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Evaluation of Antioxidant and Antiproliferative Activities of *Nannochloropsis gaditana* Extracts.

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ABSTRACT

To identify a new source of natural antioxidants and anticancer agents from marine microalgae, we investigated the evaluation of phenolic compounds in *Nannochloropsis gaditana* extracts namely, acetonic, methanolic, dichloromethanic, hexanic and aqueous extracts. The antioxidant activity of these extracts was evaluated by two methods: the scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical and the ferric reducing power .The antiproliferative activity was also assessed on A549 (Human lung cancer cell line) by MTT assay. The results revealed that the aqueous extract and the methanolic extract had the highest amount of phenolic compounds and the highest ferric reducing power. In DPPH assay, the aqueous extract and the acetonic extract exhibited the highest antioxidant activity. The acetonic extract showed the best effect on A549 cell line. The obtained results suggest that the antioxidant activity of *N.gaditana* extracts is not only due to the presence of phenolic compounds, but also due to the presence of other molecules that act as scavengers of free radicals via the hydrogen atom transfer and the electron transfer. Moreover, others molecules with an anticancer effect must be present in the acetonic extract, giving it an anti-proliferative activity on A549 cell line.

Keywords: *Nannochloropsis gaditana*, phenols, DPPH, reducing power, antiproliferative activity.

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INTRODUCTION

Marine algae have been historically and exceptionally considered rich source of pharmacologically active metabolites with antineoplastic [1-3], antimicrobial and antiviral effects [1, 3]. Moreover, because of phototropic life of microalgae and their permanent exposure to high oxygen and radical stresses, they have a high capability for production of numerous efficient protective chemicals against oxidative and radical stressors [4].

Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease [5, 6]. Nowadays, there are restrictions on the use of synthetic antioxidants, are suspected to be carcinogenic [7]. Natural antioxidants, therefore, have gained importance. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms [8]. Furthermore phenolic compounds may act in different stages of the development of malignant tumors by protecting cellular biomolecules from oxidative damage and preventing carcinogenesis process [9]. It was demostrated that detoxifying the body from free radicals, improve responses to anticancer treatment [10]. In this context, we propose to study different extracts of *Nannochloropsis gaditana* as free radical scavengers, by the evaluation of phenolic compounds and the measurement of antioxidant capacity by two methods: the scavenging of DPPH• (1,1-diphényl-2-picrylhydrazyl) free radical and the ferric reducing power. In order to highlight the effect of *N.gaditana* extracts on cancer cells, we investigated the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to study the antiproliferative activity of these extracts on A549 cell line.

MATERIAL AND METHODS

Biological material

Nannochloropsis gaditana is a microalgae that belongs to the class Eustigmatophycea [11]. This marine microalgae presents only chlorophyll a [12] and the main accessory pigments, violaxanthin and vaucheriaxanthin esters play a major role in light harvesting [13]. Some minor xanthophylls (canthaxanthin, anteraxanthin, zeaxanthin) and carotenes (β-carotene) are also present in much lower amounts [14]. The microalgae *Nannochloropsis gaditana* used in this study was obtained from Partisano Biotech Company, Algeria.

Sample preparation

The extraction was performed according to the method described by Li and *al.* [15] with some modifications. 2g of dried algal biomass of *Nannochloropsis gaditana* were macerated in 40ml of the extraction solvents namely: acetone, methanol, dichloromethane, hexane and purified water. After centrifugation at 45000g for 10min, the supernatant of aqueous extract was lyophilized while the supernatants of the others solvents were purged to dryness using nitrogen. The extracts were stored at 0°C before use. For the antioxidant assays, the aqueous extract was appropriately diluted in water, while the others extracts were diluted with ethanol and immediately used the tests [15].

Determination of total phenolics content

Total phenolics content was estimated by the Folin–Ciocalteu method [16]. 200µl of diluted sample (50mg/ml) were added to 1 ml of 1:10 diluted Folin–Ciocalteu reagent. After 4 min, 800 µl of saturated sodium carbonate (75 g/l) was added. After 2h of incubation at 20°C, the absorbance at 765 nm was measured. Gallic acid (0–180 µg/ml) was used for the standard calibration curve. The results were expressed as µg gallic acid equivalent /g dry weight of microalgae (µg GAE/g D.W) from the gallic acid standard curve, and calculated as mean value ± standard deviation (n = 3).

