J Appl Phycol (2015) 27:839–848
DOI 10.1007/s10811-014-0369-4

Botryococcus braunii and *Nannochloropsis oculata* extracts inhibit cholinesterases and protect human dopaminergic SH-SY5Y cells from H_2O_2-induced cytotoxicity

Luísa Custódio · Fernando Soares · Hugo Pereira · Maria João Rodrigues · Luísa Barreira · Amélia Pilar Rauter · Fernando Alberício · João Varela

Received: 25 March 2014 / Revised and accepted: 17 June 2014 / Published online: 3 September 2014

Abstract Extracts of *Botryococcus braunii* and *Nannochloropsis oculata* were evaluated for inhibitory activity against acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and tyrosinase (TYRO) and capacity to attenuate hydrogen peroxide (H_2O_2)-induced injury in the human dopaminergic cell line SH-SY5Y. We also report the antioxidant activity, the total phenolic content (TPC) and the fatty acid (FA) profile of these microalgae. Both species had low levels of TPC and considerable amounts of polyunsaturated fatty acids (PUFA). The highest radical scavenging activity (RSA) against 1,1-diphenyl-2-picrylhydrazyl (DPPH) was observed in the acetone extract of *B. braunii* and in the diethyl ether extracts of both strains. The acetone extract of *B. braunii* had the highest RSA against 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). The extracts had a higher capacity to chelate iron than copper, and the highest iron chelation was achieved with the hexane extract of *N. oculata*. The diethyl ether and water extracts of the latter species also displayed the highest copper chelation. Except for the acetone extract of *B. braunii* and the water extract of *N. oculata*, all samples inhibited AChE, especially the hexane extract of *N. oculata*. Samples had moderate BChE inhibition and no effect towards TYRO. Almost all samples effectively protected neuronal cells against oxidative stress induced by H_2O_2. These results suggest possible novel applications of biomass from those microalgae in the pharmaceutical industry and/or as functional foods.

Keywords ChE inhibitors · Functional foods · Microalgae · Neuroprotection · Oxidative stress

L. Custódio (✉) · F. Soares · H. Pereira · M. J. Rodrigues · L. Barreira · J. Varela
Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal
e-mail: lcustodio@ualg.pt

H. Pereira
Department of Chemistry and Science of Materials, Faculty of Experimental Sciences, University of Huelva, Avda. Fuerzas Armadas s/n, 21007 Huelva, Spain

A. P. Rauter
Center of Chemistry and Biochemistry, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Lisbon, Ed. C8, Piso 5, 1749-016 Campo Grande, Lisboa, Portugal

F. Alberício
Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain

F. Alberício
CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain

F. Alberício
Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1-11, 08028 Barcelona, Spain

Introduction

Microalgae are an almost unlimited resource of bioactive compounds, such as fatty acids (FA), carotenoids and vitamins (Guedes et al. 2011; Borowitzka 2013). These organisms, which can be grown in large photobioreactors, are able to rapidly produce biomass and can be enriched in a particular compound upon exposure to abiotic stresses (Coesel et al. 2008). These features make microalgae extremely attractive for the search of novel molecules, with applications in the cosmetic, pharmaceutical and food industries (Borowitzka 2013).

Alzheimer's disease (AD) affects more than 35 million people worldwide and accounts for 50–60 % of the overall

cases of dementia in the elderly population (Filho et al. 2006; Prince et al. 2012). AD has no cure and is characterized by the deterioration of multiple cognitive functions, such as memory, thinking and comprehension. AD is associated with reduced levels of the neurotransmitter acetylcholine (ACh), due to hydrolytic reactions catalyzed by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Filho et al. 2006). The use of cholinesterase inhibitors (ChE-I) in AD patients, especially AChE inhibitors (AChE-I), results in the increase of ACh levels at the nerve synapse with the consequent relief of the neuropsychiatric symptoms and improvement of the cognitive ability (Filho et al. 2006). The most prescribed drugs for the treatment of AD act on this principle and can also be useful in the treatment of other neurological disorders associated with the reduction of ACh levels, including Parkinson's disease (PD) (Orhan et al. 2007; Pulok et al. 2007).

PD affects about 1 % of the population older than 50 years of age and is the second prevalent form of dementia in aged people, after AD (Ebadi and Sharma 2006). PD leads to a progressive lack of control over voluntary movement, and its clinical symptoms may include tremor, slow movements, rigidity, loss of balance and muscle pain. PD is linked to the selective degeneration of dopamine (DA)-containing pigmented neurons mainly located in the substantia nigra pars compacta of the midbrain (Hasegawa 2010). Those neurons are particularly vulnerable to degeneration, and their depigmentation is considered a hallmark of the advanced stages of the disease (Hasegawa 2010). The degeneration of DA-containing neurons can result from the activity of oxidized metabolites of dopamine known as dopamine quinone derivatives, which are usually a product of the autoxidation of catecholamines (Hasegawa 2010). Tyrosinase (TYRO) is a multifunctional copper-containing enzyme involved in neuromelanin formation in the human brain. Due to its oxidase activity, TYRO can potentially accelerate the induction of catecholamine quinone derivatives, contributing to dopamine neurotoxicity and, thus, to the neurodegeneration associated with PD (Khan 2007). In this sense, TYRO inhibitors have become an attractive target for the treatment of PD. AD and PD are also associated with high levels of oxidative stress and with the accumulation of metal ions, namely, iron and copper (Qureshi and Parvez 2007; Uttara et al. 2009; Weinreb et al. 2011). Compounds with antioxidant properties, including metal chelating activity, are considered effective in the prevention of cerebral oxidative stress and neuronal loss which are implicated in the onset of several neurodegenerative disorders (Gaeta and Hider 2005; Weinreb et al. 2011).

Hydrogen peroxide (H_2O_2), one of the main reactive oxygen species (ROS), is produced during the redox process and is considered a messenger in cellular metabolism and proliferation (Stone and Yang 2006). H_2O_2 can induce apoptosis in many different cells and may be involved in the activation of AChE and BChE (Xiao et al. 2000; Kim et al. 2005). Moreover, H_2O_2 is generated during catecholamine oxidation and during the decay of redox-active quinones, which are products of the autoxidation of DA (Hasegawa 2010). These links between the cholinergic signal, H_2O_2 and DA degeneration provide additional therapeutic targets for neuroprotective compounds.

