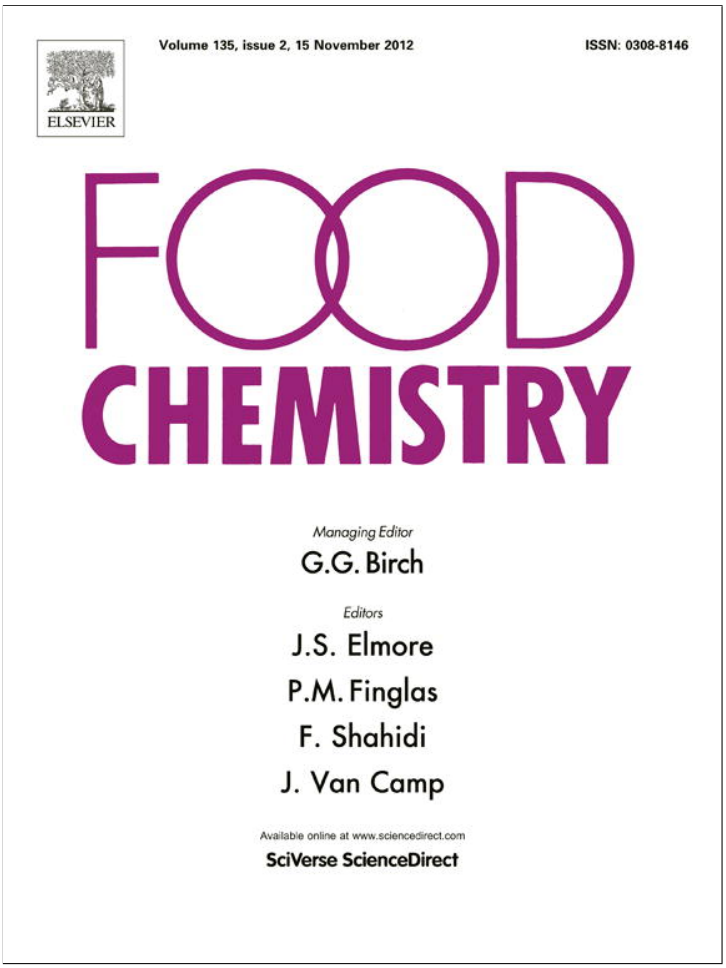

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The neuroprotective potential of phenolic-enriched fractions from four *Juniperus* species found in Portugal

Lucélia Tavares [a], Gordon J. McDougall [b], Sofia Fortalezas [a], Derek Stewart [b,c], Ricardo B. Ferreira [a,d], Cláudia N. Santos [a,e,*]

[a] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal
[b] Environmental and Biochemical Science Group, Enhancing Crop Productivity and Utilisation Theme, The James Hutton Institute, Dundee DD2 5DA, Scotland, UK
[c] School of Life Sciences, Heriot Watt University, Edinburgh EH14 4AS, UK
[d] Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal
[e] Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal

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ABSTRACT

The increase in population lifespan has enhanced the incidence of neurodegenerative diseases, for which there is, as yet, no cure.

We aimed to chemically characterize phenolic-enriched fractions (PEFs) from four wild *Juniperus* sp. found in Portugal (*Juniperus navicularis*, *Juniperus oxycedrus badia*, *Juniperus phoenicea* and *Juniperus turbinata*) and address their potential as sources of natural products for treatment of neurodegenerative diseases.

Leaves from the four *Juniperus* sp. evaluated contained a range of phenolic components which differed quantitatively between the species. The PEFs obtained were rich sources of phenolic compounds, exhibited acetylcholinesterase (AChE) inhibitory activity and also displayed effective intracellular radical scavenging properties in neurons submitted to oxidative injury but showed a different order of effectiveness compared to AChE inhibition. These properties made them good candidates for testing in a neurodegeneration cell model. Pre-incubation with *J. oxycedrus badia* PEF for 24 h protected neurons from injury in the neurodegeneration cell model.

1. Introduction

In the developed world, the increase in population lifespan has been accompanied by an increase in the incidence of many age-related diseases such as neurodegenerative disorders (Lau, Shukitt-Hale, & Joseph, 2006). These diseases are associated with enormous economic and social costs (Beking & Vieira, 2010; Wimo et al., 2011): it has been estimated that the European healthcare costs of for Alzheimer's disease (AD) in 2007 were €117 billion and that the number of cases are predicted to rise to 81.1 million by 2040 (Wimo et al., 2011). This, in conjunction with the lack of any cure, has made the development of new therapies for halting, retarding or reversing such disorders imperative.

Research in recent years has provided substantial evidence supporting the theory that oxidative stress plays a major role in the pathogenesis of neurodegenerative disease (Mulero, Zafrilla, & Martinez-Cacha, 2011; Schmitt et al., 2011; Shadrina, Slominsky, & Limborska, 2010). Oxidative stress is primarily caused by deviation of the cells redox balance from the norm and generally this is associated with an excessive accumulation of reactive oxygen species (ROS) in cells; a process previously implicated in the development of many neurodegenerative diseases including Parkinson's disease (PD), Huntington's disease, amyotrophic lateral sclerosis and AD (Gandhi & Wood, 2005; Lin & Beal, 2006; Shadrina et al., 2010). Furthermore, a consistent neuropathological occurrence associated with memory loss is a cholinergic deficit, which has been correlated with the severity of AD. Acetylcholinesterase (AChE) inhibition has been reported to ameliorate the symptoms of some neurodegenerative diseases and has been used as a rationale to develop drugs to treat AD (Williams, Sorribas, & Howes, 2011).

Natural products have made significant contributions towards the treatment of degenerative diseases (Newman & Cragg, 2007). Since plants constitute a rich and diverse source of secondary metabolites they have long contributed to the development of small-molecule based therapeutics; the class of compounds called phenolics, which display a significantly diverse combination of

* Corresponding author at: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal. Tel.: +351 214469651; fax: +351 214433644.

E-mail addresses: ltavares@itqb.unl.pt (L. Tavares), gordon.mcdougall@hutton.ac.uk (G.J. McDougall), fortalezas@itqb.unl.pt (S. Fortalezas), derek.stewart@hutton.ac.uk (D. Stewart), rbferreira@itqb.unl.pt (R.B. Ferreira), csantos@itqb.unl.pt (C.N. Santos).