DPPH scavenging assay

The antioxidant assay was investigated by the DPPH method [17]. 50µl of each extract at three concentrations (10- 30 and 50mg/ml) were added to 1,95 ml of DPPH methanolic solution (0,025g/l). A negatif

control was prepared with 50µl methanol and 1,95 ml DPPH methanolic solution. After incubation for 30 min in the obscurity and at 20°C, the absorbance was readed at 517nm with a blank prepared for each concentration of each extract. Ascorbic acid and BHT(butylated hydroxytoluene) (20-300µg/ml) were used as references. Percentage of free radical scavenging activity was expressed as percent inhibition:

% = [(A517nm of control - A517nm of sample)/A517nm of control] x 100.

The extract concentration providing 50% inhibition (EC_{50}) was calculated from the linear regression. The experiment was performed in triplicate.

Ferric reducing power assay

The reducing power was determined according to the method described by Oyaizu [18]. 500 µl of each extract at three concentrations (10- 30 and 50mg/ml) were mixed with 500µl of 0.2 M sodium phosphate buffer (pH 6.6) and 500µl of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After incubation, 500µl of 10% trichloroacetic acid were added to the mixture, followed by centrifugation at 650g for 10 min. The upper layer (500µl) was mixed with 500µl of deionized water and 100 µl of 0.1% of ferric chloride. The mixture absorbance was measured at 700 nm (higher absorbance indicates higher reducing power). Ascorbic acid and BHT (butylated hydroxytoluene) (20-300µg/ml) were used as references. The experiment was performed in triplicate. Extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance against extract concentration.

Antiproliferative activity assay

The inhibition of cell viability was assessed by modified MTT colorimetric assay [19]. Each extract was firstly dissolved in DMSO 1%. The final concentration was equal to 1mg/ml. The extracts were then dissolved in RPMI1640 medium to obtain different concentrations ranging from 5 to150 µg/ml.

A549 cell line (ATCC n°CCL-185) (10^5cells/ml) were seeded in 96-well plates using RPMI 1640 containing 10% FBS (Fetal Bovine Serum) supplemented with 1% antibiotics (10000 U penicillin.ml^{-1} and 10mg streptomycin.ml^{-1}). After 24 h of incubation, cells were synchronized and attached to plates, after which the medium in the plates was discarded and replaced with 100 µl fresh medium containing different concentrations of *N. gaditana* extracts. The plates were incubated at 37°C for 72h under 5% CO_2 and 99% humidity. The medium containing various tested extracts were discarded and 100µl MTT at a final concentration of 5 mg/ml in PBS was added to each well. The cells were then incubated for 3 h and then 100µl isopropanol were added. Absorbance at a test wavelength of 550 nm and a reference wavelength of 620 nm was measured with a microplate reader (TECAN Sunrise™). The test was performed in triplicates. Percentage of cell viability was calculated from the flowing equation:

Cell Viability (%) = [A 550nm sample / A 550nm control] x 100.

The concentration of 50% inhibition (IC_{50}) was the concentration that achieved 50% cytotoxicity against culture cells.

Statistical analysis

The statistical analysis of the outcome data was carried out using one way analysis of variance (ANOVA) followed by Tukey test using statistics software package (Statistica). P value <0.05 were considered as significant.

RESULTS AND DISCUSSION

Total phenolic content

Phenolic compounds constitute a group of secondary metabolites that are quite widespread in nature with diverse biological activities, such as anti inflammatory, anti-atherosclerotic and anti-carcinogenic activities [20]. They exert properties such as free radical scavenging and inhibiting the generation of reactive species [21,

22].Their antioxidant activity is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, free radical scavengers, singlet oxygen quenchers and metal chelators [23].

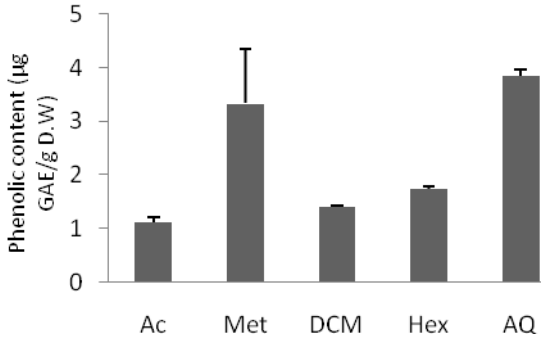

Fig 1: Phenolic content of acetonic (Ac), methanolic (met), dichloromethanic (DCM), hexanic (Hex) and aqueous extracts (AQ) of *N. gaditana*. Each value is expressed as mean ± standard deviation (n = 3).