Information on the role of microalgae as sources of neuroprotective compounds is rather scarce (Custódio et al. 2012, 2014; Aremu et al. 2014). This work evaluates the in vitro AChE, BChE and TYRO inhibitory activities of organic and water extracts of *Botryococcus braunii* (Chlorophyta, Trebouxiophyceae) and *Nannochloropsis oculata* (Ochrophyta, Eustigmatophyceae) and their capacity to attenuate H_2O_2-induced injury in the human dopaminergic cell line SH-SY5Y. The antioxidant and iron and copper ions chelating activities, the total phenolic compounds and the FA profile of both microalgae were determined as well.

Materials and methods

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), potassium persulfate, AChE (from *Electrophorus electricus*, Type V-S, EC 3.1.1.7), BChE (from horse serum, EC 3.1.1.8), TYRO (from mushroom, EC 1.14.1.8.1, 30 U) and fatty acid methyl ester (FAME) standards (Supelco® 37 Component FAME Mix) were purchased from Sigma (Germany).

Algae samples

Algae samples were provided by Necton S.A. (Portugal) in the form of a dark green solid frozen paste. Microalgae were grown in a semi-continuous cultivation system outdoors, in closed 'flat panel flow through' and 'tubular' photobioreactors. Microalgae cultures were kept sterile by mechanical and physical pre-treatment of the water used for algal production and also by weekly assessment of the presence of contaminants (e.g. *Vibrio* and total marine bacteria). The microalgal biomass was collected and concentrated by centrifugation, packed and frozen at −20ºC. Before the experiments, samples were freeze-dried, reduced to powder and stored in the dark at −20ºC.

Extracts preparation

For the preparation of the extracts, dried biomass was mixed with hexane (1:10 *w*/*v*) and homogenized using an Ultra-Turrax IKA T10B disperser at room temperature (RT). Homogenized samples were then centrifuged (5,000×*g*, 10 min, RT) and the supernatants recovered. The extraction was

repeated three times, the supernatants combined and filtered (Whatman no. 4) and the residue was sequentially extracted with diethyl ether, acetone and water. Organic extracts were dried under reduced pressure at 40ºC, while the water extracts were freeze-dried. The extracts were dissolved in DMSO at the concentration of 50 mg mL^{-1} and stored at 4 °C.

Phytochemical analysis

Determination of total phenolic content Total phenolic content (TPC) was determined on samples at the concentration of 10 mg mL^{-1} by the F-C assay (Julkunen-Tiitto 1985) as described previously (Custódio et al. 2014). The absorbances were measured at 725 nm, after 90 min incubation at RT, on a microplate reader (BioTek Synergy 4), and results were expressed as gallic acid equivalents (GAE) in μg g^{-1} extract (dry weight, DW), using a calibration curve made of gallic acid (GA) at six different concentrations (0–1,500 μg mL^{-1}).

Fatty acid composition Fatty acid (FA) was extracted and converted to the corresponding FAME by a direct transesterification method with acetyl chloride/methanol followed by extraction into hexane according to Lepage and Roy (1984) as described by Pereira et al. (2012).

Determination of antioxidant activity by radical-based assays

Radical scavenging activity on DPPH The radical scavenging activity (RSA) against DPPH radical was determined on extracts at different concentrations (0.25–10 mg mL^{-1}), according to the method of Brand-Williams et al. (1995) adapted to 96-well microplates (Moreno et al. 2006). A methanol solution of DPPH was used (120 μM), and the absorbances were measured at 515 nm (BioTek Synergy 4). Results were expressed as antioxidant activity (%), relative to a control containing DMSO and as half maximal inhibitory concentration (IC_{50}, mg mL^{-1}). BHT (E320) was used as the positive control at the same concentrations of the biological samples.

RSA on ABTS radical The RSA on ABTS radical was evaluated by the method described by Wang et al. (2007) on extracts at concentrations ranging from 0.25 to 10 mg mL^{-1}. The absorbance was measured at 734 nm (BioTek Synergy 4). Results were expressed as RSA (%) relative to a control containing DMSO and as IC_{50} values (mg mL^{-1}). BHT (E320) was used as the positive control at the same concentrations of the extracts.

Antioxidant activity by metal-related methods

Iron and copper chelating activities Iron chelating activity (ICA) and copper chelating activity (CCA) were determined according to Megías et al. (2009) on extracts at different concentrations (0.25–10 mg mL^{-1}). Change in colour was measured in a microplate reader (BioTek Synergy 4) at 562 nm (ICA) or 632 nm (CCA), and results were expressed as percentage of inhibition, relative to a control containing DMSO in place of the sample and as IC_{50} values (mg mL^{-1}). Ethylenediamine tetraacetic acid (EDTA) was used as the positive control at the same concentrations of the extracts.

Enzyme inhibition assays

AChE and BChE inhibitory activity The inhibitory effect on AChE and BChE activities was measured on samples at different concentrations (0.25–1.0 mg mL^{-1}), by the Ellman method (Ellman et al. 1961) as described by Orhan et al. (2007). The hydrolysis of acetylthiocholine or butyrylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines catalyzed by the enzyme, at 412 nm, using a 96-well microplate reader (BioTek Synergy 4). Results were expressed as AChE and BChE percentage inhibition relative to a control containing DMSO in place of the sample and as IC_{50} values (mg mL^{-1}). Galanthamine was used as a positive control at the same concentration of the samples.

Tyrosinase inhibitory activity The inhibitory activity against TYRO was determined by the method reported by Nerya et al. (2003) with modifications, using L-tyrosine as substrate. Samples were tested at concentrations ranging from 0.25 to 1 mg mL^{-1}, and the optical densities read at 492 nm using a 96-well microplate reader (BioTek Synergy 4). Results were expressed as TYRO percentage inhibition relative to a control containing DMSO in place of the sample. Arbutin was used as the positive control at the concentration of 1 mg mL^{-1}.

Cytotoxicity of the extracts and protective effect against H_2O_2-induced injury

Cell culture and viability determination The human neuroblastoma cell line (SH-SY5Y cells) was kindly provided by Dr. Eduardo Soriano (Barcelona Science Park, Spain). Cells were maintained in Dulbecco's modified eagle medium (DMEM) with 4,500 mg mL^{-1} of glucose, 10 % heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (50 U mL^{-1}) and streptomycin (50 μg mL^{-1}) and were grown in an incubator at 37 °C, 5.1 % CO_2 in humidified atmosphere. To evaluate the effect of the extracts on SH-SY5Y cells viability, they were seeded in 96-well plates at a density of 2×10^4 cells $well^{-1}$ and incubated for 24 h. Then, 100 μL of the extracts were added at concentrations ranging from 3 to 50 μg mL^{-1}, incubated for 24 h and cell viability was determined by the MTT assay (Mosmann 1983) as described previously (Custódio et al. 2014).