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chemical moieties, structures and associated potent bioactivities, are a good example of this. Indeed, before the advent of high-throughput screening and the post-genomic era ~80% of drugs were natural products derived and more recently natural products derivatives still account for more than half of the drugs approved since 1994 (Harvey, 2008). Several small molecules, including (poly)phenolics, have been reported to exhibit neuroprotective properties (Mandel, Amit, Reznichenko, Weinreb, & Youdim, 2006; Ramassamy, 2006; Williams et al., 2011). In addition, reports suggest that some phenolic compounds may target numerous pathways and protein kinases that underlie these conditions and that strengthens their utility in multifactorial diseases, such as neurodegeneration (Ramassamy, 2006).

Plants from the *Juniperus* genus have found application in different European cuisines as a spice, flavouring for alcoholic drinks, as well as in cosmetics (Loizzo et al., 2007). Furthermore, these plants have an extensively history of use in global folk medicine for various disorders, such as common colds, urinary and kidney infections and dermatological disorders (Allen & Hatfield, 2004). Many biological activities have been reported for *Juniperus* sp. These include anti-inflammatory (Akkol, Guvenc, & Yesilada, 2009; Lesjak et al., 2011), diuretic, antiseptic (bacterial and fungal) (Cavaleiro, Pinto, Goncalves, & Salgueiro, 2006; Ennajar et al., 2009; Kour et al., 2008; Kusari, Lamshoft, & Spiteller, 2009; Miceli et al., 2009), anthelmintic (Kozan, Kupeli, & Yesilada, 2006), hypoglycaemic (Ju et al., 2008), hypotensive, abortifacient, antinociceptive (Akkol et al., 2009), antiviral (Sassi, Harzallah-Skhiri, Bourgougnon, & Aouni, 2008), anticancer (Kusari, Zuhlke, & Spiteller, 2010), antioxidant (Lesjak et al., 2011; Miceli et al., 2009; Orhan, Berkkan, Deliorman Orhan, Aslan, & Ergun, 2011; Orhan, Orhan, & Ergun, 2011; Ozturk, Tumen, Ugur, Aydogmus-Ozturk, & Topcu, 2011), anticholinesterase (Orhan, Orhan, et al., 2011; Ozturk et al., 2011) and analgesic properties (Moreno et al., 1998). They have also been found to be useful in the treatment of psoriasis (Koerfgen, 1964) and neurasthenic neurosis (Jonkov & Naidenov, 1974). Although the chemical composition of the essential oil from diverse *Juniperus* sp. has attracted significant interest (Angioni et al., 2003; Cavaleiro et al., 2001, 2002, 2003; Rezzi et al., 2001; Salido et al., 2002; Tunalier, Kirimer, & Baser, 2003) much less attention has been paid to other components and there is paucity of detail on the phenolic characterization of *Juniperus* sp. Previous reports have highlighted the presence of phenolic compounds including flavonoids, neolignans and phenylpropanoids (Iida et al., 2007; Innocenti et al., 2007; Lamerzarawska, 1975; Nakanishi et al., 2004; Sakar & Friedrich, 1984), but also terpenoids (Nunez, Salabarria, Collado, & Hernandez-Galan, 2007; Okasaka et al., 2006; Seca, Silva, Bazzocchi, & Jimenez, 2008). Studies on *Juniperus* species occurring in Portugal have been even more limited and the focus again was on essential oils (Cavaleiro et al., 2001, 2002, 2003). The aim of this work is to chemically characterize wild Portuguese *Juniperus* sp., in terms of phenolic compounds and address their potential as sources of natural products for treatment of neurodegenerative diseases. The rationale of this work is the search for AChE inhibitors as well as intracellular antioxidants as candidates to be tested in a neurodegeneration cell model.

2. Materials and methods

2.1. Plant material and extract preparation

Leaves of *Juniperus navicularis* Gand., *Juniperus oxycedrus* subsp. *badia* (H. Gay) Debeaux, *Juniperus phoenicea* L. and *Juniperus turbinata* Guss. were collected, frozen and then freeze-dried. *J. navicularis* is a Portuguese endemic and threatened plant and was sampled under a license for capture (11/2008/CAPT and 12/2008/CAPT). For all species, voucher samples were authenticated and deposited at the herbarium "João de Carvalho e Vasconcelos", Instituto Superior de Agronomia, Lisbon, Portugal. The herbarium numbers are: *J. navicularis* (LISI 1064/2007), *J. oxycedrus* subsp. *badia* (LISI 1/2008), *J. phoenicea* (LISI 3/2008) and *J. turbinata* (1067/2007). Freeze-dried leaves were ground in a IKA M20 mill to pass a 0.5 mm sieve and stored at −80 °C prior to extraction. Leaf extracts were prepared using an hydroethanolic solution (ethanol 50% (v/v)) as previously described (Tavares, Fortalezas, et al., 2010). Samples were dried by centrifugal evaporation.

2.2. Fractionation by solid phase extraction

Phenolic enriched fractions (PEFs) were obtained by fractionation by solid phase extraction (SPE) using Giga tubes 2 g 12 mL^{-1}, C18-E units (Phenomenex®) as described before (Tavares, Fortalezas, et al., 2010). PEFs were dried under vacuum, to suitable phenol contents for subsequent assays.

2.3. Chemical characterization

2.3.1. Total phenolic quantification

Determination of total phenolic content was performed by the Folin–Ciocalteau method adapted to microplate reader (Tavares, Carrilho, et al., 2010). Gallic acid was used as the standard and the results were expressed as mg of gallic acid equivalents (mg GAE) per g of dry weight (DW).

2.3.2. HPLC–MS phenolic profile determination

Dried extracts were redissolved and then applied to a C-18 column (Synergi Hydro C18 with polar end capping, 4.6 mm × 150 mm, Phenomonex Ltd.) and eluted over a gradient of 95:5 solvent A:B at time = 0 min to 60:40 A:B at time = 60 min at a flow rate of 400 μL min^{-1}. Solvent A was 0.1% (v/v) formic acid in ultra pure water and solvent B 0.1% (v/v) formic acid in acetonitrile. Samples were analysed on a LCQ–DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan), as reported by Tavares, Fortalezas, et al. (2010). The LCQ–DECA system comprised a Surveyor autosampler, pump and photo diode array (PDA) detector and a Thermo Finnigan mass spectrometer iontrap.

2.4. AChE inhibitory assay

AChE inhibition was determined in 96-microtiter well plate, based on Ellman's reaction, as described previously (Tavares, Fortalezas, et al., 2012). Different concentrations of PEFs of the four species were tested (50, 100, 200, 400 and 800 μg GAE mL^{-1}). The effect on AChE activity was calculated as percentage inhibition (%) of the control activity without inhibitor.

