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RESEARCH ARTICLE

Bioactive compounds from endemic plants of Southwest Portugal: Inhibition of acetylcholinesterase and radical scavenging activities

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Abstract

Context: Natural products are reported to have substantial neuroprotective activity due to their radical scavenging capacity, and also acetylcholinesterase (AChE) inhibitory capacity, both activities important in neurodegeneration.

Objective: The undesirable side effects of compounds in pharmacological use make it important to identify natural neuroprotective molecules. This work assesses the potential of five endemic Portuguese plants as sources of neuroprotective compounds.

Materials and methods: Antioxidant capacity for peroxyl radical was determined by Oxygen Radical Absorbance Capacity method and for hydroxyl by Electron Paramagnetic Resonance, as well as AChE inhibitory capacity of the plant hydroethanolic extracts. The molecules responsible for these valuable properties were also tentatively identified by HPLC.

Results and discussion: Armeria rouyana and *Thymus capitellatus* presented some of the highest phenolic contents (76.60 ± 7.19 and 12.82 ± 0.24 mg GAE g^{-1} dw, respectively) and antioxidant capacities (592 ± 116 and 449 ± 57 µmol TE g^{-1} dw, respectively). The flavonoids were identified as the phytomolecules related to the antioxidant capacity of these plant extracts; in the case of *A. rouyana,* L-ascorbic acid also made an important contribution (3.27 ± 0.26 mg g^{-1}dw). Plant extracts clearly demonstrated effective AChE inhibitory activity (480 ± 98 and 490 ± 46 µg mL^{-1}, respectively), that could be associated to polyphenols.

Conclusions: The extracts of *A. rouyana* and *T. capitellatus* and their active components, especially polyphenols, demonstrate interesting neuroprotective potential. They, therefore, deserve further study as their phytomolecules are promising sources of either natural neuroprotective products and/or novel lead compounds.

Keywords: Portuguese endemic plants, polyphenols, antioxidant capacity, acetylcholinesterase inhibitor, neuroprotective phytomolecules

Introduction

Neurodegenerative diseases are multifactorial disorders in which many biological processes become unregulated. They share a substantial number of processes, including cell signaling, oxidative stress, inflammation, apoptosis, accumulation of aggregation-prone misfolded protein, among others (Joyner & Cichewicz, 2011). A multitarget therapeutic strategy aiming at different pharmacological mechanisms might provide a more rational and improved dementia treatment approach. Natural products have made significant contributions towards the treatment of degenerative diseases (Newman & Cragg, 2007). Secondary

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metabolites have long contributed to the development of small-molecule therapeutics, due, in part, to their combination of unique chemical features and potent bioactivities; plants are a rich source of such metabolites. Several small molecules have been reported to exhibit inhibitory properties in neurodegeneration (Mandel et al., 2006; Ramassamy, 2006; Williams et al., 2011).

Alterations produced by neuropathologies include oxidative stress markers (Andersen, 2004) as well as the decline in cognitive function associated with cholinergic deficits (Bachurin, 2003). The limited resources for combating oxidative stress by the central nervous system include the following: Vitamins, bioactive molecules, lipoic acid, antioxidant enzymes and redox sensitive protein transcriptional factors. Furthermore, this defense system can be activated/modulated by natural products such as polyphenols (Mandel et al., 2008; Ramassamy, 2006).

Acetylcholinesterase (AChE) inhibition may help in the treatment of Alzheimer's disease (AD) as well as senile dementia, myasthenia gravis (Thanvi & Lo, 2004), Parkinson's disease (Soreq & Seidman, 2001) and ataxia (Mukherjee et al., 2007a) due to the associated cholinergic deficit (Bachurin, 2003). Few of this class of inhibitors have yet been approved for AD therapy (Bachurin, 2003) and most of them have short half-life and peripheral cholinergic side effects (Fang et al., 2008; Mukherjee et al., 2007b; Nordberg & Svensson, 1998; Perry et al., 2003) which significantly limit its therapeutic use. Therefore, natural sources of compounds showing AChE inhibitory capacity must be sought.

The enormous economic and social costs of neurodegenerative disorders (Beking & Vieira, 2010; Wimo et al., 2011) in conjunction with the lack of any cure, make the development of new therapies for halting or reversing such diseases urgent. The present study strived to identify plant extracts demonstrating antioxidant and AChE inhibitory activities and serving as potential novel therapies for neurodegeneration.

