1} DW of microalgae. One of the samples identified as *Scenedesmus obliquus* contained 19.7 μmol Trolox g^{-1} DW, which is twofold less than the amount measured for *Scenedesmus* sp. ME02 (47.01 ± 3.14 μmol Trolox g^{-1} DW).

Phenolics are considered to be the major contributors of antioxidant activity in plants (Dai and Mumper 2010) although there are conflicting results on the correlation of antioxidant activity and TPC of microalgal samples (Li et al. 2007; Goiris et al. 2012; Shetty and Sibi 2015). Phenolic content of the extracts depends highly on the type of solvent used. Here, we compare TPC in milligrams GAE per gram DW of *Scenedesmus* sp. ME02 extracted separately in solvents with different polarity. The TPC of samples was measured in decreasing order as ethanol/water > ethyl acetate > water > hexane. The presence of phenolics in higher amounts as extracted by the polar solvents compared to the nonpolar hexane is consistent with previous data (Hajimahmoodi et al. 2010; Goiris et al. 2012) and can be explained by the largely polar nature of the phenolic compounds in microalgae. *Scenedesmus* sp. ME02 exhibits a relatively high TPC among previously studied microalgae. Goiris et al. (2012) screened different microalgal biomass samples for their total phenolic content using the same solvents as the present study. Similar to our study, the highest TPC was found to be in ethanol/water with a range of 0.5 to 4.6 mg GAE g^{-1} DW. Three out of 32 microalgae with TPC of 3 mg GAE g^{-1} DW or higher in ethanol/water were considered to have a relatively high phenolic content. A *S. obliquus* strain that was among the microalgae studied contained 1.94 mg GAE g^{-1} DW. Yet, in another study by Custódio et al. (2014), TPC of a different *Scenedesmus* sp. was determined to be 0.05 mg GAE g^{-1} DW in hexane compared to 1.13 mg GAE g^{-1} DW in *Scenedesmus* sp. ME02. It is important to note that many factors including growth medium, pH, temperature, light intensity, and harvest time, in addition to the natural variability and the extraction procedure effect the phenolic content and antioxidant capacities of microalgae (Guedes et al. 2011; Shetty and Sibi 2015;

Table 3 The coefficient of determination values for relation between total phenolic content, total flavonoid content, and carotenoid content and antioxidant capacity

	Solvent	DPPH	FRAP
Total phenolic content	Ethanol/water	$R^2 = 0.02$; $p = 0.91$	$R^2 = 0.99$; $p = 0.02$
	Ethyl acetate	$R^2 = 0.99$; $p = 0.05$	$R^2 = 0.97$; $p = 0.11$
	Hexane	$R^2 = 0.14$; $p = 0.76$	$R^2 = 0.02$; $p = 0.97$
	Water	$R^2 = 0.90$; $p = 0.20$	$R^2 = 0.01$; $p = 0.96$
Total flavonoid content	Ethanol/water	$R^2 = 0.98$; p e = 0.07	$R^2 = 0.08$; $p = 0.82$
	Ethyl acetate	$R^2 = 0.25$; $p = 0.66$	$R^2 = 0.17$; $p = 0.73$
Total carotenoid content	Ethanol/water	$R^2 = 0.52$; $p = 0.48$	$R^2 = 0.31$; $p = 0.62$
	Ethyl acetate	$R^2 = 0.97$; $p = 0.11$	$R^2 = 0.93$; $p = 0.17$
	Hexane	$R^2 = 0.91$; $p = 0.19$	$R^2 = 0.55$; $p = 0.47$

Table 4 The phenolic compounds extracted in ethanol/water (3:1 *v/v*) and ethyl acetate identified by RP-HPLC analysis

Phenolic compound	Amount[a] (μg g^{-1} dry weight)	
	in ethanol/water	in ethyl acetate
Benzoic acid derivatives		
Gallic acid	653.6 ± 54.3	2.3 ± 0.1
Benzoic acid	ND	14.7 ± 3.6
4-hydroxy benzoic acid	441.9 ± 30.0	10.3 ± 3.3
Vanillic acid	83.2 ± 7.3	ND
Syringic acid	ND	4.5 ± 2.2
Cinnamic acid derivatives		
Cinnamic acid	ND	4.0 ± 0.7
Coumaric acid	ND	4.9 ± 0.9
Caffeic acid	67.6 ± 24.7	1.4 ± 0.3
Chlorogenic acid	352.0 ± 45.0	7.2 ± 0.2
Rosmarinic acid	ND	35.5 ± 2.5
Flavonols		
Quercetin	551.9 ± 90.9	844.5 ± 125.0
Rutin	ND	105.8 ± 8.6

