Biochemical profile and *in vitro* neuroprotective properties of *Carpobrotus edulis L.,* a medicinal and edible halophyte native to the coast of South Africa

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ABSTRACT

This work reports the nutritional profile and *in vitro* neuroprotective properties of leaves of *Carpobrotus edulis* L., a medicinal and edible succulent species native to the coast of South Africa. Biomass was evaluated for proximate composition and for contents in carotenoids, liposoluble pigments and minerals. Hexane, dichloromethane, ethyl acetate and methanol extracts were prepared by Soxhlet extraction from dried biomass and evaluated for *in vitro* inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), capacity to attenuate hydrogen peroxide (H_2O_2)-induced injury in the human dopaminergic cell line SH-SY5Y and for anti-neuroinflammatory potential on lipopolysaccharide (LPS)-stimulated microglia cells. Extracts were evaluated for antioxidant activity by four complementary methods, total content of phenolics, tannins and flavonoids. Finally the profile of the main phenolic compounds was determined by high performance liquid chromatography with diode array detection (HPLC-DAD). *C. edulis* has a high moisture content, high levels of crude protein, fibre, ash, carotenoids, calcium and iron and a low fat level. The extracts were able to efficiently scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), reduce iron and chelate copper and iron ions, and exhibited different levels of phenolic compounds in the order ethyl acetate > methanol > dichloromethane > hexane. The main compounds detected were gallic and salicylic acids and quercetin, all in the ethyl acetate extract. The extracts allowed a dual and potent inhibition of AChE and BuChE. The dichloromethane and methanol extracts had the strongest capacity to prevent cell death induced by H_2O_2, and the methanol extract had anti-neuronflammatory properties. All together our results suggest that consumption of leaves of *C. edulis* can contribute for a balanced diet, and that they may add to the improvement of cognitive functions. It also suggests possible novel biotechnological applications of *C. edulis* such as source of molecules and/or products for the food and/or pharmaceutical industries. Studies aiming to the isolation and identification of the bioactive compounds are already in progress.

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1. Introduction

Carpobrotus edulis L. (syn. *Mesembryanthemum edule* L., Aizoaceae) is a medicinal and edible succulent extremophile plant native to the coast of South Africa. It has different common names: sour fig, Cape fig, Hottentots fig (English); "ghaukum", "ghoenavy", "hottentotsvy", "kaapsevy", "perdevy", "rankvy", "suurvy", "vyerank", (Afrikaans); "ikhambi-lamabulawo" and "umgongozi" (Zulu) and "igcukuma" in Xhosa communities (Omoruyi et al., 2012). *Carpobrotus edulis* has long been used as food and in traditional medicine in South Africa. For example, it is a valued Khoi-Khoi and San remedy adopted by several ethnic groups in that area, being used in the form of leaf juice for the treatment of diarrhoea and tuberculosis, as a mouthwash for sore throat or gum infections, or applied externally to burn wounds (Van Wyk, 2008). It is also used in the Eastern Cape by the traditional healers to treat diabetes mellitus, high blood pressure, intestinal worms and constipation (Van Wyk et al., 1997; Van Der Watt and Pretorius, 2001; Thring and Weitz, 2006; Van Wyk, 2008; Martins et al., 2011; Ksouri et al., 2012; Omoruyi et al., 2012). In the past *C. edulis* was introduced in the southern and western Europe, including Portugal, to stabilize and fix coastal sand dunes, and also for landscaping. However, due to its high successful reproduction and dispersal capacity, *C. edulis* became an invasive species in several parts of the world, including Europe, Australia, California and the Mediterranean, preventing the development of native vegetation due to the acidification of the soil.

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Several *in vitro* biological activities were described for *C. edulis* including antioxidant, immune modulating, antimicrobial; anticholinesterase and anti tumoral (Van der Watt and Pretorius, 2001; Ordway et al., 2003; Chokoe et al., 2008; Bouftira et al., 2009; Buwa and Afolayan, 2009; Martins et al., 2010; Falleh et al., 2011; Martins et al., 2011; Custódio et al., 2012; Falleh et al., 2012; Omoruyi et al., 2012, 2014), Moreover, several active molecules were already isolated from this species as for example rutin, hyperoside, cactichin, ferulic acid, uvaol, β – amyrin and oleanolic acid (Van der Watt and Pretorius, 2001; Martins et al., 2010; Falleh et al., 2011; Martins et al., 2011).

Dementia is a cluster of symptoms most often related with neurological diseases. It occurs mostly in the elders and is characterized by the decline of multiple cognitive functions, such as memory, thinking and comprehension. The commonest causes of dementia are chronic neurological diseases as for example Alzheimer's (AD) and Parkinson's disease (PD), which are expected to affect 63 million people in 2030, mostly in less developed countries (Wimo et al., 2003). In fact it is estimated that by 2040 neurodegenerative disorders could be the second leading cause of death in the elderly people, after cancer (Ip et al., 2012).

Neuroprotection encompasses a set of approaches that promote the protection of the central nervous system (CNS) against neuronal damage caused by acute (*e.g.* stroke and trauma) and chronic neurodegenerative disorders (*e.g.* AD and PD). Among these strategies, herbal medicine represents a valuable resource in preventing rather than treating those diseases (Iriti et al., 2010). The World Health Organization (WHO) encourages ethnobotanic and ethnopharmacological studies aiming the valorisation of medicinal plants, which are a reliable source of molecules and/or products in traditional and modern medicine. For example in Africa approximately 80% of the population uses some form of traditional herbal medicine (WHO, 2002; Willcox and Bodeker, 2004), while the worldwide annual market for these herbal products is nearly US$ 60 billion (WHO, 2002). In a previous report it was found that *C. edulis* had the capacity to inhibit acetylcholinesterase (AChE), suggesting a potential capacity to improve cognitive features (Custódio et al., 2012). *C. edulis* is an edible plant but the complete nutritional profile of this species in a functional food perspective has never been made. Thus, this work aimed to evaluate for the first time the adequacy of *C. edulis* to be included in human diet and advances knowledge regarding its potential as a source of innovative healthcare products able to improve cognitive features. For that purpose, leaves were evaluated in terms of nutritional properties, and different extracts were assessed for *in vitro* antioxidant activity, inhibition of enzymes related with the onset of diseases affecting the CNS, namely AChE, butyrylcholinesterase (BuChE) and tyrosinase (TYRO). Extracts were also evaluated for their capacity to attenuate hydrogen peroxide (H_2O_2)-induced toxicity in the human dopaminergic cell line SH-SY5Y and for anti-neuroinflammatory activity on lipopolysaccharide (LPS)-stimulated N9 cells. SH-SY5Y is a human neuroblastoma cell line usually used as an *in vitro* model to evaluate the effect of natural compounds and/or isolated molecules on neurological diseases (Kim et al., 2005; Custódio et al., 2015, 2016). N9 is a murine microglia cell line, and the N9-LPS stimulation assay is widely used as a model for neuroinflammation (Duan et al., 2013). The content in different groups of phenolic compounds was determined in the extracts, and the phenolic profile of active samples was chemically characterized by high performance liquid chromatography with diode array detection (HPLC-DAD).