The plants selected were collected in a coastal area of Southwestern Portugal, characterized by soils which are sandy, poor in nutrients and subject to salinity. This site, which is part of Europe's Natura 2000 network, is classified of community importance for the Mediterranean biogeographical region (Comporta/Galé) and is rich in endemic flora that have been poorly explored. Few pharmacological or phytochemical studies have been carried out on the species of this ecosystem.

Extracts were assayed for antioxidant properties for two of the most important biological free radicals (peroxyl and hydroxyl) and for their inhibitory effects on AChE. HPLC profiles determined their chemical composition.

Materials and methods

Plant materials

In this study, we used leaves from Portuguese endemic plants. The plants in the study were *Anchusa calcarea* Boiss. (Boraginaceae), *Armeria rouyana* Daveau (Plumbaginaceae), *Santolina impressa* Hoffmanns. & Link (Asteraceae), *Thymus capitellatus* Hoffmanns. & Link (Lamiaceae) and *Ulex australis* Welw. ex Webb subsp. *welwitschianus* (Planch.) M. D. Espírito Santo, Cubas, M. F. Lousã, C. Pardo & J. C. Costa (Leguminosae). All samples were collected in Comporta area in 2007 and vouchers sample representative (1069/2007; 1070/2007; 1065/2007; LISI 1071/2007; 1068/2007) were authenticated by Dalila Espírito-Santo, Instituto Superior de Agronomia and deposited on Herbário "João de Carvalho e Vasconcelos", Instituto Superior de Agronomia, Lisbon. For *Armeria rouyana*, since it is a Portuguese endemic and threatened plant classified as a priority species (Directive 92/43/EEC, Annex II), we obtained a license for capture (137/2007/CAPT). Plants were collected and stored at –80°C. Prior to extraction, plants were ground.

Chemicals and materials

Acetylthiocholine iodide (ATCI), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), physostigmine, acetylcholinesterase from electric eel (type VI-S; lyophilized powder) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were purchased from Sigma (Sigma-Aldrich).

Extraction of plant phytomolecules

To each 1 g of plant, 6 mL of hydroethanolic solvent were added and extraction was performed as described earlier (Tavares et al., 2010a).

Fractionation by solid phase extraction

Hydroethanolic extracts were fractionated by Solid Phase Extraction (SPE) using a Giga tubes 2 g 12 mL^{-1}, C18-E units (Phenomenex®) as described before (Tavares et al., 2010b).

Total phenolic content

Determination of total phenolic compounds was performed by the Folin-Ciocalteau method adapted to microplate reader as described in previous work (Fortalezas et al., 2010). Gallic acid was used as the standard and results are expressed in mg of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} dw) of plant material.

Total flavonoid content

Measurement of total flavonoid content was performed by a modification of the $AlCl_3$ complexation method as described before (Tavares et al., 2010a).

Free radical scavenging assays

Peroxyl radical scavenging capacity

Peroxyl scavenging capacity of plant extracts was determined by the ORAC method adapted to microplate as described earlier (Fortalezas et al., 2010). Trolox was used as standard and results are expressed in μmol of trolox equivalents per gram of plant dry weight (μmol TE g^{-1} dw).

Hydroxyl radical scavenging assay

The ability of the plant extracts to scavenge the hydroxyl radicals in the presence of hydrogen peroxide was evaluated by electron paramagnetic resonance (EPR) using the spin-trapping agent DMPO, as described previously (Tavares et al., 2010a).

HPLC profile

Phytochemical profile was obtained with a LiChrospher C18 column (Merck) on a Thermo Finnigan Surveyor instrument (San Jose, CA, USA) equipped with a diode array detector (Thermo Finnigan-Surveyor), and an electrochemical detector (Dionex, ED40) as previously described (Tavares et al., 2010a).

Quantification of ascorbic acid

Ascorbic acid content was quantified in the extract prepared with potassium phosphate buffer (pH 3.5) by an enzymatic method, using the kit K-asco from Megazyme, according to the manufacturer instructions. To confirm and obtain an accurate quantification, it was performed with a new plant extraction using phosphoric acid (6 mL g^{-1} fw). Ascorbic acid was then determined by a HPLC method validated for organic acids quantification (Tavares et al., 2010a).

