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Determination of the Nutritional and Functional Metabolites of *Nannochloropsis gaditana* Produced in Algeria and Evaluation of Its Antioxidant Activity

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ABSTRACT

The aim of this work was to evaluate the microalgae Nannochloropsis gaditana grown in Algeria. The different analyzes of the primary metabolites showed an interesting nutritional profile. The microalgae has a fresh matter content of 9.33 ± 1.15%. The amount of ash (6.4 ± 0.23%) was high and reflects a mineral richness. Total sugars represent 7.13 ± 0.23%. Proteins and lipids represent 13.38 ± 0.2% and 7 ± 1%, respectively. The Ethanolic (EENg) and Methanolic (MENg) extracts obtained from 10 g of lyophilisates of N. gaditana represent respectively 24 ± 1.65% and 14.2 ± 0.7%. The evaluation of the polyphenols in these extracts shows interesting rates representing 41.53 ± 0.41 mg gallic acid equivalent (GAE)/g of EENg dry extract and 38.72 ± 0.33 mg GAE/g for the MENg. The flavonoid contents were 19.82 ± 1.65 and 18.61 ± 1.45 mg quercetin equivalent (QE)/g respectively for the ethanolic and methanolic extracts of these microalgae. The condensed and hydrolyzable tannins represent respectively 0.35 ± 0.04 and 0.63 ± 0.09 mg of tannic acid/g of dry EENg. They also represent respectively 0.50 ± 0.08 and 0.18 ± 0.02 mg of tannic acid/g of dry MENg. The concentrations of the EENg and MENg extracts allowing the reduction or inhibition (IC_{50}) of the 2,2-Diphenyl-1-Picrylhydrazyl radicals ($DPPH^{\bullet}$) were comparable and represent 1.16 ± 0.06 and 2.02 ± 0.04 mg/ml, but remain significantly higher than that of ascorbic acid (0.1 ± 0.02 mg/ml). Evaluation of the antioxidant activities of the EENg, MENg and ascorbic acid extracts by the reducing power of iron yielded respectively 0.031 ± 0.03, 0.022 ± 0.009 and 1.67 ± 0.035 units of optical density at a concentration of 0.15 mg/ml and 0.349 ± 0.037, 0.414 ± 0.012 and 2.66 ± 0.007 units of optical density at the concentration of 5 mg/ml.

Keywords: Microalgae, *Nannochloropsis gaditana*, Primary metabolites, Nutritional profile, Antioxidant activities

INTRODUCTION

Microalgae are known as sources of structurally new and biologically active metabolites [1,2]. They can produce lipids, proteins vitamins, pigments and other molecules exploited for health, food and feed additives, cosmetics and for energy production [3,4]. There is a wide range of compounds that could potentially be used as antioxidants such as phenolic compounds, long-chain polyunsaturated fatty acids, terpenoids, amino acids and carotenoids [5-7]. Antioxidants are increasingly being used in food supplements as bioactive compounds and in functional foods to increase their shelf life and prevent unwanted lipid oxidation. Nearly all commercially available natural antioxidants are derived from terrestrial plants [8]. Furthermore, the qualities of the microalgal cells can be controlled, so that they contain no herbicides and pesticides, or any other toxic substances, by using clean nutrient media for growing the microalgae [9]. *Nannochloropsis* sp., on the other hand, is a unicellular green alga, spherical in shape, with diameter of about 2-5 µm, belonging to the Eustigmatophyceae class. It has five species and they are *N. gaditana*, *N. salina*, *N. oculata*, *N. oceanica* and *N. limnetica* [10-14].

It plays an important role in the food chain system and is also commonly used as live feed. Thus, it is widely cultivated in fish hatcheries and shrimp farm [15]. Until now *Nannochloropsis* species were known to occur almost exclusively in marine or saline habitats [16]. *Nannochloropsis gaditana* was used in aquaculture for the cultivation of fish, either directly or via rotifers [17]. *N. gaditana* is also recognised as a good potential source of Eicosapentaenoïc Acid (EPA), an important polyunsaturated fatty acid for human consumption for prevention of several diseases [18].