2. Experimental

2.1. General experimental procedures

HPLC-DAD analysis was conducted on an Agilent 1100 Series LC system (Germany). Data acquisition and instrumental control were performed by the software LC3D ChemStation (version Rev.A.10.02[1757], Agilent Technologies). Absorbance readings were carried out using a microplate reader (Biotek Synergy 4). Minerals were analysed by atomic absorption spectrometry-AAS (GBC Avanta Sigma, Australia).

2.2. Chemicals

Sodium nitrite, sodium acetate, 1,1-diphenyl-2picrylhydrazyl (DPPH), electric eel AChE (type-VI-S, EC 3.1.1.7), horse serum BuChE (EC 3.1.1.8), galanthamine, acetylthiocholine iodide (ATChI) and butyrylthiocholine chloride (BTChI) 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB), LPS from *Escherichia coli*, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT), tyrosinase (EC 1.14.1.8.1, 30 U, mushroom tyrosinase), N-(1-Naphthyl) and ethylenediamine dihydrochloride (NED) was were purchased from Sigma-Aldrich (Germany). Merck (Germany) supplied Folin-Ciocalteu (F-C) phenol reagent, phosphoric acid and ferrospectral (iron reagent). Lonza (Belgium) provided Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), trypsin, L-glutamine and penicillin/streptomycin. Dimethyl sulfoxide (DMSO), ethylenediamine tetraacetic acid (EDTA), Copper (II) sulphate and additional reagents and solvents were obtained from VWR International (Belgium).

2.3. Plant material

Leaves of *C. edulis* were collected in 'Praia de Faro' (coordinates: 37°00′17.6″N 7°59′25.4″W), in Southern Portugal, during 2010 summer. Samples were cleaned from extraneous matter, dried at 40 °C for 3 days, powdered and stored at −20° until needed.

2.4. Nutritional properties

2.4.1. Proximate composition

Crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method according to Uslu et al. (2013); the ash content was determined by incineration at 515 ± 15 °C for 5 h, and crude fat was determined by the Bligh and Dyer method with a few modifications as described in Pereira et al. (2011). Neutral-detergent fibre (NDF) was analysed on a Fibretherm FT 12 Fibre Analyser according to the AOAC Official Method 2002.04. Results are expressed as g per 100 g of dry weight (DW).

2.4.2. Carotenoids and liposoluble pigments

Total carotenoids were determined after extraction with acetone, and quantified spectrophotometrically according to Uslu et al. (2013). Liposoluble pigments were determined according to Nagata and Yamashita (1992). Contents of β-carotene, lycopene, and chlorophyll *a* and *b*, were calculated according to the following equations and further expressed in mg/100 g DW.

$$\beta-\text{carotene (mg/100 mL)} = 0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453};$$

$$\text{lycopene (mg/100 mL)} = -0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453};$$

$$\text{chlorophyll } a \text{ (mg/100 mL)} = 0.999 \times A_{663} - 0.0989 \times A_{645};$$

$$\text{chlorophyll } b \text{ (mg/100 mL)} = -0.328 \times A_{663} + 1.77 \times A_{645}.$$

2.4.3. Minerals

Dried biomass (300 mg) was mixed with 6 ml of HNO_3 (65%), 1 ml of $HClO_4$ (70%), and 1 ml of H_2O_2 (30%) in high-pressure Teflon vessels and microwave digested (Milestone Ethos Touch). A procedural blank was prepared and included in each digestion batch of 10 samples. Minerals were analysed by atomic absorption spectrometry-AAS (GBC Avanta Sigma, Australia) provided with a deuterium background correction.

The accuracy of the analytical procedure was assessed by the analysis of certified reference material (CRM), BCR60 (*Lagarosiphon major*). Procedural blanks always accounted for less than 1% of the metal concentrations in samples. Results are expressed per g of dry weight (DW).

2.5. Extraction

The extracts were prepared sequentially in a Soxhlet apparatus. The plant powder (20 g) was first extracted with hexane (250 ml), followed by dichloromethane, ethyl acetate, and methanol. Each extraction was performed for 6 h. The extracts were filtered with paper filter Whatman no 4, and then evaporated under reduced vacuum pressure and temperature (< 40 °C). Dried extracts were weighed, dissolved in DMSO to obtain a final concentration of 50 mg/ml and stored at 4 °C.

2.6. Phytochemical characterization of the phenolic fraction of the extracts

2.6.1. Spectrophotometric assays: total phenolics (TPC), flavonoids (TFC) and condensed tannins contents (CTC)

The extracts at the concentration of 10 mg/ml were used for the evaluation of TPC (F-C assay), TFC (AlCl₃ method), and CTC (DMACA-hydrochloric acid assay) by previously reported spectrophotometric methods (Rodrigues et al., 2015). Results were expressed as gallic acid equivalents (GAE) in milligrammes per gramme of extract (DW) for TPC; as milligrammes of rutin equivalents per gramme of dried sample (mg RE/g, DW) for TFC and as milligrammes of catechin equivalents per gramme of dried sample (mg CE/g, DW) for CTC.

2.6.2. HPLC-DAD analysis

The extracts at the concentration of 10 mg/ml in ultrapure water were analysed by HPLC-DAD as described previously (Rodrigues et al., 2015). For identification, the retention parameters of each assay were compared with the standard controls and the peak purity with the UV–visible spectral reference data. The levels of the different compounds were extrapolated from calibration standard curves. Commercial standards of gallic acid, p-hidroxybenzoic acid, catechin, vanillic acid, cafeic acid, syringic acid, epigallocatechin gallate, coumaric acid, salicylic acid, ferulic acid and rosmarinic acid, 4-hidroxybenzaldehyde, apigenin, BHT, chlorogenic acid, epicatechin, epigallocatechin, flavone, gentisic acid, m-hidroxybenzoic acid, oleanolic acid, quercetin, resveratrol, rutin hydrate, transcinnamic acid and uvaol were prepared in methanol (1000 mg/L) and diluted with ultrapure water in desired concentration.

2.7. Antioxidant activity

2.7.1. Radical scavenging activity (RSA) of DPPH

The DPPH assay was performed according to the method of Brand-Williams et al. (1995), as described by Moreno et al. (2006). The samples (22 μl) at the concentrations of 1, 5 and 10 mg/ml, were mixed with 200 μl of a methanol DPPH solution (120 μM) in 96-wells microplates and incubated for 30 min. at room temperature (RT) in the dark. The absorbance was measured at 517 nm using a Biotek Synergy 4 microplate reader and RSA was expressed as percentage of inhibition, relative to a control, containing DMSO in place of the sample. Butylated hydroxytoluene (BHT, 1 mg/ml) was used as a positive control.