AChE inhibitory assay

AChE inhibition was determined in a 96-microtiter well plate, based on Ellman's reaction (Ellman et al., 1961). Briefly, 5 µL of extracts (2, 4, 8, 40, 80, 400, 800, 1000, 1500, 2000, 3000 and 4000 µg mL^{-1}) resuspended in methanol containing DMSO 8% were added to 25 µL of 15 mM ATCI in H_2O, 125 µL of 3 mM DTNB in 50 mM Tris-HCl buffer containing 0.1 M NaCl and 0.02 M $MgCl_2$ (pH 8.0) and 70 µL of 50 mM Tris-HCl buffer with 0.1% (w/v) BSA (pH 8.0). Changes in absorbance were measured during an 8-min period to assess the spontaneous hydrolysis of the substrate (at $\lambda = 405$ nm). Then, 25 µL of 0.226 U mL^{-1} AChE from electric eel (type VI-S; lyophilized powder) in 50 mM Tris-HCl buffer containing 0.1% (w/v) BSA (pH 8.0) was added and immediately after, microplates were shaken for 15 s and the measurement was repeated during 8 min at $\lambda = 405$ nm. The experiments were carried out at 25°C. Effect on AChE activity was calculated as an inhibition percentage (%) of the maximum activity (registered on control wells without inhibitor). Physostigmine was dissolved in methanol and used as positive control. Results were transformed using a nonlinear regression with Origin Pro 6.1 software (OriginLab©, USA) and the IC_{50} values were determined.

Statistical analysis

Results reported in this work are the average of at least three biological replicates and are represented as the mean ± SD. Differences among treatments were assessed by analysis of variance (Popova et al., 2004) with Tukey HSD (Honest Significant Difference) multiple comparison test ($\alpha = 0.05$) using SigmaStat 3.10 (Systat).

Results

The Comporta/Galé site, in Southwestern Portugal, offers a wide range of little known plant biodiversity, especially where potential bioactivities are concerned. The antioxidant properties and AChE inhibitory activities of five endemic species (*Anchusa calcarea*, *Armeria rouyana*, *Santolina impressa*, *Thymus capitellatus*, and *Ulex australis welwitschianus;* Figure 1) were evaluated since both bioactivities are important features in neurodegenerative disease protection.

Total phenolic quantification of crude extracts (Table 1) identified *A. rouyana* as the richest species, followed by *T. capitellatus*, *U. australis welwitschianus*, *S. impressa,* and, finally, *A. calcarea*. The total flavonoid content was also determined since flavonoids are a class of polyphenols strongly associated with antioxidant capacity (Table 1).

Peroxyl (ROO·) and hydroxyl (·OH) are two of the most important free radicals for the human body. These plants' capacity to scavenge ROO· and ·OH was measured by the Oxygen Radical Absorbance Capacity (ORAC) method and Electron Paramagnetic Resonance (EPR), respectively (Table 1). *A. rouyana* was the most effective scavenger of both radicals; *S. impressa* presented a lower antioxidant capacity for peroxyl radical than *A. rouyana*. However, its hydroxyl radical scavenging capacity was similar to that of the most efficient scavenger, *A. rouyana*.

High Performance Liquid Chromatography with Diode Array Detection and Electrochemical Detection (HPLC-DAD-ED) was used to better understand the antioxidant capacity of these leaf extracts. The resulting phytochemical profile (Figure 2A) identified some of the major compounds by way of a study of their UV spectra and comparison with standards, such as those proposed by Robards and Antolovich (1997) for classes of flavonoids. Flavones typically show maximum absorption rates in an intense Band II (310–350 nm) with a shoulder or low intensity Band I (250–280 nm). Flavonols absorb at 250–280 nm (Band II) and 350–385 nm (Band I), while hydroxycinnamic acids demonstrate no Band I and absorb at 227–245 nm and at 310–332 (Band II). Some of the peaks identified by DAD correspond to those detected in ED (Figure 2A). The electrochemical detector identified peaks corresponding to reactive species with strong electron donating capacity. A positive relation between the total area of electrochemical chromatogram peaks (Figure 2B) and the antioxidant properties of the extracts was, therefore, expected. The largest ED total peak area was found in the *A. rouyana*, followed by *T. capitellatus*, *U. australis welwitschianus*, *S. impressa* and *A. calcarea* (Figure 2B). The former case may be explained by the *A. rouyana*'s higher flavonoid content (Table 1). The ED peaks for *U. australis welwitschianus*, *S. impressa* and *A. calcarea* were hardly detectable (Figures 2A and B).