The total phenolic content of *N.gaditana* (fig.1) was determined in order to assess its effect on the extract's antioxidant activity, and to identify a natural source of phenolic compounds. The antioxidant compounds of microalgae could be of very different polarity, and as a result were extracted into different polarity solvents.

Table 1: Phenolic content of *N. gaditana* extracts. Each value is expressed as mean ± standard deviation (n = 3).

Extract	Phenolic content µg GAE/g D.W
Acetonic (Ac)	1.12 ± 0.09
Methanolic (Met)	3.33 ±1.00
Dichloromethanic (DCM)	1.40 ± 0.02
Hexanic (Hex)	1.74 ± 0.04
Aqueous (AQ)	3.84 ± 0.12

The variation of phenolic content was quite large but statistically insignificant (P>0.05). The aqueous extract and the methanolic extract showed the highest amount of this compounds (table 1) with respectively 3.84 ± 0.12 µg GAE/g D.W, and 3.33 ±1.00 µg GAE/g D.W followed by the hexanic extract (1.74 ± 0.04µg GAE/g D.W) and dichloromethanic extract (1.40 ± 0.02 µg GAE/g D.W). The acetonic extract showed a low phenolic content with 1.12 ± 0.09 µg GAE/g D.W.

Results of the present study showed that among all the extraction solvents; the aqueous extract had the highest phenolic content. This may be due to the fact that phenolics are typically polar compounds [24], and are often extracted in higher amounts in more polar solvents [25-27].

Scavenging of DPPH radicals

The scavenging activity on DPPH radicals has been widely used to determine the free radical scavenging activity of different matrices [28-31]. Antioxidant molecules can quench DPPH free radicals by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule, and convert them to a colorless/bleached product (2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm [32].

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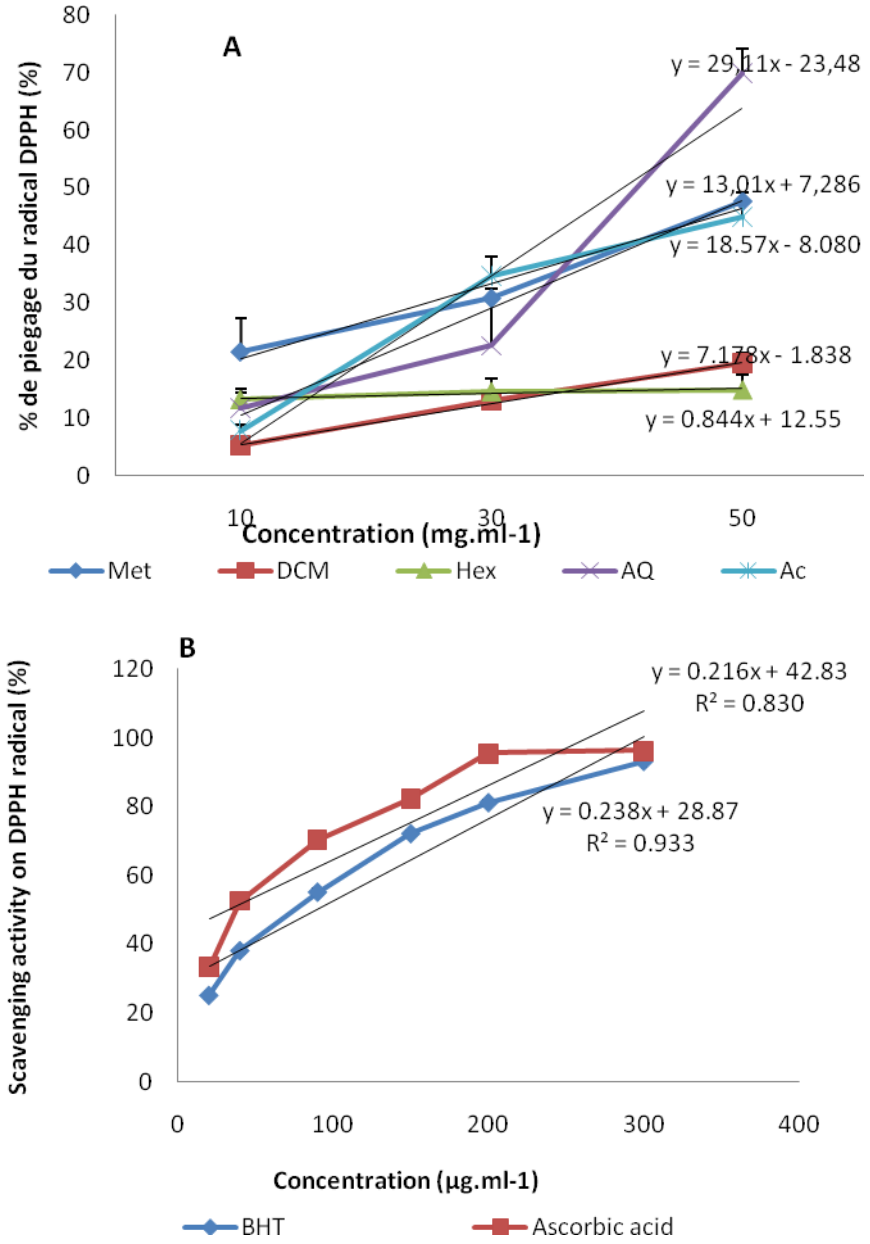