2.7.2. Iron (ICA) and copper (CCA) chelating activities

ICA was determined by the method described previously (Rodrigues et al., 2015). Briefly, samples (30 μl at the concentrations of 1, 5 and 10 mg/ml) were mixed in 96-well microplates with 200 μl of distilled water and 30 μl of a FeCl₂ solution (0.1 mg/ml in water) for 30 min. Then 12.5 μl of ferrozine solution (40 mM in water) was added and change in colour was measured in a microplate reader at 562 nm. CCA was evaluated by a previously described method (Rodrigues et al., 2015). In short, different concentrations of the extracts (30 μl, 1, 5 and 10 mg/mL) were mixed in 96-well microplates with 200 μl of 50 mM Na acetate buffer (pH 6.0), 6 μl of 4 mM pyrocatechol violet dissolved in the latter buffer and 100 μl of CuSO₄.5H₂O (50 μg/ml, *w/v*, in buffer). Change in colour was measured at 632 nm using a microplate reader. Results were expressed as percentage of inhibition relatively to a negative control solution containing DMSO in place of the sample. EDTA was used as a standard at a concentration of 1 mg/ml.

2.7.3. Ferric reducing activity power (FRAP)

The ability of the extracts to reduce Fe^{3+} was determined by the method reported by Rodrigues et al. (2015). Different concentrations of the extracts (1, 5 and 10 mg/ml; 50 μl) were mixed with distilled water (50 μl) and 1% potassium ferricyanide (50 μl) in 96 well plates and incubated at 50 °C for 20 min. Then, 50 μl of 10% trichloroacetic acid (*w/v*) and ferric chloride solution (0.1%, *w/v*) were added, and absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates better reducing power. BHT was used as a positive control at the concentration of 1 mg/ml.

2.8. In vitro neuroprotective properties

2.8.1. Inhibition of enzymes related with neurological diseases: AChE and BuChE

The inhibitory activity on AChE and BuChE was measured by the Ellman method (Ellman et al., 1961) as described previously (Custódio et al., 2016). In short, the extracts (20 μl at the concentrations of 1, 5 and 10 mg/ml) were mixed in 96- well microplates with 140 μl of 0.1 mM sodium phosphate buffer (pH 8.0) and 20 μl of AChE or BuChE solution (0.28 U/ml in the latter buffer), and incubated for 15 min. at RT. The reaction was initiated by adding 10 μl of the enzyme substrates, namely ATChI for AChE and BTChI for BuChE (4 mg/ml in 0.1 mM sodium phosphate buffer, pH 8.0)) and 20 μl of DTNB (1.2 mg/ml in 0.1 mM sodium phosphate buffer, pH 8.0). Hydrolysis of ATChI or BTChI was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines catalysed by the enzymes, at 412 nm, using a 96-well micro-plate reader. Results were expressed as AChE and BuChE percentage of inhibition relative to a negative control, containing DMSO instead of the sample. Galanthamine, an anticholinesterase alkaloid-type drug isolated from the bulbs of snowdrop (*Galanthus* sp.) was used as a positive control at the concentration of 1 mg/ml.

2.8.2. Cell-based assays

2.8.2.1. Protective effect of selected extracts on H₂O₂-induced cytotoxicity on neuronal cells.
Neuroblastoma cells (SH-SY5Y cell line) were kindly provided by Dr. Eduardo Soriano (Barcelona Science Park, Spain), and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with glucose (4500 mg/ml), 10% heat inactivated foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were grown at 37 °C in an incubator with 5.1% CO₂, in humidified atmosphere.

Extracts were first evaluated for their effect on cellular viability. For that purpose, cells were seeded in 96-well plates at a density of 2×10^4 cell per well, incubated for 24 h and exposed to different concentrations (3 to 125 μg/ml) of selected extracts. Cells were further incubated for 24 h and cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983) as described previously (Custódio et al., 2015). In brief, 20 μl of MTT (5 mg/ml in phosphate-buffered saline) were added to each well 2 h before the end of the incubation period. Plates were further incubated at 37 °C and 150 μl of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 590 nm (Biotek Synergy 4) and results were expressed as percentage of cell viability. Next, non-toxic concentrations of the extracts, that is, those allowing cell viability ≥85% were used to evaluate their protective effect on H₂O₂-

induced cytotoxicity. SH-SY5Y cells were seeded in 96-well culture plates at a density of 20×10^4 cells per well, left to attach for 16 h and treated with selected samples for 24 h. Samples were then removed and cells were treated with H_2O_2 (100 μM in DMEM) for 30 min. (Kim et al., 2005; Custódio et al., 2015). Negative control cells were treated with DMSO at the highest concentration used in test wells (0.5%, *v*/v), and cell viability was determined by the MTT assay.

2.8.2.2. In vitro anti-neuroinflammatory activity on LPS-stimulated microglia cells. The murine microglia cell line N9 was provided by Dr. João Malva (Center for Neurosciences and Cell Biology, University of Coimbra). Cells were maintained in DMEM culture medium supplemented with 1% of ʟ-glutamine, 1% of penicillin/streptomycin and 10% of heat-inactivated foetal bovine serum (FBS), at 37 °C in humid atmosphere with 5% of CO_2. To determine the effect of the extracts on the viability of N9 cells, they were seeded in 96-well plates at a density of 2×10^4 cell per well, incubated for 24 h and exposed to different concentrations (3 to 125 μg/ml) of *C. edulis* for 24 h. Cell viability was then determined by the MTT assay, as described on Section 2.8.2.1. Samples allowing cell viability higher than 80% were selected and used to assess their anti-inflammatory activity, through the study if their inhibition of NO production on LPS-stimulated cells. Cells were treated with non-toxic concentrations of the extracts, in serum- and phenol-free culture medium containing 100 ng/ml of LPS, and NO content was measured by the Griess assay (Rodrigues et al., 2016). A calibration curve was prepared with different concentrations (1.5–100 μM) of sodium nitrite as standard. Results were expressed as a percentage relative to a control containing culture medium alone, and as IC_{50} values (μg/ml).

2.9. Statistical analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM), and the experiments were conducted at least in triplicate. Analysis of variance (ANOVA) was used to assess differences using the SPSS statistical package for Windows (release 15.0, SPSS INC), and significance between means was analysed by the Duncan HSD test ($P < 0.05$).

3. Results and discussion

3.1. Nutritional profile

3.1.1. Proximate composition

Halophytes have a long tradition of consumption for their organoleptic properties (Ventura and Sagi, 2013). Knowledge of the nutritional components of edible species is essential to ascertain its adequacy for human consumption. In this context, the macronutrient composition of *C. edulis* was evaluated and is summarized on Table 1. *C. edulis* is a succulent plant, and as expected, its moisture content (92.3%) was higher than the values reported for other edible halophytes, as for example *Sporobolus virginicus* (seashore dropseed) and *Salicornia bigelovii* (dwarf saltwort) (Alhadrami et al., 2010; Lu et al., 2010). Crude protein

Table 1
Moisture (g/100 g of wet weight, WW), nutrients (g/100 g of dry weight, DW), chlorophylls and carotenoids (mg/100 g DW) content of leaves of *C. edulis*.