Protective effect on H_2O_2-induced cell cytotoxicity SH-SY5Y cells were seeded at a density of 20×10^4 cells well^{-1} in 96-well culture plates, left to attach overnight, treated with different concentrations of the extracts in culture medium and incubated for 24 h. Then, the extracts were gently removed, cells were treated with H_2O_2 (100 μM) for 30 min (Kim et al. 2005) and cell viability was determined by the MTT assay. The stock solution of H_2O_2 was prepared on phosphate-buffered saline (PBS, pH 7.4) and diluted with DMEM without FBS immediately before use. Negative control cells were treated with DMSO at the highest concentration used in test wells (0.5 %, *v*/*v*).

Statistical analysis

Results are expressed as mean±standard error of the mean (SEM), and experiments were conducted at least in triplicate ($n=9$). Significant differences were assessed by analysis of variance (ANOVA) or using Duncan's new multiple range test when parametricity of data did not prevail. SPSS statistical package for Windows (release 15.0, SPSS INC) was used.

Results

Fractions yield and TPC Both species have a high content in polar (water-extractable) compounds, as revealed by the higher yields obtained in the water extraction (Table 1). *N. oculata* had the highest TPC (578 μg GAE g^{-1}), present in higher concentration in the diethyl ether extract followed by acetone, water and hexane (Table 1). In *B. braunii*, the TPC was 431 μg GAE g^{-1}, and the highest values were detected in the acetone extracts followed by water, diethyl ether and hexane (Table 1).

FA profile The FA profile of *B. braunii* is mainly composed of palmitic (C16:0), oleic (C18:1) and linoleic acids (C18:2n-6), which accounted for more than 77 % of the total fatty acids (TFA) detected (Table 2). In comparison, palmitic, palmitoleic (C16:1) and eicosapentaenoic acids (C20:5n-3; EPA) were the most abundant FA detected in *N. oculata* and represented 84 % of the total FA of this microalga (Table 2). Therefore, *B. braunii* displayed a lower percentage of polyunsaturated fatty acids (PUFA) (32 %) when compared with *N. oculata* (46 %). Although both strains had considerable amounts of PUFA with a ratio of PUFA/SFA of 1.56 for *B. braunii* and 2.11 for *N. oculata*, *B. braunii* had only 3 % of n-3 FA, compared to nearly 42 % of EPA registered in *N. oculata*.

Antioxidant activity The highest RSA against the DPPH radical was achieved after application of the acetone extract of *B. braunii* (IC_{50}=3.96 mg mL^{-1}), and with the diethyl ether extracts, with IC_{50} values of 4.02 and 4.19 mg mL^{-1} for *B. braunii* and *N. oculata*, respectively (Table 1). The acetone extract of *B. braunii* had the highest capacity to scavenge ABTS• with an IC_{50} value of 1.19 mg mL^{-1} (Table 1). The extracts exhibited a higher ability to chelate Fe^{2+} than Cu^{2+} (Table 3). *N. oculata* had the highest metal chelation potential, and the utmost results were observed with the hexane extract for iron (IC_{50}=0.70 mg mL^{-1}) and with the water extract for copper (IC_{50}=3.23 mg mL^{-1}) (Table 3).

Table 1 Extraction yields (%), total phenolic content (TPC, μg GAE g^{-1} microalgae) and radical scavenging activity (RSA) on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radicals (IC_{50}, mg mL^{-1}) of organic and water extracts of *Botryococcus braunii* and *Nannochloropsis oculata*

Species/compound	Extract	Yield (%)	TPC	DPPH	ABTS
B. braunii	Hexane	0.76	11±6^{f}	8.55±0.58^{e}	8.32±0.38^{g}
	Diethyl ether	0.48	100±3^{e}	4.02±0.14^{a}	3.8±0.37^{d}
	Acetone	1.06	170±66^{b}	3.96±0.27^{a}	1.19±0.05^{a}
	Water	2.67	150±21^{c}	>10	4.60±0.04^{c}
	Total	*2.30*	431±69		
N. oculata	Hexane	1.35	18±3^{c}	4.93±0.37^{b}	6.93±0.16^{f}
	Diethyl ether	0.93	261±6^{a}	4.19±0.17^{a}	1.80±0.16^{b}
	Acetone	1.50	177±5^{b}	6.70±0.44^{c}	2.02±0.03^{c}
	Water	2.89	122±6^{d}	7.31±0.71^{d}	4.05±0.18^{e}
	Total	*3.78*	578±10		
BHT*				0.07±0.00	

Values represent the mean±standard error of mean (SEM) of at least three experiments performed in triplicate ($n=9$). For the same column, different letters are significantly different for all extracts of both species (Duncan's new multiple range test, $p<0.05$)

*Butylated hydroxytoluene (BHT, E320): positive control

Italic is used to distinguish partial from total values

Table 2 Fatty acids methyl esters (FAME) composition, expressed as percentage of total fatty acids, of *Botryococcus braunii* and *Nannochloropsis oculata*

	Common name	*B. braunii*	*N. oculata*
C10:0	Capric acid	nd	0.33±0.01
C12:0	Lauric acid	nd	0.51±0.01
C14:0	Myristic acid	nd	4.60±0.18
C15:0	Pentadecanoic acid	nd	0.35±0.02
C16:0	Palmitic acid	18.05±0.27	15.67±0.88
C17:0	Margaric acid	nd	0.35±0.02
C18:0	Stearic acid	0.84±0.06	0.34±0.05
C22:0+	Behenic acid	1.21±0.05	nd
C24:0	Lignoceric acid	0.89±0.01	nd
∑ *SFA*		*20.98±0.39*	*22.17±1.16*
C16:1	Palmitoleic acid	9.71±0.04	26.89±0.49
C18:1	Oleic acid	35.85±2.84	4.26±0.28
C20:1	Eicosenoic acid	0.66±0.10	nd
∑ MUFA		*46.22±2.97*	*31.16±0.77*
C16:2(n-6)	Hexadecadienoic acid	2.24±0.05	0.32±0.01
C18:2(n-6)	Linoleic acid	23.15±0.30	4.48±0.10
C16:3(n-3)	Hexadecatrienoic acid	3.14±0.07	nd
C16:3(n-6)	Hexadecatrienoic acid	0.91±0.01	nd
C18:3(n-3)	α-Linolenic acid	3.38±0.09	nd
C20:5(n-3)	Eicosapentaenoic acid	nd	41.88±2.01
∑ PUFA		*32.81±0.25*	*46.68±2.11*
∑n-3		*3.14±0.07*	*41.88±2.01*
∑n-6		*29.67±0.18*	*4.80±0.10*
∑n-6/∑n-3		*9.51*	*0.15*
PUFA/SFA		*1.56*	*2.11*