Fig 2: Scavenging activity (%) on DPPH radicals: (A) by *N.gaditana* extracts, (B) by ascorbic acid and BHT. Each value is expressed as mean ± standard deviation (n = 3).

Table 2: EC_{50} values (mg/ml) of *N.gaditana* extracts in DPPH scavenging assay and ferric reducing power assay

Extract	DPPH (EC_{50} [a])	Reducing power (EC_{50} [b])
Acetonic	3.12	1.22
Methanolic	3.28	1.06
Dichloromethanic	7.22	2.72
Hexanic	44.37	15.5
Aqueous	2.52	1.03
Ascorbic acid	0.03	0.10
BHT	0.08	0.08

[a]EC_{50} (mg/ml): effective concentration at which 50% of DPPH radicals are scavenged.
[b]EC_{50} (mg/ml): effective concentration at which the absorbance is 0.5.

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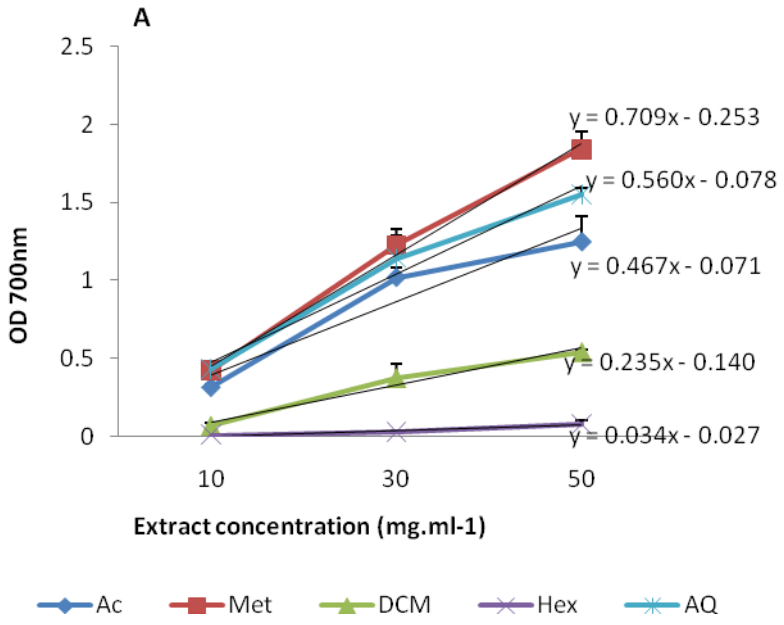
Fig.2 (A) shows the scavenging activity of *N.gaditana* extracts. In Table 2, we present the EC_{50} values corresponding to that activity. At 50mg/ml, the scavenging action was higher in aqueous extract (69.97%) than methanolic extract (47.59%) and acetonic extract (44.90%). At this concentration, the dichloromethanic extract (19.47%) and hexanic extract (14.90%) showed a weak antioxidant activity. These extracts do not show a significant difference in their activity (P= 0.97). However, the aqueous extract exhibited a significant difference in relation to all these extracts (P=0.0018). It must be noted that all these extracts had a lower scavenging action than ascorbic acid and BHT which present a very strong antioxidant effect at 300µg/ml (fig. 2B), with respectively 96.23% and 93%. The five extracts of *N.gaditana* exhibited a significant difference compared to the standards (P<0.05).