Besides the higher flavonoid content in *A. rouyana* (Figure 2A) which contributes to antioxidant capacity,

L-ascorbic acid (Figure 2A, Peak 3) also play an important role in *A. rouyana*'s antioxidant capacity due to its redox reactivity (ED signals). An enzymatic method, using the K-asco kit from Megazyme (1.98 ± 0.33 mg g^{-1} dw) confirmed L-ascorbic acid presence and subsequently quantified it by a HPLC validated method (3.27 ± 0.26 mg g^{-1} dw). Rutin derivative (Figure 2A, Peak 4), a flavonol, is also an abundant compound in this plant extract.

T. capitellatus demonstrated high levels of the flavone luteoline glucoside (Figure 2A, Peak 7) which is one of the main compounds corresponding to the reactive peak in ED detection and may therefore constitute an antioxidant. Hydroxycinnamic acid (Peak 1, Figure 2A) and myrcetin glucoside (Peak 2, Figure 2A) displayed peaks in *A. calcarea*. Ferulic acid (Peak 5, Figure 2A) and its derivatives (Peak 6, Figure 2A) were the principal components of *S. impressa*. Finally, only a protocatechuic acid derivative (Peak 8, Figure 2A) and an apigenin glucoside (Peak 9, Figure 2A) were identified in *U. australis welwitschianus*.

The AChE inhibitory activity of hydroethanolic extracts, using a fixed amount of extract (2 mg mL^{-1}) and using AChE from electric eel (type VI-S; lyophilized powder) is presented in Table 2, as a percentage of AChE inhibition. This screening revealed that two of the extracts, *A. rouyana* and *T. capitellatus*, inhibited the enzyme markedly (>60–70%). Further assays for a range of concentrations (0–4000 μg mL^{-1}) allowed us to estimate the IC_{50} for both extracts (Figure 3); similar IC_{50} (Table 2 and Figure 3) arose. We performed a SPE fractionation for *A. rouyana* and *T. capitellatus*. This provided a polyphenols-enriched fraction (bound fraction) capable of discarding free sugars and organic acids

