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Evaluation of the antioxidant properties of microalgae naturally isolated from Mediterranean Morocco

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ABSTRACT

This study aimed to investigate the antioxidant potentials of four locally-isolated microalgae. The following four microalgae; *Phaedactylium tricornutum*, *Nannochloropsis gaditana*, *Nannochloris* sp. and *Tetraselmis suecica*, were screened for their antioxidant activity using DPPH free radical scavenging assay. Natural antioxidants, including carotenoids and phenolics content were measured in three solvents with different polarities; ethyl acetate, hexane, and water extract biomass. Maximum and minimum concentrations of these compounds were found in *Phaedactylium tricornutum* and *Nannochloropsis gaditana*, respectively. Therefore, the total polyphenols and carotenoid content measurement were performed in three solvents extract. The highest antioxidant activity, total polyphenols contents, and carotenoids contents were registered for *Phaedactylium tricornutum* and *Tetraselmis suecica*, respectively. The results showed that there is a strong correlation between total polyphenol, carotenoid content, and the total antioxidant activity of the microalgae.

INTRODUCTION

The antioxidant is a molecule that protects organisms against free radicals and it is used in food industries as an additive to prevent lipid oxidation (Lin *et al*., 2014). Nowadays, the consumption of antioxidants aims to improve the quality of life, explains

their commercialization as a nutraceutical and food supplement. At present, there is a tendency in food industry, to replace synthetic antioxidants, such as butylated hydroxytoluene, with natural alternative (rosemary, green tea, and grape seed). Microalgae are a new and reproducible source for natural antioxidants (Morowvat & Ghasemi, 2016). Due to its essential polyunsaturated fatty acids, phytosterols, sulfated polysaccharides, pigments, and polyphenols compounds; the microalgae are already used in commercial production of antioxidant carotenoids; *Dunaliella* species used for the production of beta-carotene and *Haematococcus* for production of astaxanthin. Also, the microalgae represent a source for tocopherols (vitamin E), ascorbic acid (vitamin C) or polyphenols compounds (Cuellar-Bermudez *et al*., 2015). The polyphenol compounds, found in the terrestrial plants such as tea and raisins, constitute an important class of antioxidants. Several studies at laboratory conditions and photobioreactors have been conducted to exploit the bioactive antioxidant and polyphenols contents from microalgae (Hajimahmoodi *et al*., 2010; Custódio *et al*., 2012; Tibbetts *et al*., 2015). The free radicals are generated by uncontrollable aerobic metabolisms. Also, the free radicals production can be influenced by external causes like cigarettes and pollutions, but due to their biochemical composition, microalgae can reduce and clean the free radicals by scavenging the radical initiators(Saha *et al*., 2004).

In this study, during a screening program, four microalgae were isolated and identified using morphological and molecular methods. Culture medium Guillard F/2 was used for the growth and preservation of microalgae during this study. The antioxidant properties and total polyphenols content of the intracellular extracts from four microalgae were examined using different solvent extracts that have different polarities; ethyl-acetate, n-hexane, and water.

MATERIALS AND METHODS

Strain, media, and culture condition

Microalgae species investigated in this experiment were isolated from seawater at M'diq Bay (west of the Mediterranean, Morocco). Morphological studies and taxonomical approaches were conducted to identify and isolate microalgae. The isolation and culture procedures were performed in Guillard F/2 medium.

The four species of microalgae; *Nannochloropsis gaditana*, *Nannochloris sp*, *Tetraselmis suecica*, and *Phaedactylium tricornutum* were cultivated in the same conditions (Erlenmeyer flasks of 1000 mL, temperature of 21°C, illumination of 18/6 h and agitation/aeration with filtered compressed air).

Sample preparation

Microalgae species were collected after seven days in stationary phase, and subsequently centrifuged at 5.000×g for 10 min at 4 °C. The pellet that represents the intracellular

content was conserved in a refrigerator overnight at (4°C). Approximately 2 mL of n-hexane, the non-polar solvent was added to 0.1 g of dried samples and incubated for 30 min at room temperature (25°C). Then, the mixture was centrifuged at 4500×g for 10 min and the supernatant was collected. Approximately 2 mL of n-hexane was added to the pellet, the above steps were repeated and the two supernatants obtained were mixed together. The remaining compounds were extracted using ethyl acetate (2 mL) as a polar solvent (polarity index 4.4) for 30 minutes as described above. Then, the obtained supernatants were collected. Finally, two subsequent extractions, using hot water (80°C) were performed to collect the residual antioxidant and polyphenols compounds in the investigated samples, as the most polar solvent (polarity index 10.2). The compounds obtained were stored at 0°C until investigation. The water fraction was used directly in the next analysis. However, the extracts isolated with the other solvents (n-hexane and ethyl acetate), were dried before analysis(Li *et al*., 2007).