It is important to highlight that the free radical scavenging by antioxidants is dependent on two types of mechanisms : the hydrogen atom transfer from hydroxyl group (a rapid kinetics from some phenolic acids and derivatives) and the electron transfer (slow kinetics from derived glycosylated and anthocyanins) [33, 34]. The aqueous extract had exhibited the highest amount of phenolic compounds (3.84 ± 0.12 µg GAE/g D.W) and the highest scavenging effect on DPPH free radical (tab.2) (EC_{50}=2.52mg/ml), so we can suppose that phenolic compounds are the source of this activity via the hydrogen atom transfer. It was suggested that the ability of these phytochemicals to scavenge free radicals depended on types and volume of phenolic compounds [35]. The difference in their chemical structure was proposed for their different scavenging property. The type of solvent might be another factor affecting the antioxidant efficiency of the extract [36].

However, the acetonic extract had not a high concentration of phenolics (1.12 ± 0.09 µg GAE/g D.W), therefore it had a good scavenging effect on DPPH (tab.2). It should be noted that other antioxidant compounds such as carotenoids, polyunsaturated fatty acids and polysaccharides may play an important role in the antioxidant activity [37, 38].

Ferric reducing power

To further characterize the antioxidant properties in *N.gaditana* extracts, we tested the antioxidant capacity using the ferric reducing power assay. This technique measures the ability of extracts to reduce the ferric iron (Fe^{3+}) present in the K_3 [Fe(CN)6] complex to Ferrous iron (Fe^{2+}) [18].



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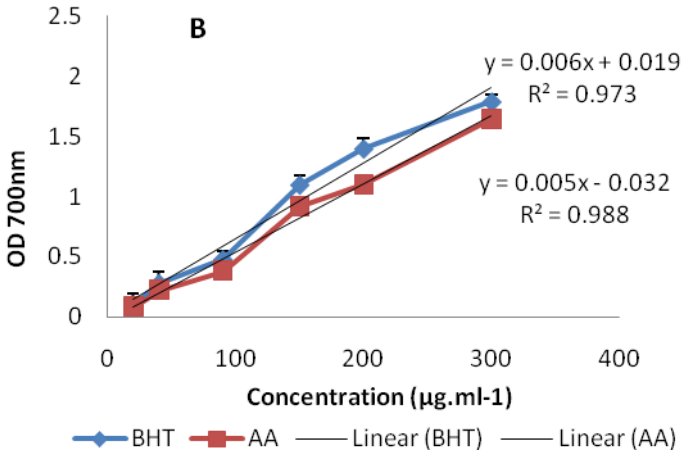

Fig 3: Ferric reducing power: (A) of *N. gaditana* extracts, (B) of ascorbic acid and BHT. Each value is expressed as mean ± standard deviation (n= 3).

Fig.3 (A) shows the reducing power of *N.gaditana* extracts as a function of their concentration. At 30 and 50mg/ml the methanolic extract exhibited the highest ferric reducing power with OD700 values equal to 1.22 and 1.84, followed by the aqueous extract which exhibited at these same concentrations, OD700 values equal to 1.14 and 1.52. These two extracts do not show a significant difference in their activity (P> 0.05). Dichloromethanic extract and hexanic extract exhibited a very weak activity than the other extracts. The ferric reducing power of the five extracts was lower than that of BHT and ascorbic acid (fig. 3 B) and statistically very significant (P=0.0001).

The EC_{50} related to this activity are reported in table 2. It is obvious that the reducing power of aqueous extract and methanolic extract is due to the presence of phenolic compounds that are considered as reducer and inactivators of oxidants, because of the presence of hydroxyl group that can serve as an electron donor [25].

Antiproliferative activity

The MTT assay works as an indicator of the activity of mitochondriale succinate deshydrogenase in living cells. This enzyme convert the MTT into crystal of formazan by cleaving the tetrazolium ring [19].