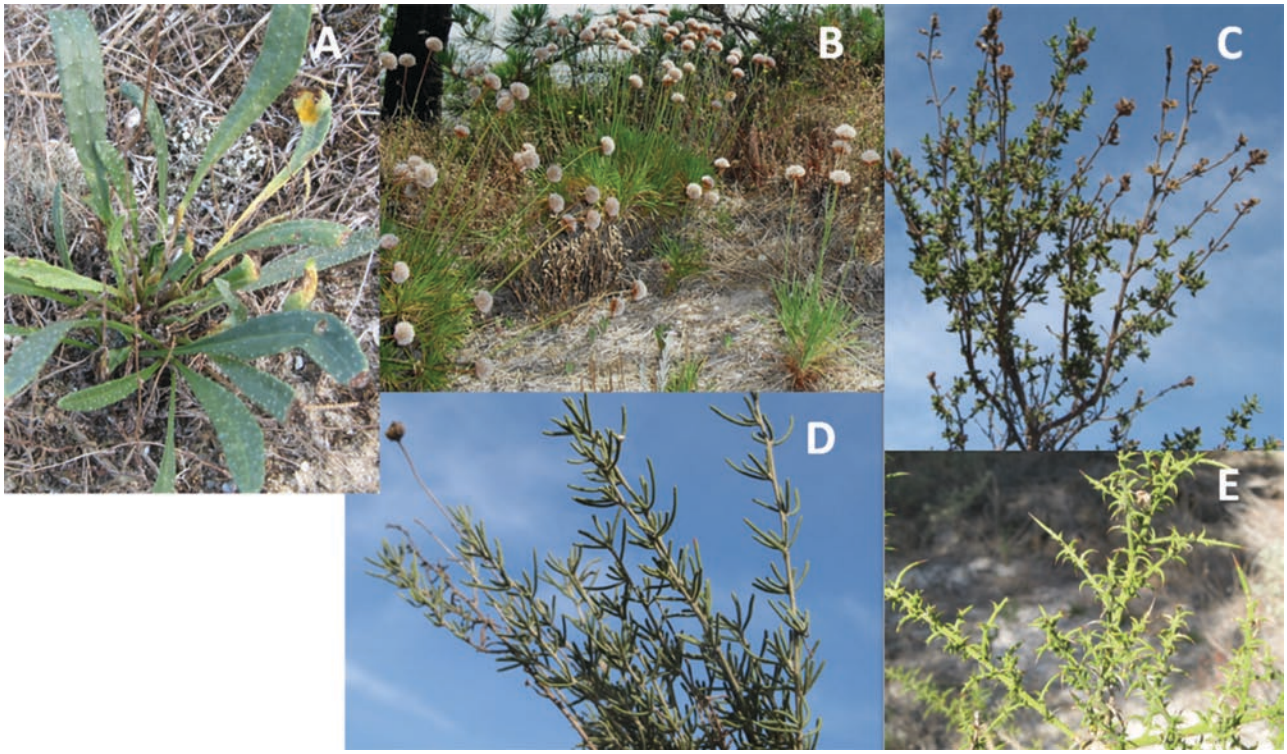

Figure 1. Studied plants from the region of Comporta in the Southwest of Portugal in their natural habitat. A- *A. calcarea*, B- *A. rouyana*, C- *T. capitellatus*, D- *S. impressa*, and E- *U. australis welwitschianus*.

Table 1. Total phenol content, total flavonoid content and antioxidant capacity (peroxyl radical scavenging and hydroxyl radical scavenging capacities) for the hydroethanolic extracts of the five endemic plants from the Southwest of Portugal under study.

Species	Total phenol content (mg GAE g^{-1} dw)	Total flavonoid content (mg CE g^{-1} dw)	Peroxyl radical scavenging capacity (μmol TE g^{-1} dw)	Hydroxyl radical scavenging capacity (% signal reduction)
A. calcarea	2.95 ± 0.49^{d}	0.41 ± 0.07^{d}	231 ± 13^{b}	28.5 ± 5.1^{c}
A. rouyana	76.60 ± 7.19^{a}	14.11 ± 1.84^{a}	592 ± 116^{a}	98.5 ± 0.1^{a}
S. impressa	4.36 ± 0.32^{cd}	1.13 ± 0.07^{cd}	210 ± 8^{b}	96.6 ± 4.5^{a}
T. capitellatus	12.82 ± 0.24^{b}	6.09 ± 0.28^{b}	449 ± 57^{a}	71.2 ± 3.8^{b}
U. australis welwistschianus	8.64 ± 0.31^{bc}	2.66 ± 0.20^{c}	417 ± 22^{a}	94.3 ± 4.9^{a}

Note: Values are the mean of three independent replicates ± SD. Different superscript letters (a–d) denote statistically significant difference ($p < 0.05$).