2.5. Cell culture

Human neuroblastoma SK-N-MC cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured in EMEM (Eagle Minimum Essential Medium, Sigma) supplemented with 2 mM L-glutamine (Sigma), 10% (v/v) heat inactivated foetal bovine serum (FBS, Gibco), 1% (v/v) non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma) and containing 50 U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin. The cells were maintained at 37 °C in 5% CO_2 and harvested at sub-confluence of 70–80% using 0.05% trypsin/EDTA (Gibco).

2.6. Cytotoxicity profile

PEFs were dissolved in cell medium for measuring cell viability as previously described (Tavares, Figueira, et al., 2012). Briefly, SK-

N-MC neuroblastoma cells were seeded in a 96-well plate using 1.25×10^5 cells mL^{-1} and grown for 48 h prior to addition of PEFs. Cytotoxicity was assessed using 24 h incubation with PEFs in the range 0–500 μg GAE mL^{-1} medium. Cell viability was assessed using the CellTiter-Blue® Cell Viability Assay (Promega), according to the manufacturer's instructions.

2.7. Intracellular ROS production

To evaluate the ability of PEFs to reduce cellular ROS levels, the conversion of 2′,7′-dichlorofluorescein diacetate (H_2DCFDA, Invitrogen) to fluorescent 2′,7′-dichlorofluorescein (DCF) was monitored, as already described (Tavares, Figueira, et al., 2012). Briefly, SK-N-MC neuroblastoma cells were seeded in a 96-well plate (1.25×10^5 cells mL^{-1}), grown for 24 h prior to treatment then pre-incubated with PEFs prepared in medium (0.5% (v/v) FBS) for 2 or 24 h. Cells were then incubated with 25 μM H_2DCFDA for 30 min at 37 °C, followed by addition of H_2O_2 (200 μM) and the fluorescence determined (λ_{ex}: 485 nm, λ_{em}: 530 nm) over 1 h at 37 °C. ROS generation was calculated as an increase in fluorescent signal between the control and H_2O_2-treated cells.

2.8. Determination of antioxidant enzyme activity

Cells were seeded in 6-well plates and grown for 24 h before treatment with PEFs. After 24 h, cells were treated with H_2O_2 (200 μM, 1 h), which has been previously reported as inducing oxidative stress without cell death (Tavares, Figueira, et al., 2012). Cells were washed with PBS and lysed with 200 μL of lysis buffer, containing 50 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.5 mM EGTA and 0.05% (v/v) Triton X-100. Cells were incubated for 20 min on ice with vortexing every 5 min, then centrifuged 15 min at 9000*g*. The supernatant was used to determine the activities of superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6). The activity of both enzymes was normalized for protein content determined by the Lowry method (Bensadoun & Weinstein, 1976).

2.8.1. Superoxide dismutase (SOD) assay

SOD activity was measured based on the extent of inhibition of amino blue tetrazolium formazan formation and was adapted from Mockett, Bayne, Sohal, and Sohal (2002). Briefly, cell extracts were incubated in 100 mM PBS (pH 7.8) containing 0.1 mM EDTA, 0.1 mM xanthine, 100 μM nitro blue tetrazolium (NBT) and 2.5 nM xanthine oxidase (EC 1.1.3.22, CAS No.: 9002-17-9, purified from bovine milk, Grade I, Sigma). The reduction of NBT was measured at 560 nm. One unit of SOD activity was determined as the rate of absorbance change per min.

2.8.2. Catalase (CAT) assay

CAT activity was measured by the method of Aebi (1984) in which the rate of H_2O_2 decomposition was determined spectrophotometrically at 240 nm. The reaction mixture contained 30 mM of H_2O_2 in 50 mM potassium phosphate buffer, at pH 7.0. The reaction was initiated by adding 50 μL of cell lysate and the decrease in absorbance per min was calculated from the initial linear portion of the curve, using a molar extinction coefficient of 0.0394 cm^{-1} mM^{-1}. The enzyme activity was expressed as U mg^{-1} protein, with 1 U being the amount of enzyme consuming 1 μmol of H_2O_2 per min.

2.9. Neuroprotective effect against oxidative stress

Fractions were evaluated for their neuroprotective effect in a neurodegeneration cell model described previously (Tavares, Figueira, et al., 2012). The model involved the treatment of SK-N-MC neuroblastoma cells with H_2O_2 to induce cell death. Briefly, cells were seeded at 7.4×10^4 cells mL^{-1} and grown for 24 h. After 24 h of pre-incubation with medium supplemented with non-toxic concentrations of PEFs, cells were treated with medium containing H_2O_2 (300 μM). After 24 h, the medium was removed and cells were washed with PBS and collected by trypsinisation. Cells were then incubated with two fluorescent probes for 30 min at 37 °C. Mitochondrial transmembrane potential ($\Delta\Psi m$) was assessed using 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, 20 nM, Invitrogen) and cell viability was determined by assessing plasma membrane integrity using propidium iodide (PI, 1 μg mL^{-1}, Invitrogen) (Queiroga et al., 2010). These parameters were analysed by flow cytometry using a blue solid state laser (488 nm) with FL1 green fluorescence channel for $DiOC_6(3)$ at 530 nm and a FL3 red fluorescence channel for PI detection at 650 nm. The acquisition and analysis of the results were performed with FlowMax® (Partec) software.

2.10. Statistical analysis

The results reported are the averages of at least three independent experiments and are represented as the mean ± SD. Differences among treatments were detected by analysis of variance with Tukey HSD (Honest Significant Difference) multiple comparison test ($\alpha = 0.05$) using SigmaStat 3.10 (Systat).

3. Results

3.1. Chemical characterization

Hydroethanolic extracts were obtained from the four Juniper species and chemically characterized (Table 1). The phenolic content was highest for the extract obtained from *J. oxycedrus badia*, followed by *J. turbinata*, *J. navicularis* and *J. phoenicea*, respectively. Following SPE fractionation, enriched phenolic fractions (PEFs) were obtained for all species with similar phenolic contents. They were used at an equivalent phenolic load for all subsequent comparative bioactivity assessments.

All four *Juniperus* sp. gave LC–MS–PDA profiles, in which some shared peaks presented quantitative differences (Fig. 1 and Table 2). Their phenolic composition is also in agreement with the phenolic identifications found in the literature for *Juniperus* sp. Until now the identifications of *Juniperus* sp. compounds are spread across different reports, in which different species were used and for each only a few number of compounds were identified (Dzharullaeva, 2009; Iida et al., 2007, 2010; Lesjak et al., 2011; Lim et al., 2002; Martz et al., 2009; Miceli et al., 2009; Nakanishi et al., 2004; Orhan, Orhan, et al., 2011; Sakar & Friedrich, 1984; Stassi, Verykokidou, Loukis, & Harvala, 1998).