Antioxidant capacity assay

In our study, to determine the antioxidant capacity in the intracellular components, DPPH assay was performed using three solvents with different polarities. In brief, a methanolic DPPH solution was prepared daily (0.1 mmol/mL). Ethyl acetate and n-hexane fractions were diluted in ethanol (500 µL for each solvent) before use in the antioxidant activity analysis, while the aquatic fraction was used immediately after thawing. The aliquots were mixed with 2 mL of DPPH solution, and the extracts were vortexed for 10 seconds, then incubated, in the dark at room temperature (25°C), for 40 min(Hajimahmoodi *et al*., 2010). Ascorbic acid was used as a standard calibration curve ($y = 5.849x-0.02$; $R^2 = 0.994$).

Analysis of different classes of antioxidant

Determination of total polyphenol compounds

In this study, the Folin-Ciocalteu reagent method was used to determine the total polyphenol content(Li *et al*., 2007). This method is based on the reduction of Folin reagent, which is a mixture of phosphotungstic (WO_4^{-2}) and phosphomolybdic (MoO_4^{-2}), leading to the formation of blue color by the oxidizable group ofpolyphenol compounds. Briefly, a volume of 200 µL of each diluted sample was added to 1.0 mL of Folin–Ciocalteu reagent (1:10, *v/v*). 800 µL of saturated sodium carbonate with a concentration of 75 g/l was added after 4 min. The absorbance was measured at λ_{765} nm after 2 hours incubation at 25°C(Amoussa *et al*., 2015). Acid Gallic was used as a calibration curve ($y = 0.011 \times -0.002$, $R^2 = 0.999$). The median of three values was calculated and the results expressed using µg of Gallic Acid Equivalents (GAE)/g of extract.

Determination of total flavonoids

The flavonoids procedure was determined by adding 100 µg/mL of each extract to 3 mL of methanol, 0.2 mL of potassium acetate 1M, 0.2 mL of 10% aluminum chloride and 5.6 mL of distilled water. The components were then incubated for 30 minutes at room temperature(Nadhiya & Vijayalakshmi, 2014). The absorbance was measured at λ_{415} nm using a UV-Vis spectrophotometer (Rayleigh UV-1800). For the standard curve (y = 0.0271 ×0.0129; R^2 = 0.996), Quercetin (QE) was used as a positive control to evaluate total flavonoid content (µg quercetin equivalent/g of sample).

Determination of total carotenoids

Carotenoids and chlorophylls (Chla, Chlb) were quantified in the three solvent extracts as previously described(Fazeli *et al*., 2006). The reading by UV-Vis spectrophotometer was performed at three wavelengths: $\lambda_{663.2}$, $\lambda_{646.8}$ and λ_{470} nm. Linchtenthaler HK (1987) equations were used to calculate the concentration of photosynthetic pigments (µg/g).

Chlorophyll a (Chla) = (12.25 $A_{663.2}$) - (2.79 $A_{646.8}$)

Chlorophyll b (Chb) = (21.5 $A_{646.8}$) - (5.10 $A_{663.2}$)

Total Carotenoids = (1000 A_{470}) - (1.82 Chlorophyll a (Chla)) - (85.02 Chlorophyll b (Chlb)) / 198

Statistical analysis

One-way ANOVA with a 5% level of statistical significance and the Tukey TSD test of the IBM SPSS for multiple comparisons were used in this study to evaluate the difference between solvents.

RESULTS AND DISCUSSION

Determination of antioxidant capacity

The small subunit ribosomal RNA sequences of *Nannochloropsis gaditana* (MN625926), *Phaeodactylum tricornutum* (MN625939), *Nannochloris Sp,KMMCC161* (MN625923) and *Tetraselmis suecica* (MN625941) are published in NCBI database under the GenBank accession numbers mentioned between parentheses.

The four microalgae were stored at controlled conditions for preservation and future cultivation.