The purpose of this work is to evaluate the nutritional and functional principles of *N. gaditana* a species of microalgae produced in Algeria and to evaluate the antioxidant activity of its ethanolic and methanolic extracts by the radical 2,2-Diphenyl-1-Picrylhydrazyl ($DPPH^{\bullet}$) and Ferric Reducing Antioxidant Power (FRAP).

Biological material

N. gaditana is a biomass produced by the company Partisano Biotech Algeria (PBA) in Sidi Bel Abbès and the samples were supplied to us in the form of powder obtained after cultivation and freeze-drying.

Biochemical analyzes

Water content was determined by weight difference after drying of sample, following the official method of [19]. The inorganic matter content was conventionally the residue of the substance after mineralization of dry matter sample in a muffle furnace (Heraeus Instruments). It was obtained by burning at 500-600°C [19]. Total carbohydrates were determined by phenol-sulfuric acid method [20]. The total lipid of *N. gaditana* was extracted using a Soxhlet extractor system. Approximately 3 g of biomass powder was weighed into a cellulose thimble inside the extraction chamber. A total of 150 ml pure n-hexane was used to extract the lipid for 6 h at the rate of 10 refluxes per hour to achieve maximum extraction efficiency. The extracted lipid was measured after removing the solvent using vacuum rotary evaporator (Heidolph instruments) to evaporate the n-hexane at 35°C for 60 min and then lipid content was calculated [21]. The crude proteins of samples were determined by the Kjeldahl method and were calculated using a nitrogen conversion factor of 5.95 [22]. The results were expressed as percent of dry weight.

Preparation of antioxidants extracted from the *N. gaditana*

The ethanolic and methanolic extracts were prepared from 10 g of the sample, macerated in 100 ml of 70% ethanol or methanol at ambient temperature and protected from light for 24 h, with magnetic stirring. The resulting extract was filtered with Whatman paper. The procedure was repeated a second time with the dry residue. The resulting filtrates were added and evaporated to dryness using a rotary evaporator (Heidolph instruments) at a temperature of 60°C. The dry extract was stored at 4°C until use.

Determination of secondary metabolic compounds

Determination of total polyphenols

The total polyphenols were determined by the method of Singleton et al. [23] using the Folin Ciocalteu reagent. 0.4 ml of each extract was mixed with 2 ml of Folin-ciocalteu reagent diluted (1:10 with distilled water) and 1.6 ml of Na_2CO_3 7.5%. After 30 min incubation, optical density of the samples with blue color was measured at 765 nm. Gallic acid was used as a standard. Results were expressed as gallic acid equivalent (GAE)/g dry weight of *N. gaditana* and calculate as mean value ± Standard Deviation (n=3).

Determination of total flavonoids

The total flavonoids of extracts were measured using a colorimetric assay slightly modified according Zhishen et al. [24]. In a test tube, 0.1 ml of the extract was diluted with water, mixed with 0.2 ml of 5% $NaNO_2$ and left to stand for 6 min, then added 0.2 ml of 10% $AlCl_3$ and mixed. After 6 min, 2 ml of 4% NaOH was added. The mixture was then made to 5 ml with distilled water. The absorbance of the mixture was measured at 510 nm against a prepared blank using a spectrophotometer UV-VIS (Shimadzu Scientific Instruments). The results were expressed in mg equivalent quercetin per gram of dry matter (mg EQ/g DM).

Determination of hydrolysable tannins (*Gallic tannins*)

The method of Mole and Waterman [25] was based on a reaction with ferric chloride. The mixture of tannic extract with ferric chloride reagent results in the formation of purple-red color complex with Fe^{3+} ion formation [26]. A solution of 0.01 M $FeCl_3$ was mixed with a solution of 0.001 M HCl (v/v). Add 3.5 ml of this reagent in 1 ml of the extract. After 15 s, read the absorbance at 660 nm. The hydrolysable tannins were expressed by the relationship: T%=OD (MV/Emoles P), with OD: optical density, Emoles: 2169 of gallic acid, M: 300, V: Volume of extract used, W: Weight of sample and T%: Percentage of hydrolysable tannins.