Catechins, procyanidins, flavonol derivatives, flavones and biflavones (Fig. 1 and Table 2) were putatively identified in the *Juniperus* sp. Two major compounds that have been previously reported in *J. communis* as unknown (Martz et al., 2009) were present and identified as psydrin (peak 1) and procyanidin trimmer (peak 9). Psydrin was the major peak of *J. oxycedrus badia*, *J. phoe-*

Table 1
Total phenolic content of crude extracts and PEFs obtained from the four *Juniperus* sp. Values are expressed as mg GAE g^{-1} of dried powder. Statistically significant differences at $p < 0.05$ are denoted with different letters (a–c). All values are mean ± SD, $n = 3$.

Species	Crude extract	PEF
J. navicularis	247 ± 28^{bc}	310 ± 40
J. oxycedrus badia	354 ± 10^{a}	414 ± 90
J. phoenicea	221 ± 5^{c}	330 ± 24
J. turbinata	274 ± 3^{b}	401 ± 55

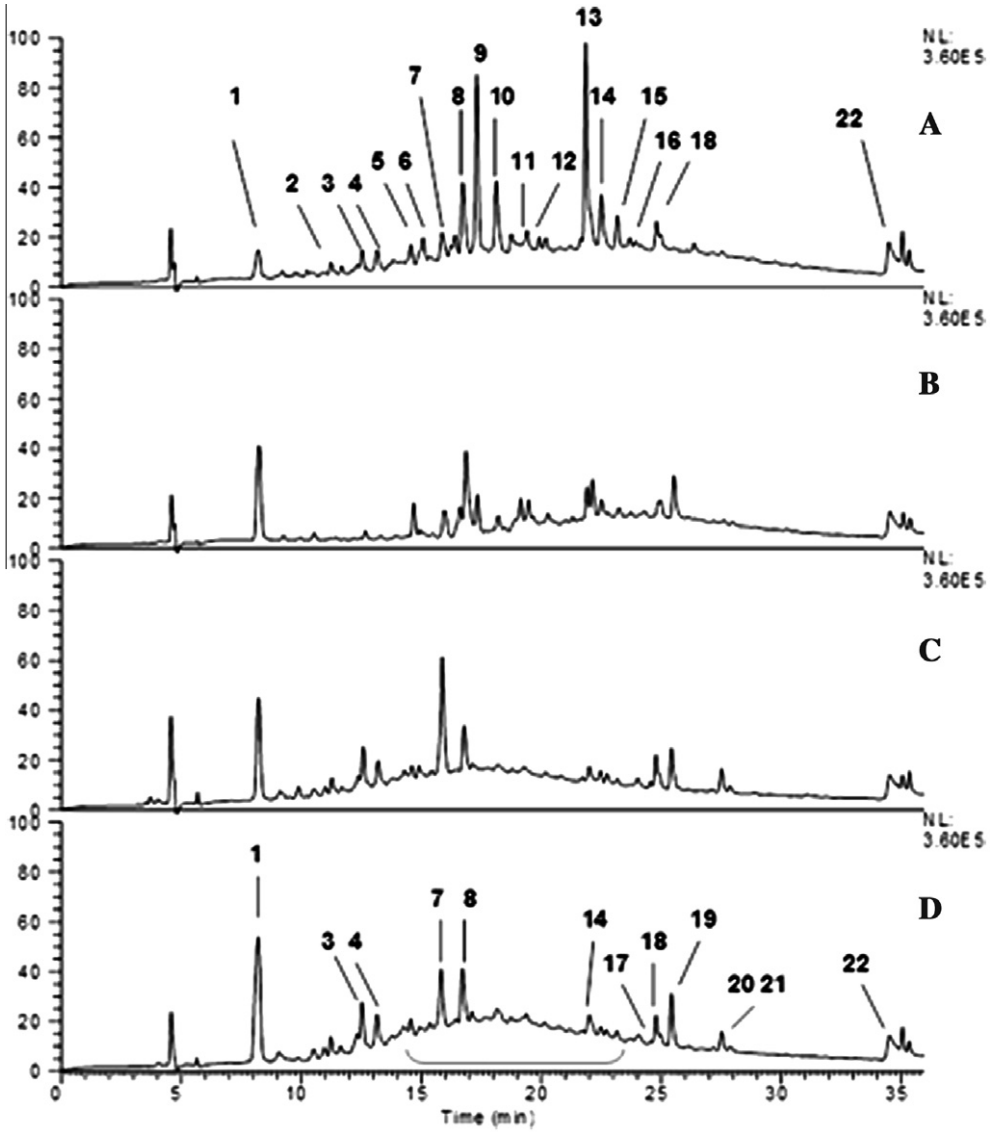

Fig. 1. Representative PDA profiles for *J. navicularis* (A), *J. oxycedrus badia* (B), *J. phoenicea* (C) and *J. turbinata* (D). Peak numbers correspond to their putative identifications based on MS fragmentation data presented in Table 2. The full scale deflection for each trace is shown in the top right corner. The area denoted by the bar is discussed in the text.

nicea and *J. turbinata*, but significantly less prevalent in the *J. navicularis* LC–MS–PDA profile. Amongst the major compounds of *Juniperus* sp. were catechins, a range of flavonol derivatives (such as quercetin rutinoside and quercetin rhamnoside), flavones (such as isoscutellarein and luteolin glycosides) and biflavones. Procyanidins were present in all PEFs, but in variable amounts. Some procyanidins components eluted as discrete peaks (e.g. peaks 2, 4, 5 and 7), but they were also presented as a poorly resolved smear across RTs 17–25 min (Fig. 1, annotated by bar).

All four species contained flavonol derivatives mainly as quercetin, but also as myricetin, glycosides, but they varied in content and composition between the species. For example, quercetin rutinoside (peak 13) was a major component of *J. navicularis* but it was present in much lower amounts in the other species. Another major difference between *J. navicularis* and the other species was the abundance of procyanidin trimmer (peak 9, the unidentified phenolic component reported by Martz et al. (2009)).