Values represent average±standard error (n=4)

nd not detected, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

Numbers in italics are ratios or sums that depend on the other values

Cholinesterases and TYRO inhibitory activity The inhibitory activity (%) against AChE, BChE and TYRO was classified as potent (>50 %), moderate (30–50 %), low (<30 %) or nil (<5 %) as described in Vinutha et al. (2007). In accordance with this classification, all extracts exhibited potent AChE inhibitory activity at the highest concentration tested, except for the acetone extract of *N. oculata*, which had moderate inhibition, and the water extract of the same species, with nil activity (Fig. 1a, c). The highest AChE inhibition was obtained with the hexane extract of *N. oculata*, with an IC_{50} value of 0.31 mg mL^{-1} (Table 3). Except for the diethyl ether extract of *B. braunii*, which had moderate inhibitory activity on BChE (32 % at 1 mg mL^{-1}), all extracts exhibited low inhibitory activity on this enzyme (Fig. 1b, d). None of the extracts was effective against TYRO (data not shown).

Neuroprotective effect against H_2O_2-induced cytotoxicity on SH-SY5Y cells The neuroprotective effect of the microalgae extracts was evaluated against H_2O_2-induced cytotoxicity on SH-SY5Y human neuroblastoma cells. Extracts were tested at non-toxic concentrations, that is, at concentrations that did not reduce SH-SY5Y cells viability to values lower than 80 % of the control. For these extracts, this means that a range of 3–50 μg mL^{-1} had to be used. The extracts exhibited different cytotoxicity, depending on their nature and concentration applied, and generally, extracts from *N. oculata* were less toxic (data not shown). The pre-treatment of cells with the acetone extract of *B. braunii* at the concentration of 25 μg mL^{-1} significantly reduced the cytotoxicity induced by exposure to H_2O_2, increasing cell viability from 60 to 75 % (Fig. 2a). Regarding *N. oculata*, only the diethyl ether extract did not protect neuronal cells from H_2O_2 toxicity, and the water extract, applied at the concentrations of 6 μg mL^{-1}, allowed the highest cell viability (76 %, Fig. 2b).

Table 3 Iron and copper chelating activity and AChE inhibitory activity (IC_{50}, mg mL^{-1}) of organic and water extracts of *Botryococcus braunii* and *Nannochloropsis oculata*

Species/compound	Extract	Iron	Copper	AChE
B. braunii	Hexane	$9.25±0.23^{f}$	>10	$0.39±0.06^{b}$
	Diethyl ether	$8.57±0.95^{e}$	>10	$0.69±0.17^{d}$
	Acetone	$7.42±0.23^{d}$	>10	>10
	Water	$5.44±0.03^{c}$	$3.73±0.17^{b}$	$0.76±0.11^{e}$
N. oculata	Hexane	$0.70±0.03^{a}$	$5.99±0.30^{c}$	$0.31±0.02^{a}$
	Diethyl ether	$5.04±0.26^{c}$	$3.52±0.06^{a}$	$0.49±0.02^{c}$
	Acetone	$2.70±0.14^{b}$	$5.42±0.29^{c}$	$1.53±0.82^{f}$
	Water	$2.53±0.14^{b}$	$3.23±0.06^{a}$	>10
EDTA*	–	0.10±0.00		
Galanthamine*				0.14±0.03

Values represent the mean±standard error of mean (SEM) of at least three experiments performed in triplicate (n= 9). For the same column, different letters are significantly different for all extracts of both species (Duncan's new multiple range test, P< 0.05)