Parameters	Contents
Moisture	92.3 ± 1.10
Protein	4.55 ± 0.11
Fat	4.43 ± 0.48
Neutral-detergent fibre (NDF)	30.4
Ash	21.4 ± 0.30
Β-carotene	25.8 ± 2.08
Lycopene	35.7 ± 0.63
Chlorophyll a	17.1 ± 0.78
Chlorophyll b	12.2 ± 3.37

Values represent mean $\pm$ SEM (n = 3).

(4.55%) and ash levels (21.4%) were also higher than those of *S. bigelovii* (Lu et al., 2010), while ash was similar to the value reported for *Portulaca oleracia* (common purslane, Aberoumand and Deokule, 2009). Halophytes have in general a higher ash contents than other edible plants (Borah et al., 2009). Ash content is related to the total mineral levels, and thus the high ash contents of *C. edulis* leaves is most probably related to the saline environment in which it grows and to its capacity to accumulate minerals (Redondo-Gómez et al., 2010; Díaz et al., 2013).

The crude fat level was low (4.43%), but higher that the values reported for other edible halophytes, as for example *S. virginicus* and *S. bigelovii* (Alhadrami et al., 2010; Lu et al., 2010) and also for *Asparagus officinalis* (asparagus; USDA, 2016). In a previous work it was found that the fatty acids (FA) contents of leaves of *C. edulis* collected in the same location was characterized by a relevant polyunsaturated fatty acids (PUFAs) content (32.5%, Custódio et al., 2012). PUFAs, saturated fatty acids (SFA) and monounsaturated fatty acids and (MUFA) are the three key types of FA that humans can obtain through food. Animals and plants can synthetize MUFA and SFA, but not PUFA that thus must be provided in the diet (Schmitz and Ecker, 2008). PUFA allow for the increase of cholinergic neurotransmission, thus lightening AD symptoms (Willis et al., 2009). *C. edulis* also contains stearic acid (Custódio et al., 2012), a FA acid with AChE inhibitory properties (Fang et al., 2010). These data suggest that *C. edulis* has potential as a nutritional supplement to improve cognitive functions.

Halophytes usually contain appreciable levels of fibre (Díaz et al., 2013). In this work it was determined the content of Neutral Detergent Fibre (NDF) to estimate the amounts of cellulose, hemicellulose, lignin, cutin and tannins. NDF is generally used for the assessment of feed quality, but it is considered to be a reliable analytical tool for the estimation of the insoluble portion of dietary fibre in food (Dhingra et al., 2012). *C. edulis* had a higher content of NDF (30.4%) than those of most vegetables, but similar to the levels found in *Beta vulgaris* (beetroot) or *Solanum melongena* (eggplant, Dhingra et al., 2012) or in other halophytes like *Bassia hyssopifolia* (five-horn smotherweed, Díaz et al., 2013). A daily intake of 7 g of fibre of cereal or vegetable origin is considered enough to significantly reduce the risk of cardiovascular and coronary heart diseases (Threapleton et al., 2013). In this sense the consumption of 100 g of fresh portions of *C. edulis* would cover approx. 90% of such recommended daily dose.

Vegetables are a valuable source of phytochemicals like chlorophylls and carotenoids, the latter with recognized health-promoting properties, as for example antioxidant and immunomodulator (Bernal et al., 2011; Butt and Sultan, 2011). As can be seen on Table 1 *C. edulis* is a good source of β-carotene with a value (25.8 mg/100 g DW) similar to that found in *Salicornia* and *Sarcocornia* species (Ventura et al., 2011). The lycopene levels (35.7 mg/100 g DW) were similar to those of raw and canned *Solanum lycopersicum* (tomato), but more than 4-fold lower than other tomato products, such as sauce (USDA, 2016). Chlorophylls, although not particularly important in nutritional terms, provide a measure of the green vegetable colour and an estimation of senescence for consumers (Ventura et al., 2011). *C. edulis* leaves had low chlorophyll levels than other halophytes (Ventura et al., 2011) which may be less attractive to consumers.

Halophytes have several adaptations to saline environments such as the accumulation and compartmentalization of compatible solutes and ions for osmotic adjustment. They can also regulate transpiration and store essential nutrients, such as K in the presence of high levels of Na and Cl (Flowers et al., 2010), making halophytes valuable sources of essential minerals. The most abundant macroelement in *C. edulis* leaves was Na (43.8 mg/g, Table 2), higher than that of some food products rich in such mineral, as for example seaweeds (El-Said and El-Sikaily, 2013). Na is an essential nutrient but its excessive consumption is associated to several pathologies such as hypertension and cardiovascular diseases (Kotchen et al., 2013). Therefore, the World Health Organization (WHO) recommends that the daily intake of Na does not exceed

Table 2
Mineral amounts (per gramme of sample, DW) of leaves of
C. edulis.

Minerals	Contents
Macroelements	
Na (mg/g)	43.8 ± 0.15[a]
K (mg/g)	5.31 ± 0.02[c]
Ca (mg/g)	27.1 ± 0.26[b]
Mg (mg/g)	9.27 ± 0.11[c]
Microelements	
Fe (µg/g)	80 ± 0.002[a]
Mn (µg/g)	66.4 ± 0.98[b]
Zn (µg/g)	20.1 ± 0.12[c]
Toxic elements	
Cr (µg/g)	0.41 ± 0.03[a]
Pb (µg/g)	nd[1]
Ni (µg/g)	nd[2]
Cd (µg/g)	nd[3]

Values represent mean ± SEM (n = 3). In each group
of minerals, different letters mean significant differences
(P < 0.05).
[1] nd: not detected, LOD − 0.03 µg/g.
[2] LOD − 0.33 µg/g.
[3] LOD − 0.11 µg/g.

2000 mg (WHO, 2012). The consumption of 100 g of fresh leaves of
C. edulis would represent an intake of 337.26 mg of Na, and hence,
care must be taken so that the maximum allowed daily dose recom-
mended by WHO is not exceeded.