Putative flavone derivatives were detected, but whether these were isoscutellarein and/or luteolin glycosides could not be completely confirmed by LC–MS. However, these flavone derivatives (especially peaks 20 and 21) were more abundant in *J. oxycedrus badia, J. phoenicea* and *J. turbinata*. Such flavone derivatives have already been identified in *J. communis* var. *depressa* (Iida et al., 2007; Nakanishi et al., 2004). Biflavone derivatives were putatively identified in all four species (peak 22, RT ~35 min). However, these apolar compounds eluted late in the gradient and were not sufficiently separated to allow us to distinguish between the three biflavones already identified in *J. drupacea* as amentoflavone, cupressuflavone and hinokiflavone (Sakar & Friedrich, 1984).

3.2. AChE inhibitory assay

AChE inhibition by the PEFs was tested at the following concentrations: 50, 100, 200, 400 and 800 μg mL^{-1} (Table 3). *J. turbinata* was the most potent species. Using 800 μg mL^{-1} of *J. turbinata* PEF the inhibition was 72.65%. This species was followed by *J. phoenicea, J. navicularis* and *J. oxycedrus badia*. The last species was the only not able to inhibit more than 50% at the concentrations tested.

Table 2
Putative identities of major peaks in Juniper fractions.

Peak No.	RT	PDA	*m/z* [M−H]	MS^2	Putative identity
1*	8.22	271	**275.1** [321.1]	**161.1**, 113.1	Psydrin,a furanone glycoside (Comte, Allais, Chulia, Vercauteren, & Delage, 1996)
			+**277.1**, 115.1	+**115.1**, 87.1	
2	11.23–11.41	280–310	**593.2**, 425.4, **289.8**	549.1, **289.1**	Epicatechin–epigallocatechin (EC–EGC)
3	12.54	285	**305.1**	179.1	Gallocatechin
4	13.17	269	**896.7**, **593.0**, 425.3, **289.1**	710.9, **593.1**, 425.0	Procyanidin (EC–EGC–EGC)
5	14.58	277	**577.1**, 289.2	451.0, 407.1, **289.1**	Procyanidin dimer (EC2)
6	15.07	283–314	**305.2**	219.1, **179.0**	Epigallocatechin
7	15.87	275	**577.0**, 289.1	**289.1**	Procyanidin dimer (EC2)
8	16.74	279	**289.2**	245.1, 179.2	Epicatechin
9	17.32	275	**865.1**, 577.0	695.1, **577.1**	Procyanidin trimer (EC3)
10	18.14	275	**881.2**	593.1, 695.1	Procyanidin trimer (EC–EGC–EC)
11	19.42	275, 378	**479.2**, 317.1	317.1	Myricetin hexose (Miceli et al., 2009)
12	19.93	270, 357	**625.3**, 317.0	317.1	Myricetin rutinoside
13	21.86	255, 354	**609.2**, 301.1	301.2,	Quercetin rutinoside (Lesjak et al., 2011)
14	22.06	270–365	**449.2**, 317.1	317.1	Myricetin pentose
15	22.51	221, 269	**755.0**	**569.0**, 289.1	Proanthocyanidin derivative
16	23.17	280–365	**181.5** **463.7**, 301.2	None 301.2	Unknown compound Quercetin hexose
17	24.56	280–340	**447.1**, 285.0	285.1	Isoscutellarein hexoside
18	24.81	276, 342	**433.2**, 301.2	301.2	Quercetin pentoside
19	25.43	280–340	**447.1**, 301.1	301.1	Quercetin rhamnoside
20	27.57	280–340	**417.0**, 285.2	285.2	Luteolin/isoscutellarein pentoside
21	27.88	280–340	**431.1**, 285.1	285.1	Luteolin/isoscutellarein rhamnoside
22	35.06, 35.34	272, 329	**537.9**	375.2	Amentoflavone or Cupressuflavone or Hinokiflavone (Sakar & Friedrich, 1984)

Figures in bold are the main m/z or MS^2 signals and were those used for MS^2 fragmentation.
Compound 1 is equivalent to the unknown compound as detected previously by Martz et al. (2009). Figures in parantheses are formate adducts. +denotes [M + H] signals.
Other *m/z* signals for larger proanthocyanidins (e.g. 1153 for epicatechins tetramers) are present but do not elute as discrete peaks.
* - Exact mass data obtained on the Orbitrap system supports this identification; molecular formula $C_{11}H_{17}O_8$ in negative mode (RDB 4.5, delta amu 0.001). The difference in MS^2 fragmentation pattern between positive and negative mode is also noted by Comte et al. (1996).

3.3. Cytotoxicity profile

Cell viability was determined for SK-N-MC neuroblastoma cells submitted to increasing concentrations of PEFs (0 to 500 μg GAE mL^{-1}; Fig. 2). The four species presented different toxicity profiles. The less toxic species were *J. navicularis* and *J. oxycedrus badia*, which required more than 100 μg GAE mL^{-1} to cause complete cell death. *J. turbinata* and *J. phoenicea* PEFs were more toxic,

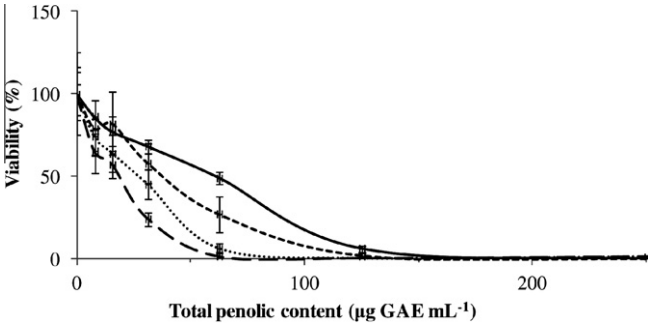

Fig. 2. Cytotoxicity profiles of *Juniperus* sp. PEFs. Cell viability was determined for SK-N-MC neuroblastoma incubated with *Juniperus* sp. PEFS (0–500 μg GAE mL^{-1}) for 24 h. *J. navicularis* (——), *J. oxycedrus badia* (———), *J. phoenicea* (– –) and *J. turbinata* (·····). All values are mean ± SD, $n = 3$.

Table 3
AChE inhibitory capacity by *Juniperus* species. Results are reported as % inhibition compared to the control activity. Values are the average of at least three replicates ± SD.

