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Microalgae of different phyla display antioxidant, metal chelating and acetylcholinesterase inhibitory activities

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ABSTRACT

Methanol and hexane extracts from *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella minutissima* and *Rhodomonas salina* were evaluated for total phenolic contents, radical scavenging activity (RSA), metal chelating potential against copper and iron ions and acetylcholinesterase (AChE) inhibition. Only the methanol extracts contained phenolic compounds. The hexane extracts had the highest RSA. The extracts had a higher capacity to chelate Fe^{2+} ions, more pronounced in the lowest concentration of the hexane extracts with values ranging from 73.3 ± 3.3% (*R. salina*) to 97.5 ± 1.1% (*N. oculata*). The highest AChE inhibitory activity was found in the hexane extracts at 10 mg/ml of *C. minutissima* (79.3 ± 1.9%), *T. chuii* (85.7 ± 0.7%) and *R. salina* (81.5 ± 7.5%). GC–MS analysis indicated polyunsaturated fatty acids and steroids as the most abundant compounds in the hexane extracts. The species under study provide a valuable source of antioxidants, metal chelators and AChE inhibitors.

1. Introduction

Dementia is a group of symptoms that may accompany neurological disorders or conditions, and occurs mainly in the elderly population. It is characterised by the deterioration of multiple cognitive functions, such as memory, thinking, comprehension, calculation and language. It is estimated that 63 million people will suffer from dementia in 2030, 65% of whom in less developed countries (Wimo, Winblad, Aguero-Torres, & von Strauss, 2003). The most common causes of dementia include degenerative neurological diseases, such as Alzheimer's (AD), dementia with Lewy bodies, Parkinson's, and Huntington's. AD is estimated to account for 50–60% of dementia cases in people over 65 years of age (Filho et al., 2006). This disease has no cure and it is terminal within 3–7 years of diagnosis. The characteristic pathology of AD includes the extracellular deposits of plasma amyloid beta peptide (Aβ) in senile plaques, intracellular formation of neurofibrillary tangles and the loss of neuronal synapses and pyramidal neurons (Weinreb, Mandel, Bar-Am, & Amit, 2011). Although the initiating factors underpinning this pathology remain to be elucidated, it is well established that AD is associated with a reduction of the levels of acetylcholine (ACh), which is the major neurotransmitter in the central nervous system (CNS) (Filho et al., 2006). Acetylcholinesterase (AChE) is considered to be the chief enzyme involved in ACh hydrolysis, as well as in the development of AD. According to the cholinergic hypothesis, the restoration of ACh levels, which are progressively lost during the progression of AD, delays the loss of cognitive function (Filho et al., 2006). Recent studies have shown that AChE inhibitors alleviate neuropsychiatric symptoms in AD patients, and provide beneficial effects on cognitive ability by increasing ACh levels within the synaptic region (Zarotsky, Sramek, & Cutler, 2003). Inhibition of AChE serves also as a strategy for the treatment of other neurological disorders, such as senile dementia, ataxia, myasthenia gravis and Parkinson's disease (Pulok, Venkatesan, Mainak, & Houghton, 2007).

Another factor considered to be pathologically important in various neurodegenerative processes is oxidative stress, which may play a key pathogenic role in AD as an early event during the progression of the disease (Qureshi & Parvez, 2007). It was shown that the absence of natural antioxidants such as vitamin D exacerbated AD in a mouse model (Mhatre & Hensley, 2007). Moreover, increased levels of free radicals contribute to the inflammatory process, which is known to enhance the development of AD (Mhatre & Hensley, 2007).

Various metals have also been implicated in the development of neurological disorders. For instance, changes in iron homeostasis

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have been noticed in AD patients, who might display altered levels of iron, ferritin and transferrin receptors in the hippocampus and cerebral cortex (Weinreb et al., 2011). Iron may promote the deposition of Aβ and, specifically, redox-active iron is known to be involved in the development of oxidative stress through the promotion of the Haber–Weiss/Fenton reaction (Weinreb et al., 2011). Thus, therapies involving the chelation of iron and other redox active metals (e.g. copper) are presently being considered as a valuable strategy in AD (Weinreb et al., 2011).

Microalgae are the most important primary producer of biomass, and one of the most diverse ecological groups of organisms. They exhibit a unique combination of features typical of higher plants, such as efficient oxygenic photosynthesis and simple nutritional requirements, and yet they display biotechnological attributes of proper microbial cells, namely fast growth in liquid medium and the ability to accumulate or secrete metabolites. In fact, microalgal biomass is a natural source of a number of biologically active compounds (e.g. carotenoids, phycobilins, fatty acids, polysaccharides, vitamins, and sterols) (Plaza, Herrero, Cifuentes, & Ibáñez, 2009) and has a very wide range of applications, from animal feed and aquaculture to human nutrition and health products. In addition, the taxonomical diversity of microalgae and the possibility of growing and harvesting them under different environmental conditions render these aquatic photosynthetic organisms particularly attractive as bioreactors, which can be enriched in a particular bioactive compound upon exposure to abiotic stresses (Coesel et al., 2008).