Determination of condensed tannins

Condensed tannins were determined according to the method proposed by the European Community [27]. 1 ml of each extract was mixed with 7.5 ml of distilled water, then added 5 ml of Folin Denis Reagent (FDR) and 10 ml of saturated solution of CO_3Na_2. This saturated solution was prepared from 43.75 g of Na_2CO_3 dissolved in 100 ml of hot water (70-80°C). After cooling the solution was filtered and then adjusted to 125 ml. After mechanical agitation, the preparation rests for 30 min; the absorbance of the mixture was measured at 760 nm using a spectrophotometer UV-Visible (Shimadzu Scientific Instruments). A tannic acid standard range was prepared in the same conditions with concentrations ranging from 0 to 0.1 g/l. A witness with distilled water instead of the extract was performed under the same conditions.

Antioxidant activity

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) scavenging assay

The hydrogen atom donation ability of chemical compounds in leaves and stems was measured on the basis to scavenge the DPPH free radical [28]. Fifty microliter of various concentrations of the extracts in methanol was added to 1950 ml of a 0.025 g/l methanol solution DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 515 nm. DPPH free radical scavenging activity in percentage (%) was calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = (A_{blanc} - A_{sample}/\text{Ablanc}) \times 100$$

Where, A blank was the absorbance of the control reaction (containing all reagents except the test compound). A sample is the absorbance of the test compound. The positive control was represented by a standard solution of an antioxidant; ascorbic acid, whose absorbance was measured in the same conditions as the samples and for each concentration the test was repeated 3 times. Graphically the Effective Concentration 50 (EC_{50}) was calculated by linear regression corresponding to that in antioxidants needed to reduce by half the amount DPPH initially present in the medium [29,30].

Ferric-reducing antioxidant power analysis

The reducing power of the extract was determined according to the method described by Oyaizu [31]. 1 ml of various concentrations of the extracts (mg/ml) in distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% of Potassium Ferricyanide ($K_3[Fe(CN)_6]$) water solution (2.5 ml).

The mixture was incubated at 50°C for 20 min. Aliquots of Trichloroacetic Acid (TCA) (2.5 ml, 10% aqueous solution) were added to the mixture which was then centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared $FeCl_3$ solution (0.5 ml, 0.1%). Reading the absorbance of the reaction medium was carried out at 700 nm against a blank prepared similarly by replacing extract with distilled water which was used to calibrate the apparatus (UV-visible spectrophotometer Shimadzu Scientific Instruments). The positive control was represented by a standard solution of an antioxidant; ascorbic acid, whose absorbance was measured, under the same conditions as the samples. An increase in absorbance corresponding to an increase of the reducing power of the extracts tested [32].

RESULTS AND DISCUSSION

The analysis of the chemical composition of the microalga *N. gaditana* showed a moisture content of 9.33% (Table 1) corroborated by that of Eryalç et al. [33], which yielded 9.56% for the same species. These same authors obtained an ash rate of 7.21% approximately comparable to our result (6.4%). According to Kent et al. [34], *Nannochloropsis* sp., contains 1.84% moisture and 11.32% ash. According to Bi and He [35], *N. salina* has a moisture and ash content of 2.9% and 13.8%, respectively. Also, Fernandes Seixas [36], reports a total sugar content of *N. gaditana* determined by phenol-sulfuric acid of 8.7% in accordance with our results (7.13%). Changes in crop parameters may affect the composition of carbohydrates in microalgae. Shifrin and Chisholm, Harrison et al. [37,38], showed that nitrogen deficiency leads to an increase in carbohydrates.