Calcium was the second most abundant macrolement in *C. edulis*
(27.1 mg/g), higher than the levels detected in different edible wild
plants, as for example *P. oleracia* and *A. officinalis* (Aberoumand and
Deokule, 2009), and similar to some vegetables rich in Ca, such as
Brassica rapa (mustard spinach), *Brassica oleracea var. acephala* (kale
scotch) and *Glycine max* (soybean; USDA, 2016). Calcium is an essential
element for human health, being involved in muscle functions, intracel-
lular signalling, nerve transmission vascular contraction, vasodilation
and hormonal secretion (Beto, 2015). The most common sources of Ca
in the diet are dairy products, as for example milk, yogurt, cheese, and
also commercially fortified foods, including orange juice, cereals and
soy (Beto, 2015). The recommended Ca intakes varies significantly
with several factors, including age, genetics and geographical area
(WHO, 2004). In general, the average recommended intake of Ca
for children is 600 mg, for adults is 1000 mg, while for the elderly
(>50 years old) and adolescents and menopausal women that value
rises to 1200 and 1300 mg, respectively (WHO, 2004; Beto, 2015).
Thus, a consumption of 100 g of fresh leaves of *C. edulis* would represent
an intake of 209.05 mg of Ca, thus covering 34.8%, 20.0%, 17.4% and
16.0% of the recommended daily doses for children, adults, elders and
adolescents/menopausal women, respectively.

Regarding micronutrients Fe was the most abundant (80 µg/g),
followed by Mn (66.4 µg/g) and Zn (20.1 µg/g, Table 2). The Fe content
of *C. edulis* was higher than the values reported for other edible wild
vegetables, such as *A. officinalis* and *P. oleracia* (Aberoumand and
Deokule, 2009), and similar than the contents of *S. bigelovii* (Lu et al.,
2010) and some commonly used vegetables, as for example *Ipomoea
batatas* (sweet potatoes) and *Cucurbita* species (squash; USDA, 2016).
As for the toxic elements (Cr, Pb, Ni and Cd) only Cr was detected,
and its value (0.41 µg/g) was below legislated value (0.2 mg/kg wet
weight for Cd; EC Regulation 1881/2006). Halophytes can, however,
accumulate toxic metals such as Zn, Cr, Pb, Ni and Cd when located
in contaminated sites (Caetano et al., 2008). Nevertheless, the highest
levels of those elements are found in the underground organs, since
translocation to the aerial organs is generally low (Caetano et al.,
2008; Moreira da Silva, 2008; Redondo-Gómez et al., 2010). Other
elements, as for example Fe and Mn are present in aboveground or-
gans at higher levels due to their role in photosynthesis (Moreira da
Silva, 2008).

3.2. Phytochemical characterization of the phenolic fraction of the extracts

Since each plant species contains different phenolics groups its char-
acterization is most often challenging. In this sense, the use of fast-
screening colorimetric methods is very helpful for the rapid assessment
of the total contents of specific phenolic groups (Naczk and Shahidi,
2004). In this work different solvents of increasing polarities, namely
hexane, dichloromethane, ethyl acetate and methanol were used to ex-
tract dried leaves of *C. edulis,* and the obtained extracts were evaluated
for total contents in phenolics (TPC), flavonoids (TFC) and tannins
(TTC). As expected the extracted compounds are highly dependent on
the polarity of the solvent (Table 3). Methanol has a high affinity
towards phenolic compounds (Khoddami et al., 2013), and accordingly
the methanol extracts had the highest TPC (378.8 mg GAE/g DW),
followed by the ethyl acetate (260.5 mg GAE/g DW), dichloromethane
(200.1 mg GAE/g DW) and hexane extracts (196.5 mg GAE/g DW;
Table 3).

Having in mind that natural extracts are generally considered rich in
phenolics when their TPC (expressed as GAE) is higher than 20 mg/g
(Kähkönen et al., 1999), *C. edulis* leaves can effectively be considered a
promising source of those compounds. The TPC of *C. edulis* was signifi-
cantly higher than those found in the literature for the same species
(Falleh et al., 2011; Custódio et al., 2012; Rodrigues et al., 2014). These
differences are most likely due to the different extraction procedures
or harvesting time and environmental characteristics, which are factors
known to influence the levels of phenolics in different plant species
(Grujic et al., 2012; Upadhya et al., 2015). Flavonoids followed a similar
trend than TPC, with the methanol extracts exhibiting the highest levels
(587 mg RE/g DW), followed by the ethyl acetate (383 mg RE/g DW), di-
chloromethane (369 mg RE/g DW), and hexane extracts (298 mg RE/g
DW; Table 3). Regarding tannins, the highest amounts were observed
in the dichloromethane (102 mg CE/g DW) and ethyl acetate extracts
(100 mg CE/g), followed by hexane (95.3 mg CE/g), and methanol
(58 mg CE/g; Table 3).

In order to further evaluate the individual phenolic components
present in the extracts, samples were analysed by HPLC-DAD and re-
sults are summarized on Table 4. From the twenty-six standards tested,
11 were identified belonging to 2 groups, namely phenolic acids: gallic,
p-hydroxybenzoic, vanillic and salicylic acids (hydroxybenzoic acids),
caffeic, coumaric, ferulic and rosmarinic acids (hydroxycinnamic
acids) and flavonoids: epicatechin (flavanol), quercetin (flavonol) and
apigenin (flavones). Some phenolics were previously identified in
leaves of *C. edulis*, namely coumaric acid, epicatechin and quercetin
(Martins et al., 2010; Falleh et al., 2011). Other compounds such as
rutin, catechin, oleanolic acic and uvaol were described previously
in *C. edulis* (Martins et al., 2010; Falleh et al., 2011), but were not
identified in this work. Differences in the phenolic profile among ex-
tracts from the same species collected in different habitats are most
likely caused by different genetic, geographical and cultural condi-
tions. However one must keep in mind that those dissimilarities
can simply be related with technical issues, such as sample prepara-
tion, extraction and method of analysis. To the best of our knowledge,
all the other identified phenolics are here firstly described in the
species.

Table 3
Total polyphenols (TPC; mg GAE/g DW), flavonoids (TFC; mg CE/g DW), and condensed
tannin contents (TTC, mg RE/g DW) of organic extracts of leaves of *C. edulis.*

Extract	TPC	TFC	TTC
Hexane	196 ± 3.89[c]	298 ± 5.85[c]	95.3 ± 6.37[b]
Dichloromethane	200 ± 7.73[c]	369 ± 8.14[b]	101 ± 10.9[a]
Ethyl acetate	260 ± 4.30[b]	383 ± 6.56[b]	100 ± 5.59[a]
Methanol	378 ± 4.72[a]	587 ± 6.82[a]	58.0 ± 2.66[c]

Values represent means ± SE (n = 3). In the same column, values followed by the same
letter are not significantly different at P < 0.05 according to the Duncan's multiple range
test.

Table 4

Phenolic profile (mg/g dry extract) of different extracts of *C. edulis* leaves analysed by HPLC-DAD.