Table 3: IC_{50} values (mg/ml) of *N.gaditana* extracts in MTT assay

Extract	IC_{50} mg/ml
Acetonic	0.412
Methanolic	0.512
Dichloromethanic	0.521
Hexanic	1.16
Aqueous	2.308

IC_{50} (mg/ml): The concentration of 50% inhibition was the concentration that achieved 50% cytotoxicity against culture cells

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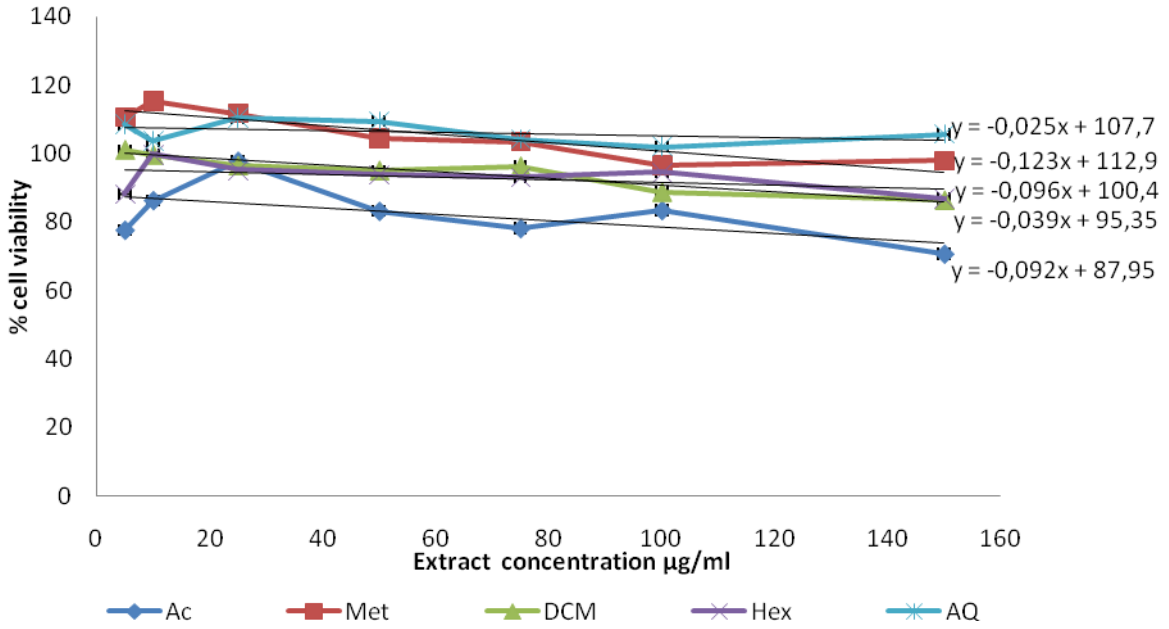

Fig 4: Viability of A549 cell line in response to *N.gaditana* extracts. Each value is expressed as mean ± standard deviation (n= 3).

Fig. 4 shows the viability of A549 cell line, after culturing with various concentrations of *N. gaditana* extracts for 72h. The acetonic extract displayed a significant ($P<0.05$) antiproliferative activity with an IC_{50} equal to 0.412mg/ml (tab.3). This extract showed the best effect at 5µg/ml and 150µg/ml with respectively 77.64% and 70.67% of cell viability. The others extracts had shown a weaker effect on cell viability, in order, methanolic extract (IC_{50}=0.512mg/ml), dichloromethanic extract (IC_{50}=0.521mg/ml), and hexanic extract (IC_{50}=1.16 mg/ml). In contrast, the cell viability was not really affected by the aqueous extract which exhibited an IC_{50} equal to 2.308mg/ml.

The antiproliferative activity of acetonic extract on A549 can be related to the presence of pigments in this extract. Because the acetone is known to extract most of the photosynthetic pigments, in a wide range of polarity and furthermore, acetone 90% is recommended for phytoplankton pigment analysis [39-41].

Various studies have been devoted to the action of microalgae extracts on different cancer cells lines. All these studies showed that the pigments, in particular carotenoids, are the most effective compounds on cancer cells.

Fucoxanthin is the carotenoid that has been most studied for its antiproliferative activity. It shows antiproliferative and apoptotic activity on lung cancer cell lines A549 and NSCLC-6 [42], on prostate cancer cell line PC-3 [43], on liver cancers [44], on the colon cancer lines Caco-2 [45] and WiDr [44], and on leukemia cell line HL-60[46,47]. The violaxanthin showed a cytotoxic and apoptotic effect on MCF-7 cancer cells line [48]. The β,β-carotene [49], lycopene, neoxanthin, lutein, cantaxanthin, also showed an antiproliferative and apoptotic activity on various cancer cell lines [43].