*Ethylenediamine tetraacetic acid (EDTA) and galanthamine: positive controls

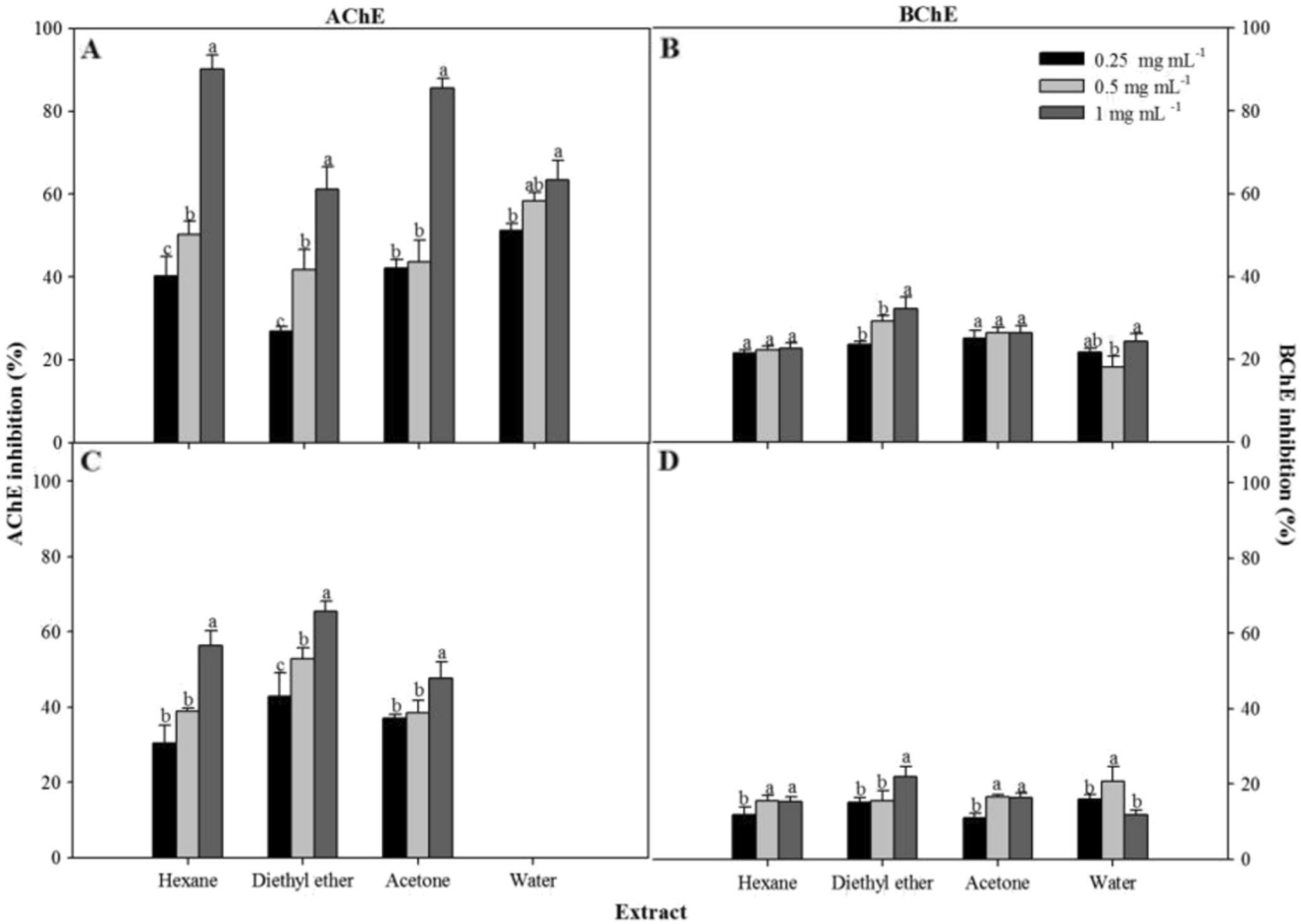

Fig. 1 AChE and BChE inhibitory activity of organic and water extracts of *B. braunii* (**a**, **b**) and *N. oculata* (**c**, **d**). *Different letters in the same extract* indicate significant differences between concentrations by the Duncan's new multiple range test at $P<0.05$. *Solid bars* and *errors bars* represent the average and standard error values, respectively ($n=9$). Galanthamine (positive control) had an inhibitory activity of 78.1 % (AChE) and 71.2 % (BChE) at the concentration of 0.25 mg mL^{-1}

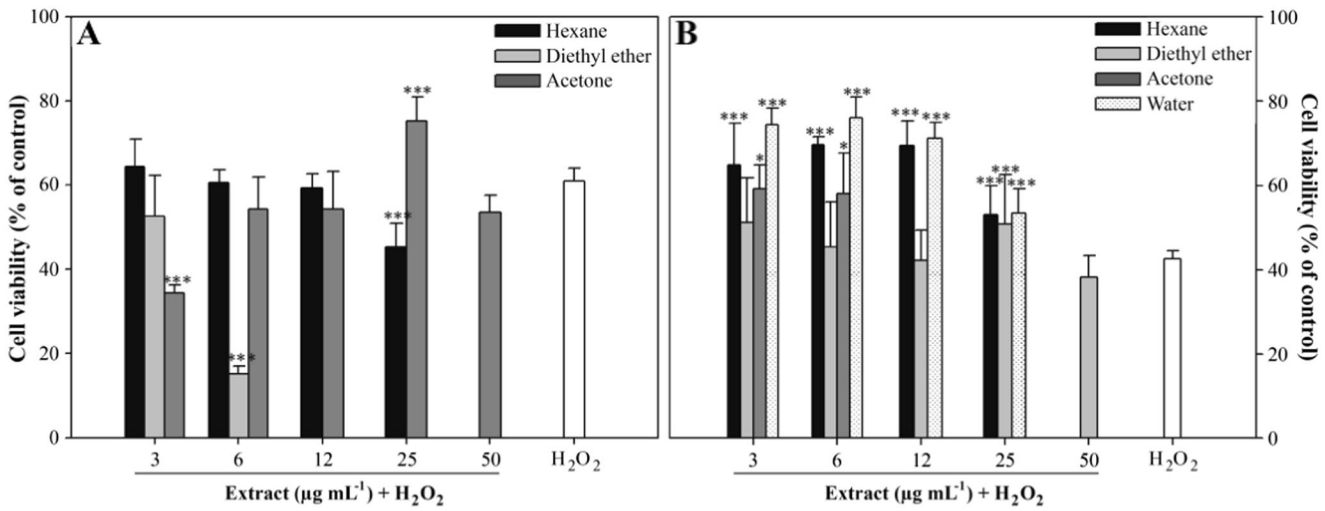

Fig. 2 Protective effect of *B. braunii* (**a**) and *N. oculata* (**b**) extracts in SH-SY5Y cells pre-treated with the extracts at different concentrations for 24 h and exposed to 100 μM of H_2O_2. Each value represents the mean ±SEM of three replicates. Significantly different than that of the H_2O_2-treated group: $*P<0.05$; $***P<0.001$

Discussion

Microalgae dried biomass was sequentially extracted with different solvents of increasing polarity index (PI), namely, hexane (PI=0.1), diethyl ether (PI=2.8), acetone (PI=5.1) and water (PI=10.1), in order to fractionate compounds between extracts and maximize its concentration, reducing the occurrence of false negative results (Tempone et al. 2011). The extracts were evaluated for their TPC, given that phenolics are one of the most common bioactive compounds, with an important role in the prevention of oxidative stress-related diseases, namely, cancer and neurological disorders (Dai and Mumper 2010). *B. braunii* and *N. oculata* presented lower TPC than other microalgal species belonging, for example, to the *Chlorella* and *Nostoc* genera (Li et al. 2007; Hajimahmoodi et al. 2010). This can be either a characteristic of the studied algae or the result of different culture conditions, which are known to have a high impact on the chemical composition of microalgae (Coesel et al. 2008).

Marine microalgae are acknowledged for the accumulation of substantial amounts of omega-3 FA (Sijtsma and Swaaf 2004; Doughman et al. 2007). The obtained FAME profiles are in accordance with the results reported previously by other authors, in the same species (Huerlimann et al. 2010; Zhila et al. 2011). Martins et al. (2013) reviewed the potential of marine microalgae for EPA production: *Nannochloropsis* strains (5–39 % of TFA) along with *Phaeodactylum tricornutum* (28–57 % of TFA) and *Porphyridium cruentum* (25–41 % of TFA) are considered the most promising strains for EPA production. The value obtained in this work was slightly higher (41 % of TFA) and may represent 7 to 8 % of the biomass DW. Considering the importance of these PUFA for a balanced diet (Martins et al. 2013), these species may be regarded as valuable ingredients in human nutrition.