Compound[a]	Extract			
	Hexane	Dichloromethane	Ethyl acetate	Methanol
Phenolic acids				
Hydroxybenzoic acids				
Gallic acid	–	0.03	1.3	–
p-Hidroxybenzoic acid	–	0.03	–	0.17
Vanillic acid	–	–	–	0.7
Salicylic acid	0.07	0.42	2.17	–
Sub total	0.07	0.48	3.47	0.87
Hydroxycinnamic acids				
Cafeic acid	–	0.11	–	–
Coumaric acid	–	–	0.49	0.11
Ferulic acid	–	0.3	0.89	0.5
Rosmarinic acid	0.03	0.11	–	–
Sub total	0.03	0.52	1.38	0.61
Total	0.1	1	4.85	1.48
Flavonoids				
Flavanols				
Epicatechin	–	0.15	–	–
Sub total	0	0.15	0	0
Flavonols				
Quercetin	–	–	1.15	–
Sub total	0	0	1.15	0
Flavones				
Apigenin	0.13	–	–	–
Sub total	0.13	0	0	0
Total	0.13	0.15	1.15	0
Total	0.23	1.15	6	1.48

[a] Identified by comparison of the retention parameters with the standard controls and peak purity with the UV–vis spectral reference data.

The extracts had different levels of phenolic compounds in the order ethyl acetate > methanol > dichloromethane > hexane. They also had a distinct phenolic profile and some compounds were identified in specific extracts, as for example, vanillic acid (methanol); cafeic acid and epicatechin (dichloromethane) and apigenin (hexane). The main compounds detected (>1 mg/g dried extract) were gallic and salicylic acids and quercetin, all in the ethyl acetate extract. Gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring low molecular weight triphenolic compound with established antioxidant and apoptosis inducing properties (Badhani et al., 2015). Salicylic acid is related with the enhancement of stress tolerance in plants, including resistance to saline stress (Singh and Gautam, 2013), and has important biological activities, as for example anti-inflammatory, analgesic, antipyretic and antiseptic (Khadem and Marles, 2010). Quercetin and other flavonols are produced by plants in severe stress conditions, thus contributing to their survival to abiotic constraints (Agati et al., 2012). Quercetin also exhibits bioactivities relevant for human health improvement, such as antioxidant, neuroprotective, antimicrobial and anti-tumoral (Maalik et al., 2014).

3.3. Antioxidant activity

With ageing the antioxidant defences decrease significantly resulting in the increase in oxidative stress which is an important underlying cause for several age-related diseases such as AD (Valko et al., 2007). Antioxidants have the capacity to protect the CNS by reducing oxidative stress and consequent damages (Guest and Grant, 2012). More specifically natural antioxidants are suggested as a promising alternative for the treatment and/or prevention of age-related neurological ailments (Guest and Grant, 2012). Moreover the use of products able to chelate iron and copper ions is indicated for the management of oxidative stress-related neurodegeneration since their accumulation significantly increases the formation of ROS through the Haber–Weiss/Fenton reaction (Gaeta and Hider, 2005; Weinreb et al., 2011).

The extracts had a dose-dependent RSA against the DPPH radical, and the highest activity was observed with the methanol extract at the concentration of 1 mg/mL (96.1%), which was significantly higher ($P < 0.05$) than the value obtained with the positive control used (BHT: 89.0%; Table 5). A lower DPPH scavenging capacity was reported for a methanol extract made with leaves from the same species (Custódio et al., 2012). These differences are most likely linked with different extraction methods used and/or periods of collection of the biomass, which are factors affecting the presence of antioxidant molecules in natural extracts (Khoddami et al., 2013).

Except for the methanol extract all samples had the capacity to reduce Fe^{3+} to Fe^{2+} in a dose-dependent way and the best result was achieved after application of the ethyl acetate extract at the concentration of 10 mg/mL (76.6%, Table 5). All samples were able to chelate iron, especially the hexane and dichloromethane extracts at the highest concentration tested (10 mg/mL), with values of 81% and 76%, respectively (Table 5). Conversely, only the ethyl acetate and methanol extracts had moderate capacity to chelate copper, and the best result was obtained with the methanol extract at the concentration of 10 mg/mL (48.2%; Table 5). Our results indicate that *C. edulis* leaves contain compounds able to act as primary antioxidants through the neutralization of free radicals and prevention of the initiation and propagation of oxidative chain reactions (Loganayaki and Manian, 2010). Moreover *C. edulis* is also endowed with secondary antioxidants capable to reduce oxidative stress-related injuries through the avoidance of radical generation (Loganayaki and Manian, 2010). Phenolic compounds also reached the maximum levels in the methanol extracts, and can thus be involved in the RSA and copper chelation activity detected in that extract (Soobrattee et al., 2005). The antioxidant capacity of phenolic compounds is well documented and is due to their basic chemical structure consisting of an aromatic hydrocarbon group attached directly to a hydroxyl group. This structure allows for the interruption of the free radical chain of oxidation through the donation of the hydrogen from the phenolic hydroxyl groups, resulting in the formation of a stable final product that does not initiate or propagate lipid oxidation (Pietta, 2000).

3.4. In vitro neuroprotective properties

Although the precise biochemical mechanisms of AD still remain unknown it is consensual that it is linked with a considerable loss of the neurotransmitter acetylcholine (ACh), which is hydrolysed by AChE and BuChE (Schifilliti et al., 2010). The use of compounds able to inhibit those enzymes, that is, cholinergic inhibitors (ChE-Is) is recognized as a reliable approach for the management of symptoms related with AD (Ahmad et al., 2006). In this work the inhibitory activity (%) of *C. edulis* extracts against AChE and BuChE was evaluated *in vitro* by the Ellman's method, and results are depicted on Table 6. Inhibition was classified as potent (> 50%), moderate (30–50%), low (<30%) or nil (<5%) (Vinutha et al., 2007). In accordance with this classification, the extracts had generally a higher capacity to inhibit AChE than BuChE, and the more polar samples, that is the ethyl acetate and methanol extracts generally resulted in a higher inhibition. The highest AChE inhibition was observed after application of the methanol extract at the concentration of 10 mg/mL (86%). This extract also allowed for a potent inhibition at the lowest concentration tested (75% at 1 mg/mL). Similar results were previously reported for methanol extracts from the same species (Custódio et al., 2012). Regarding BuChE, a potent inhibition was obtained after application of the highest concentration of the methanol (78%) and dichloromethane (64.3%) extracts. Noteworthy is the fact that except for the hexane extracts, all the samples allowed a dual and potent inhibition of both enzymes, indicating that *C. edulis* leaves contains molecules able to inhibit both AChE and BuChE, and may thus be useful for the improvement of cognitive functions. There is evidence that the inhibition of those enzymes not only rise the ACh levels, thus improving the cholinergic activity, but also avoid

Table 5
Radical scavenging activity on DPPH radical, metal chelating activity on copper (CCA) and iron (ICA) and ferric reducing activity (FRAP) activity of organic extracts of *C. edulis*. Results are expressed as percentage (%) of activity.