The screen of microalgae species is based on their, *in vivo* or *in vitro*, antioxidant competences. The four investigated species showed significantly different ($P<0.001$) antioxidant capacities between each other (Table 1).The value of antioxidant capacity varied between 0.38 and 0.41mg/g. The high flux fraction was that of water with values of 0.17 for *P.tricornutum* followed by 0.16 mg/g for *N. sp*, *T.suecica*, and *N.gaditana*

species. Thereafter, the fraction of n-hexane was 0.14 mg/g for *T.suecica* and *N. sp*, and 0.13, 0.12 mg/g for *P.tricornutum*, and *N.gaditana*, respectively. The lowest values were obtained in ethyl acetate fraction with 0.10 mg/g for all investigated species. On the other hand, another study(Morowvat & Ghasemi, 2016) showed that the fraction of ethyl acetate was found to be higher than the fractions of hexane and water with a values of 20.67±0.98 µmol of Trolox/g and 24.89±1.81 µmol of Trolox/g for *F. ambigua* and *S. rubescens*, respectively. These data suggest that the different conditions of the assay can affect the results.

The results obtained in this study, showed that *P. tricornutum*, is the diatom with the intracellular content that have the highest antioxidant activity among all the species tested in this study. With our results, we could be concluded that *P. tricornutum* as an important source of natural antioxidants, and can be used for the production of chemical syntheses as an alternative to medicinal plants(Rodriguez-Garcia & Guil-Guerrero, 2008).

Table 1. Antioxidant analysis (mg/g acid ascorbic) in three intra-substance fractions of marine microalgae.

Species	**Water fraction**	**Ethyl acetate fraction**	**n-hexane fraction**	**Total**
Nannochloropsis gaditana	0.16^{a}±0.0002	0.10^{a} ±0.0002	0.12^{a} ±0.0003	0.38^{a} ±0.001
Phaeodactylum tricornutum	0.17^{b}±0.0003	0.10^{a} ±0.0004	0.13^{b} ±0.0009	0.41^{c} ±0.001
Nannochloris Sp	0.16^{a}±0.0005	0.10^{a} ±0.0005	0.14^{bc} ±0.0003	0.40^{b} ±0.001
Tetraselmis suecica	0.16^{a}±0.0002	0.10^{a} ±0.0003	0.14^{c} ±0.0014	0.40^{b} ±0.002

Statistically different values (p = 0.05, one-way ANOVA, with Tukey TSD test, n = 3) are indicated by a different letter per antioxidant parameter for each microalgal species.

Analysis of different classes of antioxidants

Determination of polyphenols compounds

The total polyphenols content (based upon µg GAE/g) in the intracellular extract of the four microalgae, was between 23.14±0.88 and 39.68±0.60µg GAE/g(Table 2). Moreover, the total content of flavonoids varied between 5.95±0.34 and 9.02±0.91 µg/g of EQ for*T. suecica* and *N. sp* species, respectively (Table 3). The polyphenols measurement in the three solvents with different polarities were 39.68±0.60, 33.54±0.75, 28.42±1.18, and 23.14±0.88µg GAE/g for *P.tricornutum*, *N. Sp*, *T.suecica*, and *N. gaditana* species, respectively. The highest polyphenols content was found in *P.tricornutum* with a value of 24.52±0.18 µg GAE/g that was extracted using n-hexane fraction. For the ethyl acetate fraction, *N. sp* has the highest value of 8.41±0.14µg GAE/g.

Table 2. Phenolic contents (µg GAE/g) of three fractions investigated for intra-cellular substance of four naturally isolated microalgae.

Species	Water fraction	Ethyl acetate fraction	n-hexane fraction	Total
Nannochloropsis gaditana	6.19^{c} ±0.24	3.43^{a} ±0.32	13.51^{a} ±0.32	23.14^{a} ±0.88
Phaeodactylum tricornutum	8.10^{d} ±0.18	7.06^{c} ±0.23	24.52^{d} ±0.18	39.68^{d} ±0.59
Nannochloris Sp	2.65^{b} ±0.18	8.41^{d} ±0.14	22.48^{c} ±0.43	33.54^{c} ±0.75
Tetraselmis suecica	1.93^{a} ±0.33	4.12^{b} ±0.43	22.37^{b} ±0.42	28.42^{b} ±1.18

Statistically different values (p = 0.05, one-way ANOVA, with Tukey TSD test, n = 3) are indicated by a different letter per phenolic content for each microalgal species.

Table 3. Flavonoids contents (µg QE/g) of three fractions investigated for intra-cellular substance of four naturally isolated microalgae.