In an other side, a crude extract is interesting when the IC_{50} values are lower than 10 µg. ml^{-1} [48]. Crude extracts of *N.gaditana* do not reach this value. However, an extract is a complex mixture, and the active compound may be a minority molecule of the mixture. In addition, the results must be expressed in $mol.l^{-1}$ to rule on the antiproliferative potential of molecules, the active compound may have a high molecular weight [48]. Thus, a bioguided research on active molecules present in the acetonic exctract of *N.gaditana* will decide on its real anti-cancer effect.

CONCLUSION

Nannochloropsis gaditana extracts display a remarkable antioxidant activity. Overall, aqueous extract revealed the highest concentration of phenolic compounds, which is in agreement with its antioxidant

properties due to the hydroxyl groups of phenolic compounds. This result suggests that, antioxidants compounds of *N. gaditana* can be considered as promising alternative to synthetic antioxidants.

In the other hand, the acetonic extract exhibited the lowest phenolic content, but showed a good antioxidant activity. Furthermore, this extract presents the best antiproliferative activity on A549 cell line. Then, it is clear that different functional compounds would be present in this extract endowed with great antioxidant and antiproliferative activity.

In recent years, the use of photosynthetic microorganisms, such as microalgae, in life sciences has received increasing attentions due to their diverse phytometabolic contents with various chemical structures and biological activities [50]. Thus, present findings encourage for further studies for isolation and identification of active components of *N. gaditana* extracts, to consider them as antioxidants and anticancer agents.

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REFERENCES

[1] Faulkner D.J.Nat.Prod.Rep 2000; 17: 7-55.
[2] El-Baz F K, Aboul-Enein M A, Al-Baroty S G, Youssef A M, Abd El-Baky H. Hanaa. J. Med. Sci., 2002; 2(5-6):243-251.
[3] Tziveleka L A, Vagias C, Roussis V. Med Chem. 2003; 3(13):1512 -11535.
[4] Tsao R, Deng Z. J Chromatogr 2004; 812:85-99.
[5] Lee J, Koo N, Min D B. Compr Rev Food Sci Food Saf. 2004; 3: 21-33.
[6] Mantena R K R, Wijburg O L C, Vindurampulle C, Bennett-Wood V R, Walduck A, Drummond G R, Davies J K, Robins-Browne R M, Strugnell R A. Cell Microbiol 2008; 10:1058.
[7] Singh R P, Chidamara-Murthy K N, Jayaprakasha G K. J Agric Food Chem. 2002; 6: 50-81.
[8] Gulcin I, Huyut Z, Elmastas M, Aboul H Y E. Arabian J. Chem 2010; 3: 43-53.
[9] Bravo L. Nutr Rev 1998;56: 317–333.
[10] Boveris A and Navarro A. Life 2008; 60 (5): 308-314.
[11] Cheng-Wu Z, Zmora O, Kopel R, Richmond A. Aqua. 2001; 195(1,2) : 35-49
[12] Gentile M P and Blanch H W. Biotech Bio 2001; 75(1):1-12.
[13] Macías-Sánchez M. D, Mantell C, Rodríguez M, Martínez de la Ossa E, Lubián L M, Montero O. J FOOD ENG 2005 ; 66(2): 245-251.
[14] Lubián L.M, Montero O, Moreno-Garrido I, Huertas E, Sobrino C, González del Valle M, Parés G. J APPL PHYCOL 2000; 12(3/5): 249-255.
[15] Li H, Cheng K, Wong C, Fan K, Chen F, Jiang Y. Food Chem 2007; 102:771–776
[16] Singleton V L, and Rossi J A. AM J ENOL VITICULT 1965;16: 144–153.
[17] Sanchez-Moreno C, Larrauri J A, Saura-calixto F. J Sci Food Agri. 1998; 79: 270–276.
[18] Oyaizu M. Jpn J Nutr 1986; 44 :307–315.
[19] Mosmann T. J Immunol Methods 1983; 65(1-2): 55-63.
[20] Chung K. T, Wong T Y, Huang Y W, Lin Y. Crit. Rev. Food Sci. Nutr.1998; 38: 421–464.
[21] Gulcin I. Toxicology 2006; 217(2-3):213-220.
[22] Zhang Y, Seeram N, Lee R, Feng L, Heber D. J Agric Food Chem 2008; 56(3):670-675.
[23] Gilioli A M H H, Missau F C, Brighente I M C, Marques M C A, Pizzolatti M G. 30ª Reunião Anual da Sociedade Brasileira de Química 2007; 177.
[24] Goiris K, Muylaert K, Fraeye I, Foubert I, De Brabanter J, De Cooman L. J Appl Phycol 2012. doi: 10.1007/s10811-012-9804-6.
[25] Siddhuraju P. and Becker K. Food Chem. 2007; 101(1): 10-19.
[26] Anwar F, Jamil A, Iqbal S, Sheikh M A. GRASAS ACEITES 2006 ; 57 :189-197.
[27] Sultana B, Anwar F, Przybylski R. Food Chem. 2007; 104:1106-1114.