Sample	DPPH			CCA			ICA			FRAP		
	1 mg/ml	5 mg/ml	10 mg/ml	1 mg/ml	5 mg/ml	10 mg/ml	1 mg/ml	5 mg/ml	10 mg/ml	1 mg/ml	5 mg/ml	10 mg/ml
Hexane	0.78 ± 0.58[c]	7.25 ± 0.39[b]	16.7 ± 0.44[a]	na	na	na	45.7 ± 2.01[c]	72.7 ± 0.85[b]	81.3 ± 1.79[a]	6.83 ± 0.54[c]	16.5 ± 1.20[b]	28.9 ± 0.54[a]
Dichloromethane	0.39 ± 0.26[c]	13.2 ± 0.39[b]	34.2 ± 0.75[a]	3.35 ± 2.18[a]	8.12 ± 1.52[a]	7.13 ± 1.47[a]	50.7 ± 2.20[c]	68.5 ± 1.51[b]	76.3 ± 1.31[a]	11.65 ± 0.28[c]	42.8 ± 0.95[b]	63.7 ± 2.94[c]
Ethyl acetate	6.02 ± 0.66[c]	66.8 ± 0.81[b]	85.9 ± 0.31[a]	3.14 ± 0.78[c]	12.9 ± 0.96[b]	25.8 ± 1.10[a]	na	na	na	8.31 ± 0.31[c]	37.7 ± 1.16[b]	76.6 ± 1.70[a]
Methanol	96.1 ± 0.16	nt	nt	5.81 ± 2.14[c]	30.8 ± 1.48[b]	48.2 ± 0.70[a]	12.6 ± 1.91[c]	32.7 ± 0.82[b]	45.2 ± 1.31[a]	45.09 ± 0.50[a]	37.9 ± 2.24[a]	42.0 ± 1.71[a]
BHT[*]	89.0 ± 0.2	–	–	–	–	–	–	–	–	–	–	–
EDTA[*]	–	–	–	89.2 ± 0.54	–	–	89.6 ± 0.43			–	–	–

Values are mean ± SEM (n = 12). In each row values followed by different letters for the same assay and extract are significantly different according to the Tukey HSD test (*P* < 0.05). na: no activity; nt: not tested.

[*] Positive controls.

the development of Amyloid beta (Aβ) plaques, another hallmark of AD, since ChE-Is can activate α-secretase that acts on the Aβ precursor leading to Aβ plaques formation (Mesulam et al., 2002; Natarajan et al., 2009).

The drugs currently used to alleviate symptoms related with AD, as for example rivastigmine and galanthamine, are ChE-Is (Natarajan et al., 2009). They have however adverse undesired side-effects that may include hepatotoxicity and behaviour modifications, thus driving the need to search for novel and safer ChEi products (Natarajan et al., 2009; Chen-Chen et al., 2014). In this context, *C. edulis* may be a source of ChE-Is molecules with reduced side effects. Assays are already in progress aiming to identify and isolated the molecules responsible for the observed activity.

Another undeniable factor related with AD is oxidative stress, particularly caused by the accumulation the non-radical covalent molecule H_2O_2 which is formed naturally from superoxide anion ($O^{\bullet-}_2$) or through a reaction catalysed by superoxide dismutase (Qureshi and Parvez, 2007; Pan et al., 2009). Since it is highly permeable through membranes, H_2O_2 easily enters the neuronal cells and can causes injuries for example, through its involvement in the production of the highly reactive hydroxyl radical (OH$^\bullet$) (Coombes et al., 2011). Having this in mind *C. edulis* extracts were evaluated against H_2O_2-induced toxicity on a human neuroblastoma cells (SH-SY5Y cell line), which is a widely used *in vitro* model to study the effects and mode of action of drugs on neuronal diseases (Kim et al., 2005).

First, and in order to select the H_2O_2 concentration required to reduce cell viability by approximately 50–60%, cells where treated with different concentrations of H_2O_2 (0.25–1 mM) for different periods of incubation (15–30 min.) and cell viability was evaluated by the MTT assay. Treatment of SHSY5Y cells with 1 mM of H_2O_2 for 30 min. Resulted in a reduction of cell viability down to 62.4%, and was therefore selected and used in the following assays. The extracts cytotoxicity was then determined on SHSY5Y cells by the MTT method, aiming to select non-toxic samples (≥80% cell viability). None of the samples tested were toxic (data not shown), and therefore were all used in the

protective studies. For that purpose the extracts were applied to SH-SY5Y cells at concentrations ranging from 3 to 100 μg/ml for 24 h. After extract removal, cells were washed with PBS and treated with 1 mM of H_2O_2 for 30 min and cell viability was determined by the MTT method. The dichloromethane and methanol extracts had the strongest capacity to inhibit cell death induced by H_2O_2, allowing 105.1% and 143.4% of cell viability, respectively, at the concentration of 50 μg/ml (Fig. 1).

The harmful effects caused by free radicals in the brain, more specifically H_2O_2, can comprise enzyme impairment, mutations and apoptosis (Barnham et al., 2004; Harrison et al., 2005). Oxidative stress can also be responsible for mitochondrial damage that results in reduced ATP generation and protein misfolding, the latter being related with impaired degradation and possibly with the formation of Aβ plaques (Andersen, 2004). When H_2O_2 causes apoptosis, it generally occurs *via* the inhibition of ERK 1/2 phosphorylation and activation of p38 MAPK and caspase-3, coupled with the transactivation of nuclear factor (NF)-κB (Wang et al., 2003). The decrease in toxicity observed after the application of dichloromethane and methanol extracts of *C. edulis* can be related with a reduction of apoptotic injury to cells, and can be linked to their high antioxidant activity and phenolic content (Soobrattee et al., 2005; Oke and Aslim, 2011). In fact, phenolic compounds may protect neuronal cells through a selective inhibition or stimulation of key protein in the cell signalling cascades, in addition to their antioxidant capacity (Soobrattee et al., 2005). Altogether our results suggest that *C. edulis* leaves contains compounds with the potential to prevent neuronal cell damage resulting from oxidative stress in neurodegenerative diseases such as AD and complement the natural antioxidant defence system.

3.5. In vitro anti-neuroinflammatory properties

Microglia are able to remove infectious agents and damaged cells in the brain, release trophic factors and produce cytokines, thus guaranteeing the communication among neuronal and other glial

Table 6
AChE and BuChE inhibition of organic extracts of leaves of *C. edulis*.