[a] Results are mean ± standard deviation of two measurements

ND not detected

Aremu et al. 2016). For instance, Guedes et al. (2011) reported that the antioxidant compounds studied in *S. obliquus* strain M2-1 highly varied under different temperature and pH. Different growth conditions (autotrophic, heterotrophic, and mixotrophic) also significantly influenced the antioxidant capacity and phenolic content of *Chlorella vulgaris* and *S. obliquus* strains isolated from waste water in Bengaluru, India (Shetty and Sibi 2015). TPC of *S. obliquus* strain ranged from 0.11 to 0.55 mg GAE g^{-1} DW, under autotrophic and mixotrophic growth, respectively. In the present study, the lowest recorded TPC (in hexane extracts) of the mixotrophic *Scenedesmus* sp. ME02 biomass was 1.13 mg GAE g^{-1} DW. Aremu et al. (2016) reported that the harvest time, nitrogen levels, and choice of microalgae had a significant impact on the phenolic content. A single time point was selected for harvesting in the present study, to maximize the microalgal biomass for subsequent analysis; however, different harvest times may as well affect the antioxidant capacity and total phenolic content of *Scenedesmus* sp. ME02 and can be assessed in future studies.

Although the significance of flavonoids in terms of their antioxidant potential is well-recognized in plants, reports on the presence of flavonoids in microalgae are scarce (Panche et al. 2016). Recently, Goiris et al. (2014) determined the presence of distinct classes of flavonoids in different evolutionary lineages of microalgae including Chlorophyta using ultra-high performance liquid chromatography-two-dimensional mass spectrometry (UHPLC-MS/MS) analysis. Safafar et al. (2015) reported the total flavonoid content of methanolic extracts of seven different microalgae strains to be in the range of 0.84 ± 0.12 to 4.03 ± 1.10 mg QE g^{-1} DW as compared to 1.61 ± 0.76 and 0.93 ± 0.30 mg QE g^{-1} DW in ethanol/water and ethyl acetate, respectively, in the current study. Our report contributes to the limited number of studies in literature on the presence and quantification of flavonoids in microalgae.

Total carotenoid content of *Scenedesmus* sp. ME02 is similar to that of *S. obliquus* (0.44 mg g^{-1} DW in ethanol/water) studied previously by Goiris et al. (2012) and is in the lower range in comparison to other microalgae.

Different factors may collectively contribute to the antioxidant capacity of plants or microalgae. According to our data, the coefficient of determination between the FRAP assay and total phenolics extracted in ethanol/water was significant ($p < 0.05$). This was the only statistically significant correlation determined based on our data. Both FRAP assay and Folin-Ciocalteu method for determination of total phenolics rely on the same principle of metal reduction; whereas DPPH assay relies on the radical scavenging activity for assessment of antioxidant capacity. This principal difference may explain why total phenolics in ethanol/water are a significant contributor to antioxidant potential as determined by FRAP but not the DPPH assay (Hajimahmoodi et al. 2010). Li et al. (2007) found that the correlation between phenolic content and antioxidant capacity of 23 microalgae was not significant. In another study, Goiris et al. (2012) determined that phenolic content significantly correlated with Trolox equivalent antioxidant capacity (TEAC) and FRAP assays for 32 microalgal biomass samples studied. Carotenoid content also was assessed as a significant contributor to antioxidant capacity. In the present study, the coefficient of determination between carotenoid content and both DPPH and FRAP assays were high ($R^2 = 0.97$ and $R^2 = 0.93$, respectively) but the results were not statistically significant ($p > 0.05$). Shetty and Sibi (2015) reported that the coefficient of determination between phenolic content and DPPH assay of a *Scenedesmus* strain was statistically significant. Taken together, there is no established evidence on the general relation between total phenolic, flavonoid, and carotenoid content of microalgae and their antioxidant capacity in literature. Other factors such as the fatty acid composition (high levels of PUFA) may contribute to the antioxidant capacity of *Scenedesmus* sp. ME02 (Custódio et al. 2014; Aremu et al. 2016; Sonmez et al. 2016).