Table 1: Nutritional composition of *Nannochloropsis gaditana*, Values are presented as mean ± SD (n=3)

Parameters (%)	*Nannochloropsis gaditana*
Moisture	9.33 ± 1.15
Ash	6.4 ± 0.23
Total sugar	7.13 ± 0.23
Proteins (N × 5.95)	13.38 ± 0.26
Fat	7 ± 1

The protein content of *N. gaditana*, determined by the Kjeldahl method using the nitrogen conversion factor 5.95 [22] was 13.38%. This rate was considerably lower than that reported by Mert Eryalçn et al., (67.61%) for the same species. Other authors have reported that an increase in CO_2 concentration induces a decrease in protein in *Spirulina platensis* [39]. High phosphorus and nitrogen treatments as well as temperature changes induce an increase in protein levels and total lipids in cultured *N. gaditana* [40]. The lipid content of *N. gaditana* was 7%. Hu et al. [40], reported values ranging from 9 to 62% in *Nannochloropsis* sp. This variation was due to the cultivation parameters which demonstrated a nearly 4-fold increase in the lipid content of *Nannochloropsis* sp., grown in low nitrogen. According to Renaud et al. [41], salinity has a great influence on lipid content and according to Chiu et al. [42], *N. oculata* showed an increase in lipid content from 30.8%-50.4% in a medium of 2% CO_2. Slightly limited light conditions also increase the lipid content [43].

Secondary metabolic compounds of *Nannochloropsis gaditana*

Phenolic compounds such as flavonoids, phenolics and tannins were considered to be major factors in the ability of plant antioxidants. They also possess various biological activities such as anti-inflammatory, anti-atherosclerotic and anticarcinogenic [1]. These activities may be linked to their antioxidant activity. The yields of *N. gaditana*'s Ethanolic (EENg) and Methanolic (NEM) extracts were 24 ± 1.65% and 14.2 ± 0.7%, respectively. The extraction yield, the composition of the extracts obtained and their biological activity depend on the polarity and the nature of the solvents used [44,45]. In this work, the powder of *N. gaditana* was extracted with ethanol and methanol, solvents having an affinity for a great diversity of bioactive compounds, in this case the phenolic compounds [46].

The results show that the mean total phenol contents of EENg and MENg with respect to dry weight (PS) were respectively 41.53 ± 0.42 mg GAE/g and 38.74 ± 0.33 mg GAE/g (Figure 1). The phenolic content of EENg was higher than that of MENg ($P<0.05$). Hugo Pereira [47], reported a value of 83.3 mg GAE/g PS in the methanolic extract of *Nannochloris* sp., while Amal et al. [48], reported only 32 mg EAG/g PS in *N. gaditana*.

The flavonoid content of the EENg and MENg extracts revealed respective values of 19.82 ± 1.66 and 18.61 ± 1.45 QE/g PS (Figure 1). The content of these EENg compounds appears to be significantly greater than that of the MENg ($P<0.05$). According to Safafar et al. [8], the methanolic extract of *Nannochloropsis* sp., contains 6.45 mg GAE/g of polyphenols and 5.296 mg QE/g of flavonoids.

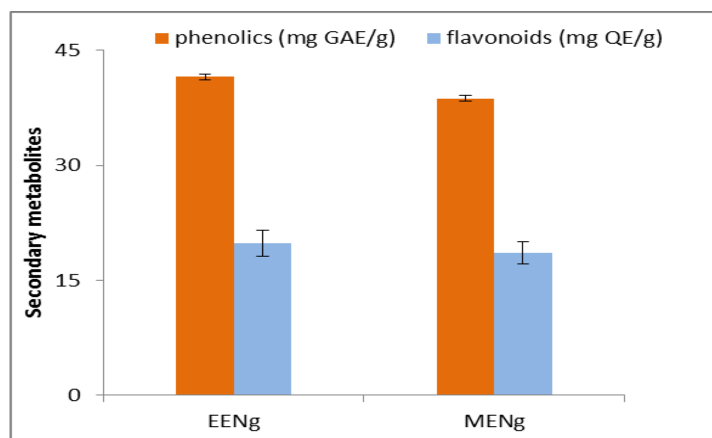

Figure 1: Contents of phenolics and flavonoids in *Nannochloropsis gaditana* (Values are expressed as mean ± standard deviation. The comparisons were made using ANOVA test with P<0.05 n=3)