Extract/compound	AChE			BuChE		
	1 mg/ml	5 mg/ml	10 mg/ml	1 mg/ml	5 mg/ml	10 mg/ml
Hexane	na	14.3 ± 4.6[b]	37.0 ± 5.1[a]	40.6 ± 1.04[a]	42.2 ± 1.2[a]	41.4 ± 3.29[a]
Dichloromethane	21.4 ± 3.1[c]	31.3 ± 3.4[b]	62.9 ± 7.5[a]	43.6 ± 1.21[c]	56.4 ± 1.69[b]	64.3 ± 1.85[a]
Ethyl acetate	50.3 ± 7.7[b]	80.7 ± 2.8[a]	75.6 ± 1.8[a]	39.5 ± 0.86[c]	64.8 ± 1.03[b]	78.8 ± 0.81[a]
Methanol	75.1 ± 0.7[b]	87.2 ± 1.0[a]	86.1 ± 0.6[a]	47.9 ± 1.21[b]	53.8 ± 2.11[ab]	59.4 ± 1.08[a]
Galanthamine[*]	89.9 ± 0.7[a]	–	–	81.3 ± 1.3[a]	–	–

Values are mean ± SEM (*n* = 12). On each row, values followed by different letters are significantly different according to the Tukey HSD test (*P* < 0.05). na: no activity.
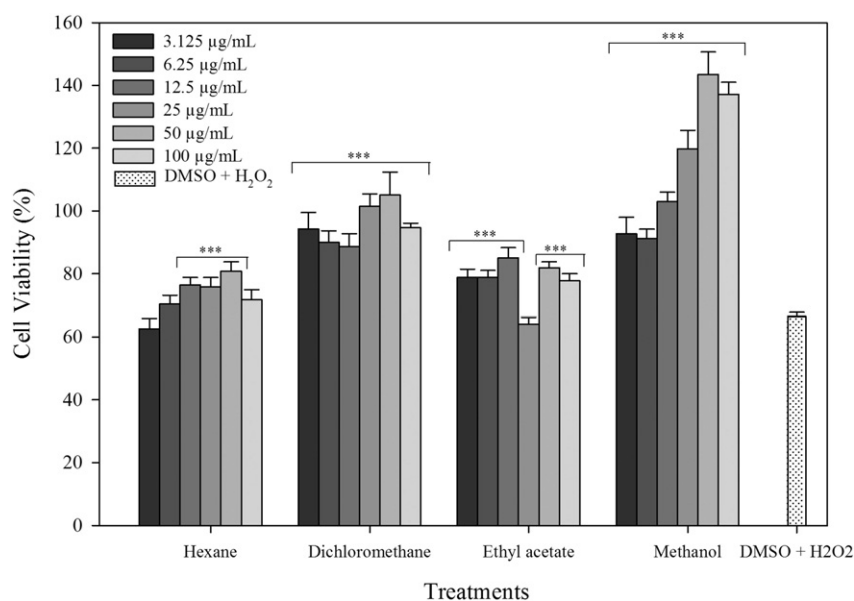
[*] Positive control.

Fig. 1. Protective effect of the *C. edulis* extracts in SH-SY5Y cells pre-treated with the extracts at different concentrations for 24 h, and exposed to 1 mM of H_2O_2 for 30 min. Solid and errors bars represent the average and standard error values, respectively ($n = 6$). Significance difference between cell viability of cells treated with DMSO (0.5%, v/v) and 1 mM H_2O_2 diluted in culture medium and those treated with the extracts are indicated as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cells (Bodrato et al., 2009). However, when microglia are constantly activated, a chronic neuro-inflammation can occur with the consequent production of neurotoxins, as for example pro-inflammatory cytokines and nitric oxide (NO), which contributes for the pathogenesis of neuro-degenerative diseases (Bodrato et al., 2009). In this context it is of utmost importance to unlock innovative molecules and/or products with anti-neuroinflammatory activity for the prevention and/or treatment of those ailments.

In this work the potential anti-neuroinflammatory activity of *C. edulis* extracts was evaluated on LPS-stimulated microglia cells. First, the cytotoxic effect of different concentrations of the extracts (3–100 μg/mL) was determined for 24 h on N9 cells by the MTT method. Similar to the observed in the previous section with the neuroblastoma cell line, an increase in cell viability occurred after application of all samples (data not shown). Thus, and having in mind that the methanol extract generally allowed the best results in the previous assays, it was selected to be tested for the anti-inflammatory activity. For that purpose, cells were treated with 200 μl of LPS during 24 h in the presence or absence of the methanol extract, and the level of nitrites in the culture medium was measured by the Griess method. The application of the extract at the concentration of 100 μg/ml significantly reduced NO production (1.53 μM) when compared with control cells (6.66 μM), corresponding to a 77% of decrease (Fig. 2), thus suggesting that this extract has compounds with anti-neuronflammatory properties. Similar results were reported by other authors for other halophyte species. For example the ethyl acetate fraction of an ethanol extract of whole plants of *S. bigelovii* suppressed the increasing production of NO on LPS-stimulated BV-2 microglial cells (Kang et al., 2013). The activity of the latter species was related with its high antioxidant activity and with the presence of several bioactive molecules, as for example triterpenoids, flavones, glycosides and saponins (Kang et al., 2013). Flavonoids have also a recognized anti-inflammatory property and since they were detected in high levels in the methanol extract may be related with the observed high *in vitro* anti-neuro-inflammatory activity (Serafini et al., 2010).

4. Conclusions

Our results show for the first time that leaves from *C. edulis*, a medicinal and edible species native to the coast of South Africa, have a

nutritional profile suitable for human consumption. Our work also confirms the antioxidant potential of this species and advances knowledge regarding its *in vitro* neuroprotective properties, namely the capacity to protect neuronal cells from oxidative stress imposed by H_2O_2 treatment and to reduce NO production in LPS-stimulated microglia cells. Altogether our results suggest that the consumption of leaves from *C. edulis* can contribute for a balanced diet and may add to the improvement of cognitive functions. Moreover, it proposes possible novel biotechnological applications for *C. edulis*, such as source of molecules and/or products to be used in the food and/or pharmaceutical industries, thus contributing for the valorisation of this medicinal plant.
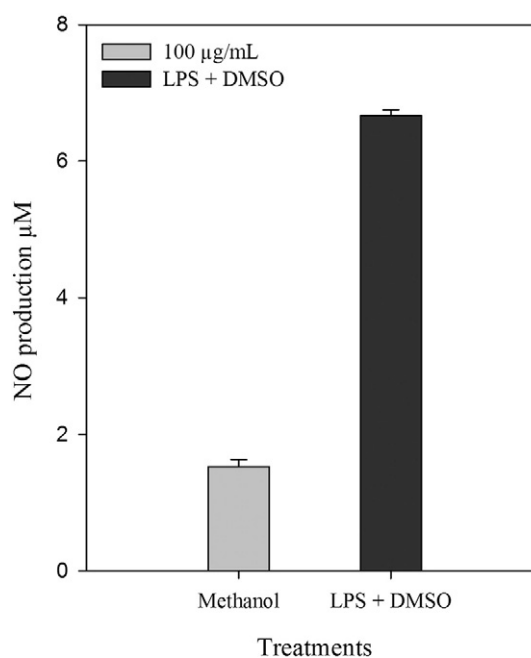

Fig. 2. NO production in the N9 cell line. Solid and errors bars represent the average and standard error values, respectively ($n = 6$). Significance difference between cell viability of cells treated with the methanol extract and LPS diluted in medium containing DMSO, 0.5%, v/v) are indicated as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Studies aiming the isolation and identification of the bioactive compounds are already in progress.

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