Species	Water fraction	Ethyl acetate fraction	n-hexane fraction	Total
Nannochloropsis gaditana	2.25^{a} ±0.074	1.77^{a}±0.11	2.27^{b}±0.07	6.29^{b}±0.26
Phaeodactylum tricornutum	2.96^{d} ±0.06	2.75^{d}±0.07	3.15^{c}±0.11	8.85^{c}±0.24
Nannochloris sp.	2.59^{b} ±0.74	2.10^{b}±0.07	4.33^{d}±0.09	9.02^{d}±0.91
Tetraselmis suecica	2.93^{c} ±0.07	2.32^{c}±0.19	0.70^{a}±0.08	5.95^{a}±0.34

Statistically different values (p = 0.05, one-way ANOVA, with Tukey TSD test, n = 3) are indicated by a different letter per flavonoid content for each microalgal species.

The intracellular polyphenol content in *P.tricornutum* extracted by hexane fraction was greater than the other solvent fractions with 24.52µg GAE/g. Indeed, Morowvat and Ghasemi (2016) study, showed that the polyphenol extracted by hexane fraction is greater than ethyl acetate and water fractions for *Dunaliella salina*. Other previous studies have demonstrated that polyphenol compounds are more soluble in a polar organic solvent, such as acetone, than in water liquid(Wang *et al*., 2012), which is the case for *N.sp* and *T.suecica* species in this study. This also suggests that the solvent used for the extraction acts directly on the intracellular phenolic content. Nevertheless, the polyphenolis composed of 8000 different compounds with different molecular structures, the transfer of an electron or a hydrogen atom can form a more stable metabolite; they serve as an essential element in microalgae cells (Safafar *et al*., 2015).

Determination of carotenoids

A highly significant difference ($P<0.01$) determined by ANOVA tests was noted between the carotenoid and chlorophyll contents extracted by the three solvents tested.

The total carotenoid content detected in this experiment (Table 4) was 5.95±0.12, 5.91±0.11, 5.44±0.05 and 3.67±0.05µg/g for *T.suecica*, *N. sp*, *P.tricornutum* and *N.gatitana*, respectively. The hexane fraction exhibited a high content of carotenoid compound 4.47±0.1, 4.33±0.02, 4.19±0.01 and 3.35±0.02µg/g in *T.suecica*, *P.tricornutum*, *N. sp*, and *N.gatitana*, respectively. The highest contents among the fractions of ethyl-acetate extracts was carotenoid content of *N.sp*, (1.52±0.11µg/g), followed by that of *T.suecica* (0.88±0.01µg/g), *P.tricornutum* (0.54±0.02µg/g), and *N.gatitana* (0.16±0.02µg/g), respectively. Water fraction obtained from *N.gaditana* (0.16±0.02 µg/g) showed the lowest contents in the three solvents. The highest chlorophyll a (Chla) and chlorophyll b (Chlb) contents were registered in *N.sp* species with value of 7.29±0.41µg/g and 5.45±0.26µg/g, respectively.

Table 4. Pigment content (µg/g) of three different studied fractions observed in intracellular substances of four naturally isolated local microalgae.

Fractions	**Pigment content**	*Nannochloropsis gaditana*	*Phaeodactylum tricornutum*	*Nannochloris Sp*	*Tetraselmis suecica*
	Chl a	0.01^{a}±0.01	0.09^{d}±0.01	0.02^{b}±0.01	0.09^{c}±0.01
n-Hexane	**Chl b**	0.02^{b}±0.01	0.15^{c}±0.01	0.02^{a}±0.01	0.16^{d}±0.01
	carotenoids	3.35^{a}±0.02	4.33^{c}±0.02	4.20^{b}±0.01	4.47^{d}±0.10
	Chl a	3.21^{c}±0.03	1.67^{b}±0.03	5.60^{d}±0.4	0.76^{a}±0.01
Ethyle-Acetate	**Chl b**	1.67^{b}±0.05	1.05^{a}±0.04	2.71^{c}±0.24	2.87^{d}±0.04
	carotenoids	0.16^{a}±0.02	0.54^{b}±0.02	1.52^{d}±0.11	0.88^{c}±0.01
	Chl a	0.73^{b}±0.02	2.03^{d}±0.02	1.68^{c}±0.01	0.72^{a}±0.02
Water	**Chl b**	0.98^{a}±0.03	1.09^{c}±0.01	2.72^{d}±0.02	1.01^{b}±0.04
	carotenoids	0.16^{a}±0.02	0.57^{c}±0.01	0.19^{b}±0.01	0.59^{d}±0.01
	Chl a	3.95^{c}±0.05	3.80^{b}±0.04	7.29^{d}±0.41	1.56^{a}±0.03
Total	**Chl b**	2.67^{b}±0.08	2.28^{a}±0.05	5.45^{d}±0.26	4.04^{c}±0.08
	carotenoids	3.67^{a}±0.05	5.44^{b}±0.05	5.91^{c}±0.11	5.95^{d}±0.12

Statistically different values ($p = 0.05$, one-way ANOVA, with Tukey TSD test, $n = 3$) are indicated by a different letter per carotenoid, chlorophyll contents for each microalgal species.