Although oxygen is vital for life, the imbalanced metabolism and the surplus production of ROS is linked with the onset of several diseases, including neural disorders (Qureshi and Parvez 2007; Uttara et al. 2009). ROS are particularly active in the brain, as a result of the excitatory amino acids and neurotransmitters, and they can damage glial cells and neurons, leading to neuronal damage (Gilgun-Sherki et al. 2001; Nunomura et al. 2006; Ebadi and Sharma 2006; Qureshi and Parvez 2007; Danielson and Andersen 2008; Kidd 2008; Uttar et al. 2009). In this sense, the use of antioxidant compounds aiming the prevention of cerebral oxidative stress and neuronal loss has gained increasing importance due to their capacity to neutralize free radicals (Konishi 2009; Uttar et al. 2009). In this work, both microalgae exhibited a considerable capacity to scavenge DPPH and ABTS radicals, a process in which different classes of molecules are likely to participate. In the polar extracts (acetone), the antioxidant activity can be partially ascribed to the presence of phenolics (Hajimahmoodi et al. 2010), and particularly, in *B. braunii*, the antioxidant activity of the acetone extract can be linked to carotenoids, namely, violaxanthin, astaxanthin, lutein, zeaxanthin, chlorophylls *a* and *b* and β-carotene which are usually present in this extract (Rao et al. 2006). However, in the non-polar extracts (hexane), the RSA detected cannot be attributed to the presence of phenolics since these compounds were present in those samples at very low levels. In this case, the antioxidant activity is probably more related to the presence of PUFAs. Oleic acid, the major FA of *B. braunii* (35 % of the total FAME) has antioxidant properties against the DPPH radical (Cerón et al. 2007), while palmitic acid, which ranged from 16 to 18 % of the total FAME profile in *N. oculata* and *B. braunii*, respectively, has high activity against the peroxynitrite anion ($ONOO^-$; Fang et al. 2010). Accordingly, the high amount of lipids of this microalga (18–20 % of DW, data supplied by Necton S.A.), of which 41 % are EPA, suggests that this PUFA might contribute or even be responsible for the activity observed in the less polar extracts.

The accumulation of Fe^{2+} and Cu^{2+} has a significant impact on ROS generation through the promotion of the Haber-Weiss/Fenton reaction (Weinreb et al. 2011). Thus, besides acting directly by scavenging free radicals, antioxidants can act indirectly through the chelation of redox metals, thus preventing free radical generation (Gilgun-Sherki et al. 2001). Metal chelation is of particular importance for the prevention of neurological diseases, and the use of Fe^{2+} and Cu^{2+} chelators is often considered a valuable strategy for the management of oxidative stress-related neurological disorders (Gaeta and Hider 2005; Weinreb et al. 2011). The extracts were tested for chelating activity on Fe^{2+} and Cu^{2+} and generally displayed a higher ability to chelate the first transition metal, and *N. oculata* had the highest metal chelation. This is in agreement with previous findings for the same species, although our results were lower than the ICA values previously reported (Custódio et al. 2012), most probably due to differences in the extraction methods used. Our results point to the presence of Fe^{2+} and Cu^{2+} chelators in the microalgae extracts under study, probably with different chemical natures. In the more polar extracts, such as acetone and water, that activity can be due to the presence of phenolics, which are known to exhibit metal chelation potential (Megías et al. 2009). In fact, phenolic compounds are considered adequate for the management of neurological disorders involving oxidative damage mediated by divalent metals (Weinreb et al. 2011). Taken together, our results on the antioxidant capacity of *B. braunii* and *N. oculata,* both in terms of radical scavenging and metal chelation potential, suggest that the biomass of both species contain molecules with potential to prevent oxidative stress-linked diseases, namely, those involving brain degeneration.

The most frequently used drugs for the treatment of AD patients are ChE inhibitors, namely, donepezil and galanthamine, which are selective AChE inhibitors, and

rivastigmine, with a dual inhibition against AChE and BChE (Dall'Acqua 2013). In spite of their recognized value in the symptomatic relief of patients with mild to moderate AD (Pettenati et al. 2003), there is still a need for new AChE inhibitory compounds with lower toxicity and higher central nervous system penetration (Dall'Acqua 2013). In spite of previous studies reporting the occurrence of bioactive compounds in microalgae, there is little information about their neuroprotective potential, except for a few studies on the cholinesterase inhibitory activity of selected species (Custódio et al. 2012, 2014; Aremu et al. 2014). In this work, *B. braunii* and *N. oculata* were evaluated for inhibitory activity in vitro on AChE, BChE and TYRO, which are enzymes implicated in the onset of neurodegenerative ailments, namely, AD and PD. The extracts had a higher capacity to inhibit AChE and were more effective against this enzyme than *Melissa officinalis* and *Salvia officinalis*, which are medicinal herbs with recognized memory-improving properties (Ferreira et al. 2006). Our results suggests a possible therapeutic value of the bioactive molecules present in those microalgae as inhibitors of AChE, with potential application in the management of neurological disorders characterized by reduced levels of ACh, such as PD and myasthenia gravis (Pulok et al. 2007; Williams et al. 2011). The FA profile of these microalgae, rich in n-3 PUFA, also indicates that the species under study have a potential interest in the field of neuroprotection. Although studies on the effect of FA on AChE and BChE inhibition are scarce, there are some indications that a diet deficient in n-3 PUFA can be related with the development of AD (Igarashi et al. 2011). PUFA are known to improve the cholinergic neurotransmission and lighten AD symptoms and are considered as potent neuroprotectors (Lauritzen et al. 2000; Willis et al. 2009). A diet rich in omega-3 PUFAs can replace the membrane fluidity, which is decreased in situations of oxidative stress, thereby overcoming the damaging effects of the free radicals on neuronal cells (Yehuda et al. 2002).