There are few studies on detection of phenolics in microalgae by HPLC to compare our results and to the best of our knowledge, this is the first study to report the amounts of selected phenolic compounds in a *Scenedesmus* strain. In a previous study by Onofrejová et al. (2010), the freshwater green microalga *Spongiochloris spongiosa* contained lower amounts of phenolic compounds, namely, chlorogenic acid, caffeic acid, coumaric acid, and cinnamic acid compared to

Scenedesmus sp. ME02 analyzed in current study. Yet, in another study by Machu et al. (2015) *Chlorella pyrenoidosa* was shown to contain 5 μg g^{-1} DW gallic acid and 20 μg g^{-1} DW 4-hydroxy benzoic acid. Gallic acid (653.6 μg g^{-1} DW) and 4-hydroxy benzoic acid (441.9 μg g^{-1} DW) amounts were much higher in the ethanol/water extracts of our sample. Quercetin, rutin, and rosmarinic acid were not among the phenolics tested in either study.

Compared to microalgae, macroalgae (i.e., seaweeds) have gained considerable attention in terms of their antioxidant potential and phenolic compounds. In a study by López et al. (2011), gallic acid was found to be the predominant polyphenol in brown alga *Stypocaulon scoparium*, and its amount highly varied in different solvent extracts; whereas quercetin and rutin were significantly lower than the amounts in the present study.

Quercetin is a flavonoid found in high amounts in vegetables and fruit such as caper, onion, cranberry, and apples. It has a wide range of health benefits including anti-obesity, anti-carcinogenic, anti-inflammatory, and antibacterial effects (Wang et al. 2016). Quercetin content of *Scenedesmus* sp. ME02 (~80 mg 100 g^{-1} DW in ethyl acetate extracts) is comparable to the quercetin levels in a selected list of vegetables, fruit and beverages (www.ImmuneHealthScience.com; USDA Database for the Flavonoid Content of Selected Foods, Release 2.1 (2007)).

Rutin, on the other hand, is a quercetin derivative and is found abundantly in cherry and spinach (Wang et al. 2016) but has been previously reported in one study with diatom *Phaeodactylum tricornutum* (Rico et al. 2013). Although different quantification methods were used, our data indicate that quercetin and rutin predominantly account for the total flavonoid content in our samples. To the best of our knowledge, this is the first study that establishes the presence of flavonoids, rutin and quercetin in high amounts in a green microalgal species.

Conclusion

The presence of vast diversity of natural compounds, ease of cultivation, and minimum requirement for land are some characteristics of microalgae that may supersede plants for industrial use. In this study, we investigated the potential of a green thermo-tolerant microalga, *Scenedesmus* sp. ME02 for use as a functional food by determining its antioxidant capacity and the presence of different phenolic compounds. The differences in methodology, solvents used, and cultivation conditions hinder direct comparison of our results with previous studies. Nonetheless, overall, *Scenedesmus* sp. ME02 stands out among other microalgae with its high total phenolic content (TPC). The statistical analysis revealed that the TPC contributed significantly to the antioxidant capacity of *Scenedesmus* sp. ME02 as measured by the FRAP assay. *Scenedesmus* sp. ME02 also showed high amounts of various phenolic compounds including gallic acid, 4-hydroxy benzoic acid, chlorogenic acid, caffeic acid, vanillic acid, and quercetin and rutin. The measurement of total flavonoid content as well as high quercetin and rutin amounts are significant contributions of this study to the present literature on antioxidant activities of microalgae.

Acknowledgments The authors would like to thank Konya Food and Agriculture University (KFAU) Strategic Products Research and Development Center (SARGEM) for technical support with the HPLC analysis and KFAU Research and Development Center for Diagnostic Kits (KIT-ARGEM) for the use of the facilities.