Knowledge on AChE inhibitors from marine photosynthetic organisms is particularly scarce. Some research has been reported for the genera *Sargassum* and *Gracilaria* (Choi et al., 2007; Natarajan, Shanmugiahthevar, & Kasi, 2009), but to the best of our knowledge there is no information on the AChE inhibitory potential or metal chelating activity of microalgae. In this context, this work evaluated the *in vitro* AChE inhibitory activities of polar (methanol) and non-polar (hexane) extracts from four species of microalgae, namely *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella minutissima* and *Rhodomonas salina* by the Ellman method. Additionally, the radical scavenging of the extracts, their capability to chelate iron and copper and their total phenolic content were assessed. A preliminary chemical characterisation of the bioactive extracts was made by Gas Chromatography coupled with Mass Spectrometry (GC/MS).

2. Materials and methods

2.1. Enzyme and chemicals

All chemicals used in the experiments were of analytical grade. Acetylcholinesterase (EC.3.1.1.7) from electrical eel with a specific activity ⩾1000 units/mg (1 unit hydrolyses 1.0 μmole of acetylcholine to choline and acetate per minute at pH 8.0 and 37 °C), acetylthiocholine iodide (ATChI), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), galanthamine, pyrocatechol violet and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma (Steinheim, Germany). Ethylenediaminetetracetic acid (EDTA), and sodium carbonate (Na_2CO_3) were from Fluka (Steinheim, Germany). Merck (Darmstadt, Germany) supplied ferrozine, copper sulphate pentahydrate and Folin–Ciocalteau (F–C), while methanol was from Fischer Scientific (Loughborough, UK). Additional reagents and solvents were purchased from VWR International (Leuven, Belgium).

2.2. Algal cell biomass

Biomass from *N. oculata* was provided by NECTON S.A (Portugal) as a solid dark green frozen paste. Microalgae were grown outdoors in closed 'Flat Panel Flow Through' and 'Tubular' photobioreactors in a semi-continuous cultivation system, using water pre-treated by mechanical and physical methods in order to ensure sterility and inorganic nutrients. Cultures were tested weekly for the presence of *Vibrio* and total marine bacteria. Microalgae were concentrated by centrifugation at controlled speed, packed under chilled conditions and frozen at −20 °C. *R. salina, T. chuii* and *C. minutissima* were grown at the LEOA (Experimental Laboratory of Aquatic Organisms, University of Algarve), in f/2 culture media using 100 L plastic bags. Cultures were maintained under artificial light (100 μmol/s/m^2) with a 12:12 h light/dark photoperiod. Microalgal cells were collected at late exponential phase, centrifuged at 2000*g* and maintained at −20 °C until further analysis.

2.3. Preparation of the extracts

For extract preparation, 1 g of lyophilised biomass was added to 40 ml of hexane or methanol, homogenised using a disperser IKA T10B Ultra-Turrax, extracted overnight at room temperature (RT) with stirring and filtered (Whatman no. 4). The extracts were dried under reduced vacuum pressure, resuspended in methanol or dimethyl sulfoxide (DMSO) for the methanol or hexane extracts, respectively, and stored at −20 °C.

2.4. Determination of total phenolic content (TPC)

TPC of the extracts was determined using the F–C colorimetric method as described by Velioglu, Mazza, Gao, and Oomah (1998). Briefly, 5 μl of the extracts were mixed with 100 μl of 10-fold diluted F–C reagent, incubated at RT for 5 min and mixed with 100 μl of sodium carbonate (Na_2CO_3, 75 g/l, w/v). Absorbance was measured at 725 nm after 90 min incubation at RT on a microplate reader (Biotek Synergy 4). TPC was calculated as gallic acid equivalents (GAE) from the calibration curve of gallic acid standard solutions, and expressed as GAE in milligrams per gram of initial dry material.

2.5. Radical scavenging activity (RSA)

RSA was evaluated by the DPPH method (Moreno, Scheyer, Romano, & Vojnov, 2006). Samples (22 μl at the concentration of 1 mg/ml) were mixed with 200 μl of methanolic DPPH solution (120 μM) in 96-well flat bottom microtitration plates, and incubated in darkness at RT for 30 min. The absorbance was measured at 515 nm (Biotek Synergy 4) and RSA was calculated as the percentage inhibition relative to a blank containing methanol or DMSO. Butylated hydroxytoluene (BHT, 1 mg/ml) was used as a positive control.

2.6. Iron (ICA) and copper (CCA) chelating activity

ICA was determined by measuring the formation of the Fe^{2+}-ferrozine complex according to the method of Megías et al. (2009). Samples (30 μl) were mixed in 96-well microplates with 250 μl of 100 mM Na acetate buffer (pH 4.9) and 30 μl of an aqueous $FeCl_2$ solution (0.1 mg/ml, w/v). After 30 min, 12.5 μl of an aqueous 40 mM ferrozine solution was added. The change in colour was measured using a microplate reader (Biotek Synergy 4) at 562 nm, and the results were expressed as the percentage inhibition, relative to a control containing methanol or DMSO in place of the sample.

The CCA was determined using pyrocatechol violet (PV) as described by Megías et al. (2009). Samples (30 μl) were mixed in 96-well microplates with 200 μl of 50 mM Na acetate buffer (pH 6.0), 6 μl of 4 mM PV dissolved in the latter buffer and 100 μl of $CuSO_4{\cdot}5H_20$ (50 μg/ml, w/v). The change in colour of the solution