Based on promising cholinesterase inhibitory results, an in vitro neuroprotective test was performed using a SH-SY5Y human neuroblastoma cell line. SH-SY5Y cells are widely used as an in vitro model to study the effects and mode of action of drugs on neuronal diseases (Kim et al. 2005). Cells were pre-treated with non-toxic concentrations of the extracts, and then oxidative stress was induced through the application of 100 μM of H_2O_2, which is one of the main ROS produced in the brain during AD progression (Pan et al. 2009). The non-radical H_2O_2 easily enter the cells due to its high membrane permeability and can originate the hydroxyl radical, one of the most unstable and damaging radicals involved in tissue injury. A significant increase in cellular viability was observed after the application of the microalgal extracts, and those from *N. oculata* were especially efficient in the protection of cells from oxidative stress induced by H_2O_2. This protective effect is not likely due to the direct antioxidant effect of the extracts, since they were removed from the cells before H_2O_2 treatment. Instead, it may be linked to the activation of intercellular and intracellular excitoprotective signalling pathways, for example, mitogen-activated protein kinase (MAPK), tumour necrosis factor (TNF) and those involving the preservation of mitochondrial function (reviewed in Mattson 2002). In fact, the treatment of cultured neurons with antioxidant compounds such as vitamin E and lipoic acid has been reported to effectively protect cells against glutamate toxicity and ischemia-like conditions (Muller and Krieglstein 1995; Gurney et al. 1996). Further work will be necessary to determine the mechanism(s) underlying the observed protective effect.

In conclusion, in this study, multiple biological activities were observed after the application of different algae extracts, indicating that *B. braunii* and *N. oculata* are endowed with different bioactive molecules. In this sense, the inclusion of biomass from *B. braunii* and *N. oculata* in the human diet can provide health benefits at different levels, either improving well-being and/or reducing the incidence of different disorders (Ibañez and Cifuentes 2013). Assays will be pursued in order to ascertain the in vivo health-promoting effects suggested by the results obtained with the in vitro studies included in this work. Those studies are mandatory before a health claim can be made regarding a particular food product (Ibañez and Cifuentes 2013). Moreover, and aiming the pharmaceutical industry, assays are now being conducted in order to isolate and identify the bioactive compounds and to elucidate their modes of action.

Acknowledgments This work was supported by the SEABIOMED project (PTDC/MAR/103957/2008), funded by the Foundation for Science and Technology (FCT) and the Portuguese National Budget. All the algal species used in this study were provided by NECTON S.A. (Portugal). The authors dedicate this article to the memory of Fernando Soares, whose untimely passing remind us all of the pressing need for novel medical treatments.

References

Aremu AO, Masondo NA, Stirk WA, Ördög V, Van Staden J (2014) Influence of culture age on the phytochemical content and pharmacological activities of five *Scenedesmus* strains. J Appl Phycol 26: 407–415

Borowitzka MA (2013) High-value products from microalgae-their development and commercialisation. J Appl Phycol 25:743–756

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. Lebensm Wiss Technol 28: 25–30

Cerón MC, García-Malea MC, Rivas J, Acien FG, Fernández JM, Del Río E, Guerrero MG, Molina E (2007) Antioxidant activity of *Haematococcus pluvialis* cells grown in continuous culture as a function of their carotenoid and fatty acid content. Appl Microbiol Biotechnol 74:1112–1119

Coesel SN, Baumgartner AC, Teles LM, Ramos AR, Henriques NM, Cancela L, Varela J (2008) Nutrient limitation is the main regulatory factor for carotenoid accumulation and for Psy and Pds steady state

transcript levels in *Dunaliella salina* (Chlorophyta) exposed to high light and salt stress. Mar Biotechnol 10:601–611

Custódio L, Justo T, Silvestre L, Barradas A, Vizetto C, Pereira H, Barreira L, Rauter AP, Alberício F, Varela J (2012) Microalgae of different phyla display antioxidant, metal chelating and acetylcholinesterase inhibitory activities. Food Chem 131:134–140

Custódio L, Soares F, Pereira H, Barreira L, Vizetto-Duarte C, Rodrigues MJ, Rauter MA, Alberício 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

Dall'Acqua S (2013) Plant-derived acetylcholinesterase inhibitory alkaloids for the treatment of Alzheimer's disease. Bot Target Ther 3:19–28

Danielson SR, Andersen K (2008) Oxidative and nitrative protein modifications in Parkinson's disease. Free Radic Biol Med 44:1787–1794

Doughman SD, Krupanidhi S, Sanjeevi CB (2007) Omega-3 fatty acids for nutrition and medicine: considering microalgae oil as a vegetarian source of EPA and DHA. Curr Diabetes Rev 3:198–203

Ebadi M, Sharma S (2006) Metallothioneins 1 and 2 attenuate peroxynitrite-induced oxidative stress in Parkinson disease. Exp Biol Med 231:1576–1583

Ellman GL, Courtney KD, Andres V, Feather-Stone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95

Fang Z, Jeong SY, Jung HA, Choi JS, Min BS, Woo MH (2010) Anticholinesterase and antioxidant constituents from *Gloiopeltis furcata*. Chem Pharm Bull 58:1236–1239

Ferreira A, Proença C, Serralheiro MLM, Araújo MEM (2006) The in vitro screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J Ethnopharmacol 108:31–37

Filho J, Medeiros K, Diniz M, Batista L, Athayde-Filho P, Silva M, da Cunha E (2006) Natural products inhibitors of the enzyme acetylcholinesterase. Braz J Pharmacogn 16:258–285

Gaeta A, Hider RC (2005) The crucial role of metal ions in neurodegeneration: the basis for a promising therapeutic strategy. Br J Pharmacol 146:1041–1059

Gilgun-Sherki Y, Melamed E, Offen D (2001) Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacology 40:959–975

Guedes AC, Amaro HM, Malcata FX (2011) Microalgae as sources of high added-value compounds—a brief review of recent work. Biotechnol Prog 27:597–613

Gurney ME, Cutting FB, Zhai P, Doble A, Taylor CP, Andrus PK, Hall ED (1996) Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosis. Ann Neurol 39:147–157

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

Hasegawa T (2010) Tyrosinase-expressing neuronal cell line as in vitro model of Parkinson's disease. Int J Mol Sci 11:1082–1089

Huerlimann R, de Nys R, Heimann K (2010) Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production. Biotechnol Bioeng 107:245–257

Ibañez E, Cifuentes A (2013) Benefits of using algae as natural sources of functional ingredients. J Sci Food Agric 93:703–709

Igarashi M, Ma K, Gao F, Kim H-W, Rapoport SI, Rao JS (2011) Disturbed choline plasmalogen and phospholipid fatty acid concentrations in Alzheimer disease prefrontal cortex. J Alzheimers Dis 24: 507–517

Khan MTH (2007) Molecular design of tyrosinase inhibitors: a critical review of promising novel inhibitors from synthetic origins. Pure App Chem 79:2277–2295