Fraction concentration (μg mL^{-1})	*J. navicularis*	*J. oxycedrus badia*	*J. phoenicea*	*J. turbinata*
50	6.44 ± 3.28	14.97 ± 8.71	14.36 ± 4.84	16.38 ± 5.76
100	14.31 ± 6.46	14.26 ± 3.60	15.93 ± 4.71	29.33 ± 3.90
200	25.76 ± 3.48	24.02 ± 5.03	33.74 ± 3.32	47.07 ± 4.88
400	30.69 ± 7.60	27.03 ± 8.47	53.44 ± 3.02	83.84 ± 7.90
800	55.60 ± 5.23	38.78 ± 4.07	65.48 ± 3.03	72.65 ± 17.06

as they required less than 100 μg GAE mL^{-1} to drop cell viability to 0%. The different toxicities were also reflected in the amount of PEFs required to attain 50% of cell viability (~20 μg GAE mL^{-1} for *J. phoenicea* and 60–70 μg GAE mL^{-1} for *J. navicularis*).

3.4. Intracellular ROS production

The effect of pre-incubation with *Juniperus* PEFs on the production of ROS by neuroblastoma cells in the presence or absence of oxidative stress, was evaluated. PEFs were applied at concentrations demonstrated to be non-toxic for cells. In the absence of stress, all four *Juniperus* sp. PEFs reduced the basal production of ROS in cells exposed for 2 or 24 h (Figs. 3A and B, respectively), and this reduction was more significant after 24 h incubation (Fig. 3B). When cells treated with PEFs for 2 h were exposed to a non-lethal stress (200 μM H_2O_2 for 1 h; (Tavares, Figueira, et al., 2012)), a significant decrease in ROS levels was recorded compared to the stress in the absence of PEFs but not for all PEF concentrations used (Fig. 3C). For example, the highest concentration of PEFs from *J. phoenicea* and *J. turbinata* (5 μg GAE mL^{-1}) did not significantly reduce ROS production although lower concentrations did. Indeed, when cells were pre-incubated with PEFs for 24 h, only *J. navicularis* and *J. oxycedrus badia* PEFs significantly reduced ROS production, for all three concentrations used (Fig. 3D).

The 24 h pre-incubation with PEFs gives sufficient time that ROS levels may be affected through alterations in indirect antioxidant defences. Therefore we decided to evaluate the activities of two important endogenous antioxidant enzymes (SOD and CAT). Their activities were determined in neuroblastoma cells pre-incubated with PEFs for 24 h in the absence or presence of non-lethal stress (200 μM H_2O_2 for 1 h). No differences were detected in SOD or CAT activities, when cells were stressed with H_2O_2 (results not shown). The lack of changes in SOD and CAT activities were also verified in cells pre-incubated with PEFs or pre-incubated with PEFs followed by the oxidative challenge.

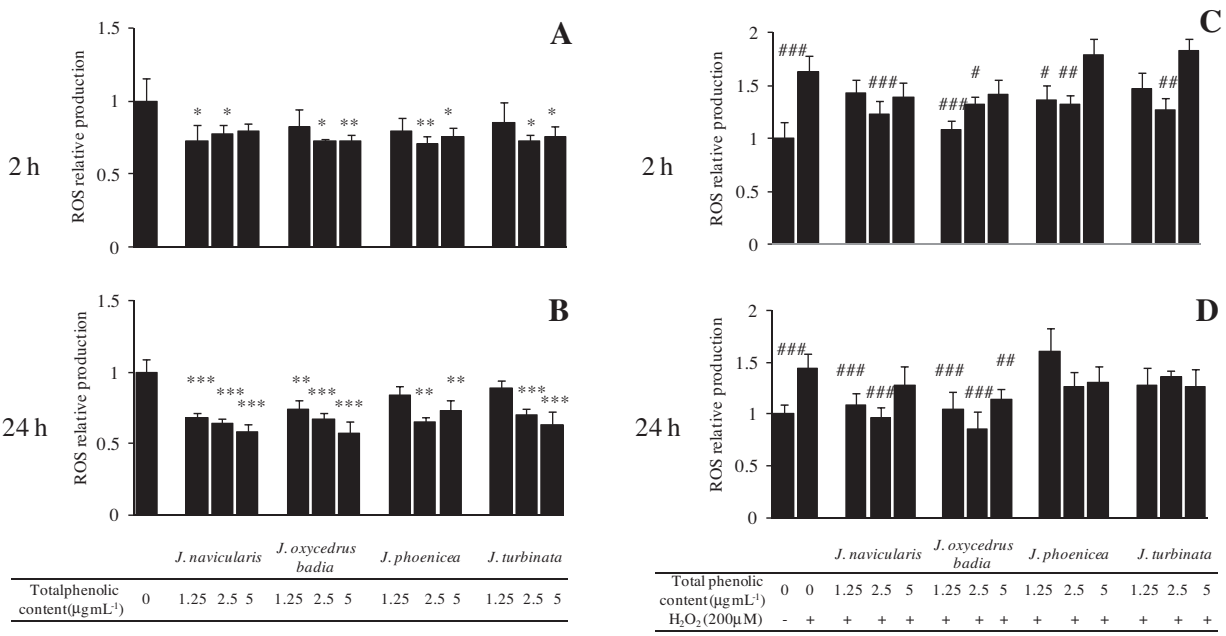

Fig. 3. Relative intracellular ROS production by SK-N-MC neuroblastoma cells pre-incubated with *Juniperus* PEFs. Neuroblastoma were pre-incubated with PEFs for 2 h (A) or 24 h (B) in the absence of stress or for 2 h (C) or 24 h (D) in the presence of stress (H_2O_2 200 μM, 1 h). ROS were detected by fluorimetry using H_2DCFDA as a probe. Statistical differences in relation with untreated cells are denoted as $^{*}p < 005$; $^{**}p < 0.01$; $^{***}p < 0.001$; statistical differences in relation with stressed cells are denoted as $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$. All values are mean ± SD, $n = 3$.

3.5. Neuroprotective effect against oxidative stress

PEFs were also tested in a neurodegeneration cell model for their protective effects. This model consists of neuroblastoma cells injured with 300 μM H_2O_2 for 24 h, a condition that routinely reduces viability to 50%. Two parameters were used to assess the protective effect of the PEFs: cell viability through the measurement of membrane integrity (Fig. 4A) and measurement of mitochondrial transmembrane potential (ΔΨm) (Fig. 4B).

All *Juniperus* sp. PEFs were tested, but only the *J. oxycedrus badia* PEF was able to protect cell viability and promote an increase in ΔΨm (Fig. 4) at the concentrations used. The others *Juniperus* sp. PEFs did not improve these parameters over the stressed conditions (results not shown). The highest concentration of *J. oxycedrus badia* PEF (5 μg GAE mL^{-1}) did not change viability, but decreased ΔΨm in the absence of H_2O_2, suggesting that it may influence cellular metabolism. Under H_2O_2 stress, 2.5 μg GAE mL^{-1} PEF was the most effective cytoprotective concentration, in both parameters (viability and ΔΨm). The use of 5 μg GAE mL^{-1} of PEF was not able produce a better protection than 2.5 μg GAE mL^{-1} PEF.