Funding This work was supported by KFAU Research Project BAP-2017/008.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

Aremu AO, Masondo NA, Molnár Z, Stirk WA, Ördög V, Van Staden J (2016) Changes in phytochemical content and pharmacological activities of three *Chlorella* strains grown in different nitrogen conditions. J Appl Phycol 28:149–159

Balasundram N, Sundram K, Samman S (2006) Phenolic compounds in plants and agri-industrial by-products: antioxidant activity, occurrence, and potential uses. Food Chem 99:191–203

Borrás-Linares I, Fernández-Arroyo S, Arráez-Roman D, Palmeros-Suárez PA, Del Val-Díaz R, Andrade-Gonzáles I et al (2015) Characterization of phenolic compounds, anthocyanidin, antioxidant and antimicrobial activity of 25 varieties of Mexican Roselle (*Hibiscus sabdariffa*). Ind Crop Prod 69:385–394

Brewer MS (2011) Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. Compr Rev Food Sci Food Saf 10:221–247

Cheng Z, Moore J, Yu L (2006) High-throughput relative DPPH radical scavenging capacity assay. J Agric Food Chem 54:7429–7436

Christen Y (2000) Oxidative stress and Alzheimer disease. Am J Clin Nutr 71:621S–629S

Custódio L, Soares F, Pereira H, Barreira L, Vizetto-Duarte C, Rodrigues MJ, Rauter AP, Alberico F, Varela J (2014) Fatty acid composition and biological activities of *Isochrysis galbana* T-ISO, *Tetraselmis* sp. and *Scenedesmus* sp.: possible application in the pharmaceutical and functional food industries. J Appl Phycol 26:151–161

Dai J, Mumper RJ (2010) Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15:7313–7352

Farvin KHS, Jacobsen C (2013) Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. Food Chem 138:1670–1681

Firuzi O, Lacanna A, Petrucci R, Marrosu G, Saso L (2005) Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant

power" assay and cyclic voltammetry. Biochim Biophys Acta 1721: 174–184
Freile-Pelegrín Y, Robledo D (2013) Bioactive phenolic compounds from algae. In: Hernández-Ledesma B, Herrero M (eds) Bioactive compounds from marine foods: plant and animal sources. Wiley, Hoboken, pp 113–129
Goiris K, Muylaert K, Fraeye I, Foubert I, De Brabanter J, De Cooman L (2012) Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. J Appl Phycol 24:1477–1486
Goiris K, Muylaert K, Voorspoels S, Noten B, De Paepe D, Baart E, De Cooman L (2014) Detection of flavonoids in microalgae from different evolutionary lineages. J Phycol 50:483–492
Guedes AC, Malcata FX (2012) Nutritional value and uses of microalgae in aquaculture. In: Muchlisin ZA (ed) Aquaculture. InTech Open, Riejecka, pp 59–78
Guedes AC, Amaro HM, Pereira RD, Malcata FX (2011) Effects of temperature and pH on growth and antioxidant content of the microalga *Scenedesmus obliquus*. Biotechnol Prog 27:1218–1224
Hajimahmoodi M, Faramarzi MA, Mohammadi N, Soltani N, Oveisi MR, Nafissi-Varcheh N (2010) Evaluation of antioxidant properties and total phenolic contents of some strains of microalgae. J Appl Phycol 22:43–50
Holt EM, Steffen LM, Moran A, Basu S, Steinberger J, Ross JA, Hong CP, Sinaiko AR (2009) Fruit and vegetable consumption and its relation to markers of inflammation and oxidative stress in adolescents. J Am Diet Assoc 109:414–421
Ito N, Hirose M, Fukushima S, Tsuda H, Shirai T, Tatematsu M (1986) Studies on antioxidants: their carcinogenic and modifying effects on chemical carcinogenesis. Food Chem Toxicol 24:1071–1082
Jagani S, Chelikani R, Kim DS (2009) Effects of phenol and natural phenolic compounds on biofilm formation by *Pseudomonas aeruginosa*. Biofouling 25:321–324
Jerez-Martel I, Garcia-Poza S, Rodriguez-Martel G, Rico M, Afonso-Olivares C, Gomez-Pinchetti JL (2017) Phenolic profile and antioxidant activity of crude extracts from microalgae and cyanobacteria strains. J Food Qual 2017:2924508
Landete JM (2013) Dietary intake of natural antioxidants: vitamins and polyphenols. Crit Rev Food Sci Nutr 53:706–721
Li HB, Cheng KW, Wong CC, Fan KW, Chen F, Jiang Y (2007) Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food Chem 102:771–776
Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. Current protocols in food analytical chemistry. Wiley, Hoboken, pp F4.3.1–F4.3.8
López A, Rico M, Rivero A, de Tangil MS (2011) The effects of solvents on the phenolic contents and antioxidant activity of *Stypocaulon scoparium* algae extracts. Food Chem 125:1104–1109
Machu L, Misurcova L, Vavra Ambrozova J, Orsavova J, Mlcek J, Sochor J, Jurikova T (2015) Phenolic content and antioxidant capacity in algal food products. Molecules 20:1118–1133
Masella R, Di Benedetto R, Varì R, Filesi C, Giovannini C (2005) Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. J Nutr Biochem 16:577–586
Onay M, Sonmez C, Oktem HA, Yucel AM (2014) Thermo-resistant green microalgae for effective biodiesel production: isolation and characterization of unialgal species from geothermal flora of Central Anatolia. Bioresour Technol 169:62–71
Onay M, Sonmez C, Oktem HA, Yücel M (2016) Evaluation of various extraction techniques for efficient lipid recovery from thermo-resistant microalgae, *Hindakia, Scenedesmus* and *Micractinium* species—comparison of lipid extraction methods from microalgae. Am J Anal Chem 7:141–150
Onofrejová L, Vašíčková J, Klejdus B, Stratil P, Mišurcová L, Kráčmar S, Kopecký J, Vacek J (2010) Bioactive phenols in algae: the application of pressurized-liquid and solid-phase extraction techniques. J Pharm Biomed Anal 51:464–470
Panche AN, Diwan AD, Chandra SR (2016) Flavonoids: an overview. J Nutr Sci 5:e47
Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Med Cell Longev 2:270–278
Pietta PG (2000) Flavonoids as antioxidants. J Nat Prod 63:1035–1042
Prior RL (2003) Fruits and vegetables in the prevention of cellular oxidative damage. Am J Clin Nutr 78:570S–578S
Prior RL, Wu X, Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53:4290–4302
Rico M, Lopez A, Santana-Casiano JM, Gonzalez AG, Gonzalez-Davila M (2013) Variability of the phenolic profile in the diatom *Phaeodactylum tricornutum* growing under copper and iron stress. Limnol Oceanogr 58:144–152
Safafar H, van Wagenen J, Møller P, Jacobsen C (2015) Carotenoids, phenolic compounds and tocopherols contribute to the antioxidative properties of some microalgae species grown on industrial wastewater. Mar Drugs 13:7339–7356
Shebis Y, Iluz D, Kinel-Tahan Y, Dubinsky Z, Yehoshua Y (2013) Natural antioxidants: function and sources. Food Nutr Sci 4:643–649
Shetty V, Sibi G (2015) Relationship between total phenolics content and antioxidant activities of microalgae under autotrophic, heterotrophic and mixotrophic growth. J Food Resour Sci 4:1–9
Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 16:144–158
Sonmez C, Elcin E, Akın D, Oktem HA, Yucel M (2016) Evaluation of novel thermo-resistant *Micractinium* and *Scenedesmus* sp. for efficient biomass and lipid production under different temperature and nutrient regimes. Bioresour Technol 211:422–428
Sørensen M, Gong Y, Bjarnason F, Vasanth GK, Dahle D, Huntley M, Kiron V (2017) *Nannochloropsis oceania*-derived defatted meal as an alternative to fishmeal in Atlantic salmon feeds. PLoS One 12: e0179907
Sugamura K, Keaney JF Jr (2011) Reactive oxygen species in cardiovascular disease. Free Radical Biol Med 51:978–992
Team RC (2013) R: A language and environment for statistical computing. (Vienna, Austria: Rfoundation for statistical computing). Available from https://www.r-project.org. Accessed 2 January 2019
Vacca RA, Valenti D, Caccamese S, Daglia M, Braidy N, Nabavi SM (2016) Plant polyphenols as natural drugs for the management of Down syndrome and related disorders. Neurosci Biobehav Rev 71: 865–877
Wang T, Jónsdóttir R, Ólafsdóttir G (2009) Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chem 116:240–248
Wang W, Sun C, Mao L, Ma P, Liu F, Yang J, Gao Y (2016) The biological activities, chemical stability, metabolism and delivery systems of quercetin: a review. Trends Food Sci Technol 56:21–38
Waris G, Ahsan H (2006) Reactive oxygen species: role in the development of cancer and various chronic conditions. J Carcinog 5:14
Zhang L, Ravipati AS, Koyyalamudi SR, Jeong SC, Reddy N, Smith PT, Bartlett J, Shanmugam K, Münch G, Wu MJ (2011) Antioxidant and anti-inflammatory activities of selected medicinal plants containing phenolic and flavonoid compounds. J Agric Food Chem 59:12361–12367
Zhishen J, Mengcheng T, Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 64:555–559