Based on the results, it could be suggested that the extraction of the carotenoids contents was influenced by using a suitable solvent able to penetrate the cell walls for carotenoids and dissolve the targeted compounds without any risk of damage (Safafar *et al*., 2015), worked with a suitable technique like that used in the Parniakov study which were used the pulsed electric field (Parniakov *et al*., 2015), and growing in appropriate conditions; nutrients, light intensity, salinity, and temperature.

Correlation between the antioxidant capacity and total polyphenol content

The correlation coefficient between the antioxidant capacity and total polyphenols contents was found to be R^2 = 0.76 (Fig. 1; 3A-B). Moreover, the highest correlation between the polyphenols contents and antioxidants activity was R^2 =0.77 in water fraction and R^2 =0.75 in hexane fraction. The correlation between the antioxidant activity and the carotenoids contents (R^2 =0.98), followed by the lowest values of flavonoids (R^2 =0.50), chlorophyll b (R^2 =0.44), and, chlorophyll a (R^2 =0.05), respectively.

Our results demonstrated that polyphenols and carotenoid contents could have a substantial contribution to the total antioxidant capacities of the investigated microalgae strains which confirmed the data published by Morowvat and Ghasemi (2016). Several other studies showed the significant contribution of polyphenols to the antioxidant capacity of microalgae; chemically, the polyphenols can be divided into different classes; such as flavonoids, isoflavonoids, stilbenes, lignans, and phenolics(Manach *et al*., 2004), these, different classes of flavonoids were produced by marine microalgae species(Natrah *et al*., 2007). In addition, the carotenoids have a direct influence on the antioxidant activity of marine microalgae(Goiris *et al*., 2014). They have an excellent antioxidant potential through the reduction of free radicals by deactivation of reactive oxygen species (ROS) formed by the exposure to light and air. There are two classes of carotenoids; the first contains only atoms of carbons and hydrogen, and the second are xanthophylls, with the oxygen atoms. The xanthophylls can be synthesized by plants, green microalgae, brown algae and diatoms. On the other hand, other researchers reported the non-significant correlation between the antioxidant activity and the phenolic, carotenoid content(Goh *et al*., 2010). Therefore, these discrepancies can be explained by the intervention of culture conditions as well as oxidative stress(Safafar *et al*., 2015).

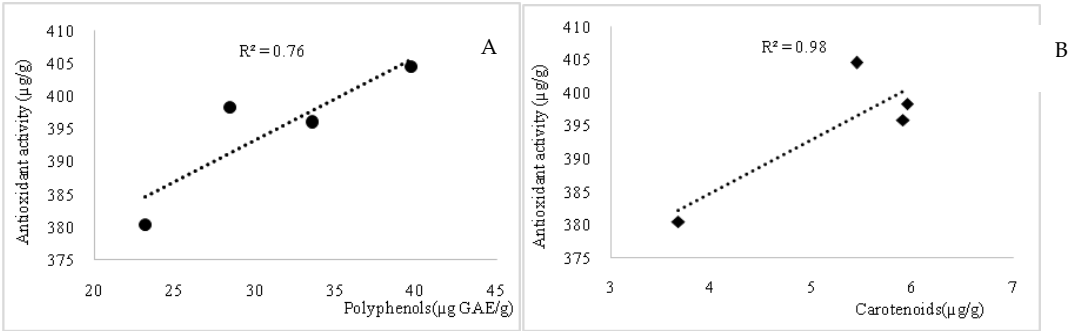

Fig. 1. Correlation between antioxidant activity and (A) phenolic contents, (B) carotenoid contents for four species of microalgae

CONCLUSION

Based upon our results of antioxidant activity and polyphenol contents, *Phaeodactylum tricornutum* showed a great promise with 0.41 mg/g and 39.68 µg GAE/g, respectively. Consequently, using this species as source of biomolecules and a complementary source will increase the demand for more antioxidants to be used as dietary supplements and related industries. So far, a small number of the microalgae were examined for their ability to produce large scale-polyphenols and antioxidant activities. Therefore, it is necessary to evaluate other strains of microalgae of different ecosystems and habitats.

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