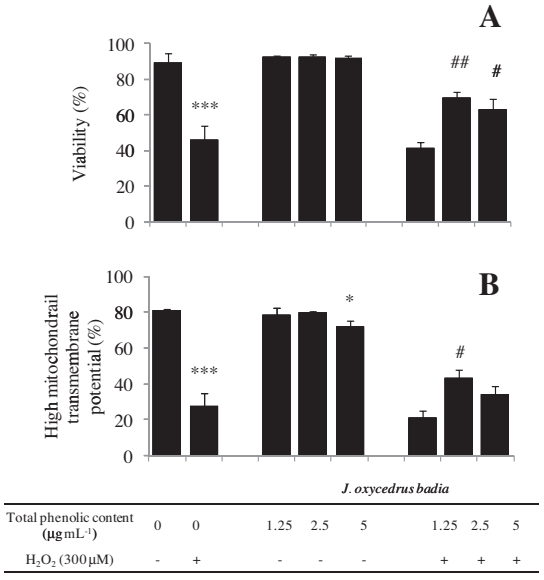

Fig. 4. Cell viability and mitochondrial transmembrane potential (ΔΨm) for cells pre-incubated with *J. oxycedrus badia* PEF. (A) Cell viability, expressed as percentage of cells containing intact membrane, using PI as fluorochrome. (B) Percentage of cells presenting high ΔΨm, using $DiOC_6(3)$ as probe. Neuroblastoma cells were pre-incubated with PEFs for 24 h and then injured by 300 μM H_2O_2 for 24 h. Statistical differences comparatively with untreated cells are denoted as $^{*}p < 0.05$; $^{***}p < 0.001$; statistical differences comparatively with injured cells are denoted as $^{\#}p < 0.05$; $^{\#\#}p < 0.01$. All values are mean ± SD, $n = 3$.

4. Discussion

Previous work has attributed antioxidant activity and acetylcholinesterase inhibitory activity to extracts of some *Juniperus* sp. These properties along with their multiple aforementioned bioactivities (see Section 1) make *Juniperus* sp. interesting plants in the search for new natural products to treat neurodegenerative diseases. Despite this, diverse species of *Juniperus*, and in particular those growing in Portugal have not been characterized with respect to their phenolic composition; the proposed source of some of their bioactivities. Botanically, *J. navicularis* and *J. oxycedrus badia* belong to section Juniperus and *J. phoenicea* and *J. turbinata* belong to section Sabina and these inter-relationships might also underlie chemical diversity (Verpoorte, 1998).

The four species yielded extracts with high total phenolic contents when compared with extracts obtained in other works (Orhan, Orhan, et al., 2011; Ozturk et al., 2011). After enrichment by SPE fractionation the fractions exhibited a higher phenolic content without organic acids, sugars and minerals, which could confound any subsequent bioactivity analyses.

This is the first time that the phenolic composition of these four *Juniperus* sp. has been described. Previous work on these species

has mainly focused on essential oils composition (Cavaleiro et al., 2006, 2001, 2003) or have described biological properties without attempting to define or chemically characterise the derivation of the bioactivity (Karaman et al., 2003; Kozan et al., 2006; Moreno et al., 1998). This work showed that the main polyphenolic compounds were common among the different species and some have been identified previously in various studies for different *Juniperus* sp. (Dzharullaeva, 2009; Lesjak et al., 2011; Lim et al., 2002; Martz et al., 2009; Miceli et al., 2009; Nakanishi et al., 2004; Sakar & Friedrich, 1984; Stassi et al., 1998). The main differences noted between the four species under study were in the relative amounts of certain compounds. For example, in *J. oxycedrus badia*, *J. phoenicea* and *J. turbinata*, psydrin was a major peak. However, in *J. navicularis*, this compound was not so dominant and other components, including the procyanidin trimmer (peak 9, RT = 17.32) and quercetin rutinoside (peak 13, RT = 21.86), were more abundant. On a equivalent phenolic basis, *J. oxycedrus badia*, *J. phoenicea* and *J. turbinata* were characterised by having greater levels of psydrin, but also enhanced levels of procyanidins and certain individual peaks such as peak 7 (a procyanidin dimmer) as well as flavone glycosides (peaks 20 and 21). Indeed, on further examination, small differences in the flavonol and flavone composition of the species could be discerned, which would not explain their different bioactivities, but could be used to distinguish them. However, this would require more extensive examination.

Among the phenolic compounds identified, biflavones such as amentoflavone, have already been described as useful in neurodegenerative diseases. It should be noted that the levels of these components were similar between the four PEFs. Amentoflavone have been reported as possessing inhibitory effect on the group II phospholipase A2 (PLA2) activity and also in inhibiting cyclooxygenase (Kim, Son, Chang, Kang, & Kim, 1998). Collective evidence from many recent studies suggests that increased PLA2 activity and PLA2-generated mediators play a central role, not only in acute inflammatory responses in the brain, but also in oxidative stress associated with neurological disorders (Sun, Xu, Jensen, & Simonyi, 2004). PLA2 reportedly contributes to the pathogenesis of these disorders by attacking neural membrane phospholipids and releasing proinflammatory lipid mediators such as prostaglandins, among others, and also by generating 4-hydroxynonenal, a well reported toxic product of lipid oxidation (Catala, 2009). Thus, inhibition of PLA2 activity provides an attractive approach for the treatment of inflammation and oxidative stress associated with acute neural trauma and some neurodegenerative disorders such as AD (Farooqui, Ong, & Horrocks, 2006).