[28] Pereira J A, Pereira, A P, Ferreira I C F R, Valentão P, Andrade P, Seabra R, Estevinho L, Bento A. J. Agric. Food Chem. 2006 ; 54: 8425–8431.
[29] Sousa A, Ferreira I C F R, Barros, L, Bento A, Pereira J A. LWT-FOOD SCI TECHNOL Journal 2008. 41 : 739–745.
[30] Oliveira I, Sousa A, Valentao P, Andrade P B, Ferreira I C F R., Ferreres F, Bento A, Seabra R, Estevinho L, Pereira J A. Food Chem 2007 ; 105 :1018–1025.
[31] Oliveira I, Sousa A, Ferreira I C F R, Bento A, Estevinho L, Pereira J A. Food chem. toxicol. 2008 ; 46: 2326–2331.
[32] Ferreira I C F R, Baptista P, Vilas-Boas M, Barros L. Food Chem 2007; 100: 1511–1516
[33] Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y. Free Radic Biol Md 1996; 21(6) : 895-902.
[34] Huang D, Ou B, Prior R L. J. Agric. Food Chem. 2005; 53: 1841-1856.
[35] Rice-Evans C A, Miller N J, Paganga G. Free Radic Biol Md 1996; 20(7):933-956.
[36] Khumpook T, Chomdej S, Saenphet S, Amornlerdpison D, Saenphet K. Chiang Mai J. Sci. 2012; 39: 1-11.
[37] Chen F. Trends Biotechnol 1996;14:421–426.
[38] Chen F., Li H B, Wong R N S, Ji B, Jiang Y. J. Chromatogr. A 2005; 1064 :183–186.
[39] Strickland J D H and Parsons T R. Bull. Fish. Res. Bd. Canada 1972; 167: 311-311.
[40] Jeffrey S W and Hallegraeff G M. Marine Ecol. Prog. Ser. 1987; 35: 293-304.
[41] Parsons T R, Maita Y, Lalli M. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Oxford, ISBN: 9780080302874, 1984, pp. 173.
[42] Moreau D, Tomasoni C, Jacquot C, Kaas R, Le Guedes R, Cadoret J-P, Muller-Feuga A, Kontiza I, Vagias C, Roussis V, Roussakis C. Environ. Toxicol. Pharmacol. 2006 ; 22(1) : 97-103.
[43] Kotake-Nara E, Asai A, Nagao A. Cancer Lett. 2005 ; 220(1) : 75-84.
[44] Das S.K, Hashimoto T, Kanazawa K. Biochim. Biophys. Acta BBA General Subjects 2008 ; 1780(4) : 743-749.
[45] Hosokawa M, Kudo M, Maeda H, Kohno H, Tanaka T, Miyashita K. Biochim. Biophys. Acta BBA General Subjects 2004 ; 1675(1-3):113-119.
[46] Hosokawa M, Wanezaki S, Miyauchi K, Kurihara H, Kohno H, Kawabata J,Odashima S. Takahashi K. Food Sci Technol Res 1999 ; 5(3), 243-246.
[47] Nakazawa Y, Sashima T, Hosokawa M, Miyashita K. J Funct Foods 2009 ; 1(1) : 88-97.
[48] Pasquet V. Bio-guided research of anticancer molecules from marine microalgae. Ph.D. thesis in biochemistry. University of La Rochelle, France, 2011.
[49] Cui Y, Lu Z, Bai L, Shi Z, Zhao W-e, Zhao B. Eur. J. Cancer 2007 ; 43(17) : 2590-2601. [50] Skulberg O M. Bioactive chemicals in microalgae. In: Richmond A (ed) Handbook of microalgal culture, biotechnology and applied phycology. Blackwell, Oxford, ISBN 1405172495, 9781405172493, 2004, pp 485–512.