Condensed tannins of the EENg and MENg were respectively 0.35 ± 0.05 and 0.50 ± 0.08 mg tannic acid/g while those which were hydrolyzable represent 0.63 ± 0.09 and 0.18 ± 0.02 mg gallic acid/g ($P<0.05$). Condensed tannins were slightly higher in EENg than in MENg ($P=0.03$). The same is true for the hydrolyzable tannins in the two extracts ($P<0.05$). This is consistent with the results reported by Rutikanga et al. [49], which showed that the green alga *Spirogyra* contains 0.399 g of tannic acid per 100 g of substrate (Figure 2).

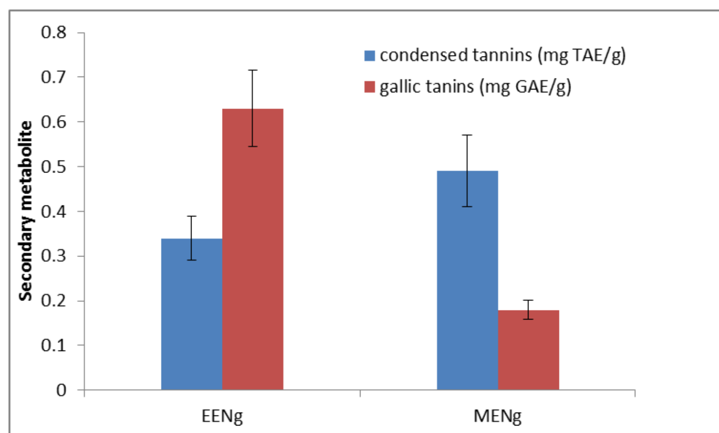

Figure 2: Contents of condensed and hydrolyzed tannins in *Nannochloropsis gaditana* (Values are expressed as mean ± standard deviation. The comparisons were made using ANOVA test with P <0.05 n=3)

Antioxidant activities

An antioxidant molecule is a substance which, even at low concentrations, can inhibit or retard the oxidation of a substrate [50]. In this study, the antioxidant capacity of *N. gaditana* was measured using the free radical scavenging assay and employing in DPPH$^{\bullet}$ its free radical form involving atom transfer of hydrogen and electron transfer. DPPH$^{\bullet}$, initially purple, discolours when the single electron pair. The activity of trapping DPPH radicals makes it possible to evaluate the antioxidant activity of the ethanolic and methanolic extracts of *N. gaditana* [51]. Based on this principle, the percentage inhibition of DPPH in our case was respectively 22.37 ± 3.5% and 12.27 ± 1.4% for EENg and MENg at the concentration of the extracts of 0.5 mg/ml (Figure 3). For the median concentration of inhibition (IC_{50}) which represents the concentration of the extracts responsible for 50% inhibition of the DPPH radicals, they were respectively 1.16 ± 0.06 and 2.02 ± 0.04 mg/ml for EENg and MENg and is lower than that of ascorbic acid (0.11 ± 0.02 mg/ml). *N. gaditana* has a moderate ability to reduce oxidative damage induced by free radicals.

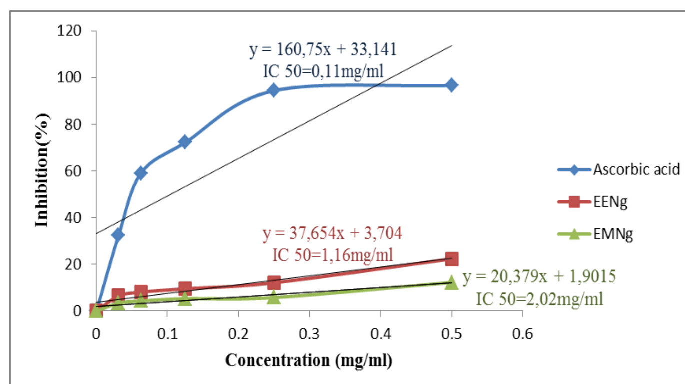

Figure 3: Evaluation by DPPH method of IC_{50} of the *Nannochloropsis gaditana* extract and standard ascorbic acid (n=3, ANOVA test, P<0. 05)