Among the possible strategies for the treatment of AD, PD, senile dementia, ataxia and myasthenia gravis is the enhancement of brain cholinergic activity by AChE inhibition (Mukherjee, Kumar, Mal, & Houghton, 2007a; Soreq & Seidman, 2001; Thanvi & Lo, 2004). The principal role of AChE is the termination of nerve impulse transmission at the colinergic synapses by rapid hydrolysis of acetylcholine. A variety of plants have been reported to show AChE inhibitory activity and constitute sources of potential new natural products in the treatment of neurodegenerative disorders (Mukherjee et al., 2007a) with diverse phytochemistries, including alkaloids such as physostigmine and galantamine (Chung et al., 2001), ursolic acid (Chung et al., 2001), lignans (El-Hassan et al., 2003) flavonoids, terpenoids and coumarins (Williams et al., 2011). Leaves of the *Juniperus* sp. used here caused similar levels of AChE inhibition as reported for other plants (Mukherjee et al., 2007a) and comparable to extracts from other *Juniperus* sp. leaves (Orhan, Orhan, et al., 2011). In that work, *J. excelsa* extracts caused 45% inhibition using 200 μg mL^{-1}, whilst here *J. turbinata* extracts inhibited 47% of AChE activity at the same concentration of PEF. The remaining three species were less effective (causing 20–30% inhibition at this concentration). Among the four species, the most effective AChE inhibition was caused by *J. phoenicea* and *J. turbinata*, which reached 50% inhibition at ~400 and 200 μg mL^{-1}, respectively. Since the PEFs, by definition, are enriched in phenolic components but free of sugars, organic acids and minerals, it is likely that AChE inhibitory activity was derived from phenolic compounds either singly or in combination. Psydrin as well as quercetin derivatives have been identified as good inhibitors of AChE (David et al., 2009; Jung & Park, 2007). It has been demonstrated that quercetin inhibits AChE by a competitive mechanism (Khan et al., 2009; Orhan, Kartal, Tosun, & Sener, 2007). However, other components must contribute to AChE inhibition since *J. navicularis* exhibited the highest amount of quercetin rutinoside (peak 13), but was the least effective PEF. The most effective PEFs for AChE inhibition (*J. phoenicea* and *J. turbinata*) had higher relative levels of procyanidins and lower levels of flavonols. Their higher effectiveness as AChE inhibitors could be related to their different composition. Due to the adverse side-effects and bioavailability problems of some AChE inhibitors already in use, there still is great interest in finding better molecules (Fang et al., 2008; Mukherjee, Kumar, Mal, & Houghton, 2007b). However, as the bioavailabilities of the juniper phenolics, are unknown, their potential as effective agents remains to be discovered.

Since many of the natural products exhibit an hormetic effect (Son, Camandola, & Mattson, 2008) it was necessary to confirm that the concentrations used were not cytotoxic before testing PEFs in neuroblastoma cells. Once again, there was a clear difference between the *Juniperus* sp., with *J. navicularis* and *J. oxycedrus badia* being the least toxic and *J. turbinata* and *J. phoenicea* more toxic. This difference in cytotoxicity must be related to differences in the polyphenol composition.

Non-toxic concentrations of PEFs (0 and 5 μg GAE mL^{-1}) from the four *Juniperus* sp. reduced basal ROS production (without H_2O_2 stress) at 2 and 24 h incubation. When a non-lethal stress was imposed (200 μM H_2O_2 for 1 h), all PEFs retained the ability to reduce intracellular ROS following pre-incubation for 2 h. However, at 24 h pre-incubation, only PEFs from *J. navicularis* and *J. oxycedrus badia* caused diminished ROS levels. Antioxidant activity in biological systems could be due to direct or indirect effects of compounds and the *Juniperus* PEFs could be acting by both direct as well as indirect antioxidant capacities. The direct antioxidant capacity could be achieved through a direct radical scavenging activity and the indirect antioxidant capacity through the induction of cellular defences generally through the Nrf2 system, such as antioxidant enzymes (Jung & Kwak, 2010). Therefore, at 2 h pre-incubation with PEFs, we assumed that changes in ROS levels were the consequence of a direct radical scavenging and at 24 h pre-incubation, the changes were the consequence of the indirect antioxidant defences. Thus, the direct scavenging that the four PEFs were able to mediate did not last for a longer time (Fig. 3C and D). At 24 h pre-incubation other molecular events should take place, but they only should occur in cells pre-incubated with *J. navicularis* and *J. oxycedrus badia* PEFs. Since the amount of phenolic compounds used to determine the intracellular ROS production was equivalent for the four species, the differences obtained must be the result of the different composition/relative amount of each compound within species. Concerning the indirect antioxidant defences, the activities of SOD and CAT, considered primary antioxidant enzymes (Halliwell & Gutheridge, 1999), were not altered as ROS production was decreased. Although both enzymes are responsible for protecting cells against ROS produced during normal metabolism and after an oxidative insult, these results suggest that alternative pathways could be regulated to cope with the imposed increase of ROS. These alternative pathways could be acting through the antioxidant response element (ARE) and/or the xenobiotic response element (XRE; (Oleaga et al., 2011)) systems with associated induction of induce genes for antioxidant and

detoxification enzymes, such as NAD(P)H:quinone oxidoreductase (NQO;1(Erlank, Elmann, Kohen, & Kanner, 2011)).

Only the PEF from *J. oxycedrus badia* could protect neurons against the injury caused by H_2O_2. This PEF enhanced values of viability and $\Delta\Psi m$, compared with cells treated with H_2O_2. A PEF concentration of 2.5 μg GAE mL^{-1} produced effective protection, enhancing viability by 51% and $\Delta\Psi m$ by 57% compared with cells only treated with H_2O_2. If considering at the same time the number of cells presenting adequate membrane integrity and a high $\Delta\Psi m$, the augmentation caused by 2.5 μg GAE mL^{-1} of *J. oxycedrus badia* PEF was about 70%.

These results, together with the data for SOD and CAT activities, suggest that reduction of intracellular ROS levels verified for 24 h pre-incubation with PEFs should be due to the induction of other intracellular antioxidant mechanisms. The involvement of ARE and XRE systems may be valid systems for investigation.

5. Conclusions

All four *Juniperus* sp. PEFs, and in particular that from *J. oxycedrus badia*, constitute potential sources of neuroprotective natural products. The four species are rich sources of phenolic compounds and contain molecules previously described with neuroprotective potentialities, such as quercetin derivatives (AChE inhibitor) and amentoflavone (effect on the group II phospholipase A2 (PLA2) inhibitor and cyclooxygenase inhibitor). Phenolic compounds from these species caused effective intracellular radical scavenging in neurons submitted to oxidative injury. Moreover, *J. navicularis* and *J. oxycedrus badia* PEFs induced mechanisms that reduced ROS formation induced by H_2O_2 treatment. Finally, *J. oxycedrus badia* PEF had a protective effect in the neurodegeneration cell model. The lack of impact of the components on the standard antioxidant enzymes CAT and SOD suggest alternative mechanisms underpin this bioactivity. Further research is merited to elucidate the source and mechanisms of these bioactivities, particularly if these are to be used in dietary-based neuroprotection strategies such as functional foods and/or functional food ingredients.

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