Kidd PM (2008) Alzheimer disease, amnestic mild cognitive impairment, and age-associated memory impairment: current understanding and progress toward integrative prevention. Altern Med Rev 13:85–115

Kim S-S, Park R-Y, Jeon H-J, Kwon Y-S, Chun W (2005) Neuroprotective effects of 3,5-dicaffeoylquinic acid on hydrogen peroxide-induced cell death in SHSY5Y cells. Phytother Res 19:243–245

Konishi T (2009) Brain oxidative stress as basic target of antioxidant traditional oriental medicines. Neurochem Res 34:711–716

Lauritzen I, Blondeau N, Heurteau C, Widmann C, Romey G, Lazdunski M (2000) Polyunsaturated fatty acids are potent neuroprotectors. EMBO J 19:1784–93

Lepage G, Roy CC (1984) Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J Lipid Res 25:1391–1396

Li H-B, Cheng K-W, Wong C-C, Fan K-W, Chen F, Jiang Y (2007) Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food Chem 102:771–776

Martins DA, Custódio L, Barreira L, Pereira H, Ben-Hamadou R, Varela J, Abu-Salah KM (2013) Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae. Mar Drugs 11: 2259–2281

Mattson MP (2002) Excitotoxic and excitoprotective mechanisms, abundant targets for the prevention and treatment of neurodegenerative disorders. NeuroMol Med 3:65–94

Megías C, Pastor-Cavada E, Torres-Fuentes C, Girón-Calle J, Alaiz M, Jua R, Julio P, Javier V (2009) Chelating, antioxidant and antiproliferative activity of *Vicia sativa* polyphenol extracts. Eur Food Res Technol 230:353–359

Moreno S, Scheyer T, Romano C, Vojnov A (2006) Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radic Res 40:223–231

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Muller U, Krieglstein J (1995) Prolonged pretreatment with alpha-lipoic acid protects cultured neurons against hypoxic, glutamate-, or iron induced injury. J Cereb Blood Flow Metab 5:624–630

Nerya O, Vaya J, Musa R, Izrael S, Ben-Arie R, Tamir S (2003) Glabrene and isoliquiritigenin as tyrosinase inhibitors from licorice roots. J Agric Food Chem 51:1201–1207

Nunomura A, Castellani RJ, Zhu X, Moreira PI, Perry G, Smith MA (2006) Involvement of oxidative stress in Alzheimer disease. J Neuropathol Exp Neurol 65:631–641

Orhan I, Kartal M, Naz Q, Ejaz A, Yilmaz G, Kan YKB, Sener B, Choudhary MI (2007) Antioxidant and anticholinesterase evaluation of selected Turkish *Salvia* species. Food Chem 103:1247–1254

Pan W, Dancik CM, Nelson VM, Jiang Z-G, Lebowitz MS, Ghanbari HA (2009) A novel neuroprotectant PAN-811 protects neurons from oxidative stress. Cent Eur J Biol 4:34–40

Pereira H, Barreira L, Figueiredo F, Custódio L, Vizetto-Duarte C, Polo C, Rešek E, Engelen A, Varela J (2012) Marine macroalgae as a source of polyunsaturated fatty acids for nutritional and pharmaceutical applications. Mar Drugs 10:1920–1935

Pettenati C, Annicchiarico R, Caltagirone C (2003) Clinical pharmacology of anti-Alzheimer drugs. Fundam Clin Pharmacol 17:659–672

Prince M, Bryce R, Ferri C (2012). World Alzheimer Report 2011. London, UK: Alzheimer's Disease International (ADI)

Pulok KM, Venkatesan K, Mainak M, Houghton PJ (2007) Acetylcholinesterase inhibitors from plants. Phytomedicine 14:289–300

Qureshi GA, Parvez HS (2007) Oxidative stress and neurodegenerative disorders. Elsevier, The Netherlands

Rao A, Sarada R, Baskaran V, Ravishankar GA (2006) Antioxidant activity of *Botryococcus braunii* extract elucidated in vitro models. J Agric Food Chem 54:4593–4599

Sijtsma L, de Swaaf ME (2004) Biotechnological production and applications of the ω-3 polyunsaturated fatty acid docosahexaenoic acid. Appl Microbiol Biotechnol 64:146–153

Stone JR, Yang S (2006) Hydrogen peroxide: a signaling messenger. Antioxid Redox Signal 8:243–270

Tempone AG, Martins de Oliveira C, Berlinck RG (2011) Current approaches to discover marine antileishmanial natural products. Planta Med 77:572–585

Uttara B, Singh AV, Zamboni P, Mahajan RT (2009) Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol 7:65–74

Vinutha B, Prashanth D, Salma K, Sreeja SL, Pratiti D, Padmaja R, Radhika S, Amit A, Venkateshwarlu K, Deepakc M (2007) Screening of selected Indian medicinal plants for acetylcholinesterase inhibitory activity. J Ethnopharmacol 109:359–363

Wang LJ, Li D, Zou L, Chen XD, Cheng QY, Yamaki K, Li LT (2007) Antioxidative activity of Douchi (A Chinese traditional salt-fermented soybean food) extracts during its processing. Int J Food Prop 10:385–396

Weinreb O, Mandel S, Bar-Am O, Amit T (2011) Iron-chelating backbone coupled with monoamine oxidase inhibitory moiety as novel pluripotential therapeutic agents for Alzheimer's disease: a tribute to Moussa Youdim. J Neural Transm 118: 479–492

Williams P, Sorribas A, Howes M-JR (2011) Natural products as a source of Alzheimer's drug leads. Nat Prod Rep 28:48–77

Willis LM, Shukitt-Hale B, Joseph JA (2009) Dietary polyunsaturated fatty acids improve cholinergic transmission in the aged brain. Genes Nutr 4:309–314

Xiao XQ, Wang R, Tang XC (2000) Huperzine A and tacrine attenuate beta-amyloid peptide-induced oxidative injury. J Neurosci Res 61: 564–569

Yehuda S, Rabinovitz S, Carasso RL, David I, Mostofsky DI (2002) The role of polyunsaturated fatty acids in restoring the aging neuronal membrane. Neurobiol Aging 23:843–853

Zhila N, Kalacheva G, Volova T (2011) Effect of salinity on the biochemical composition of the alga *Botryococcus braunii* Kütz IPPAS H-252. J Appl Phycol 23:47–52