Our results are consistent with those of Safafar et al. [8], who reported that the inhibition percentages in *Nannochloropsis limnetica* and *Nannochloropsis salina* were 19.44% and 21.26%, respectively, with a concentration of 0.5 mg/ml. Amal Maadane et al. [48], reported that the IC_{50} of the ethanolic extract of *N. gaditana* is 365 µg/ml compared to an IC_{50} of vitamin C of 2.5 µg/ml.

According to Tusevski et al. [52], an extract with low antioxidant activity cannot be considered a bad source of antioxidant because an extract is composed of chemicals with different functional groups and polarities allowing it to behave differently according to the reaction mixture. This indicates that phenolic compounds may not be a major contributor to the antioxidant capabilities of either of these microalgae, which as noted by many authors contain various antioxidant compounds such as carotenoids, polyunsaturated fatty acids and Polysaccharides [53,54]. Studies have indicated that the reaction time and concentration of DPPH influence the antioxidant activity and kinetic parameters of bioactive molecules and plant extracts and microalgae. In the reaction with the DPPH radical, the antioxidant activity measured after 2 h of reaction was significantly greater than that measured after 30 min [48,55].

Ferric reducing antioxidant power

The reducing activity of the microalgae extracts was estimated by the chemical reduction reaction of Fe^{3+} present in the $K_3Fe(CN)_6$ complex to Fe^{2+}. The antioxidant activities of the extracts EENg, MENg and ascorbic acid were respectively 0.031 ± 0.03, 0.022 ± 0.009 and 1.67 ± 0.035 units of optical density at a concentration of 0.15 mg/ml and 0.349 ± 0.037, 0.414 ± 0.012 and 2.66 ± 0.007 units of optical density at the concentration of 5 mg/ml (Figure 4).

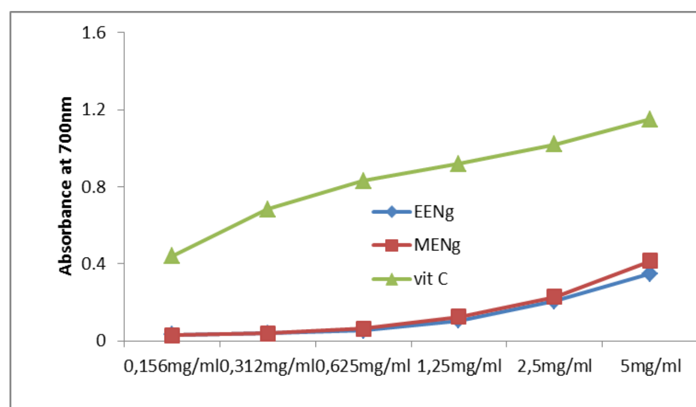

Figure 4: Antioxidant activities of ethanolic and methanolic extracts of *Nannochloropsis gaditana* and vitamin C by the reduction of iron (Values are expressed as mean ± standard deviation. The comparisons were made using ANOVA test with P<0.05 and n=3)

Safafar et al. [8] reported strong iron reduction capacity due to polyphenols of some species of microalgae grown on industrial wastewater.

CONCLUSION

The *N. gaditana* microalga produced in Algeria is rich in primary metabolites. The production unit of this microalga located in Sidi Bel Abbes, Algeria, plans to develop it on the nutritional level with a view to its use as a substitute for imported, expensive cereals intended for feeding livestock. Also, these microalgae can provide a large number of possibilities for the development of healthier food products by using them as food or as a source of functional health-promoting ingredients. The functional profile of *N. gaditana* demonstrated by the presence of phenolic compounds and antioxidant activity is promising. The beneficial effects of microalgae on human health would be numerous: anticancer, antiviral or anti-inflammatory. However, research has yet to be carried out, as few of these potential effects have been validated on humans.

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