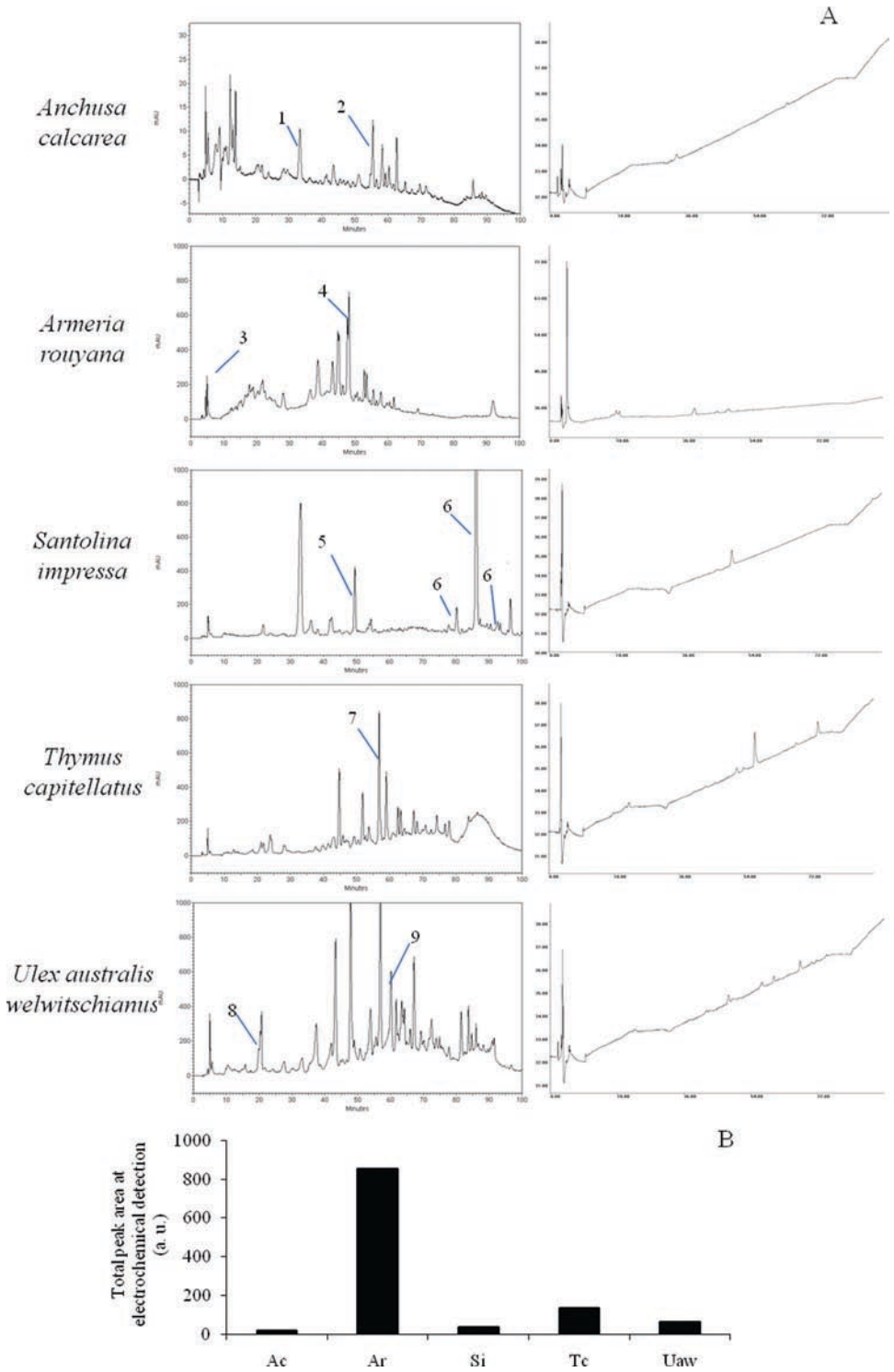

Figure 2. Phytochemical analyses of extracts. A) HPLC chromatograms and respective ED profiles B) total peak electrochemical detection for *A. calcarea* (Ac), *A. rouyana* (Ar), *S. impressa* (Si), *T. capitellatus* (Tc) and *U. australis welwitschianus* (Uaw). Tentatively, identifications are signaled in chromatograms. Legend: 1-Chlorogenic or caffeic acid; 2-Myrcetin glucoside; 3-L-Ascorbic acid; 4-Rutin derivative; 5-Ferulic acid; 6-Ferulic acid derivative; 7-Luteolin glucoside; 8-Protocatechuic acid derivative; 9-Apigenin glucoside.

such as ascorbic acid in the unbound fraction. These polyphenol-enriched fractions were identified as the cause of the AChE inhibition in both extracts (Table 3), while compounds appearing in unbound fractions did not present inhibitory capacity.

Discussion

In general, plant extracts present a positive relationship between total phenols content, flavonoids content and antioxidant capacity, with higher phenols and flavonoid

Table 2. Acetylcholinesterase inhibitory activity of hydroethanolic extracts of the five endemic plants from the Southwest of Portugal under study and AChE inhibition is presented as percentage of inhibition using 2 mg mL^{-1} of extract and for the most effective extracts was calculated the IC_{50} (μg mL^{-1}). Physostigmine was used as positive control.

	AChE inhibition	
Species	% (2 mg mL^{-1})	IC_{50} (μg mL^{-1})
A. calcarea	24.48 ± 7.31[d]	—
A. rouyana	73.65 ± 12.25[b]	480 ± 98[a]
S. impressa	40.71 ± 3.89[c]	—
T. capitellatus	95.49 ± 3.44[a]	490 ± 46[a]
U. australis welwistschianus	47.29 ± 5.95[c]	—
Physostigmine	—	0.59 ± 0.06[b]

Note: Values are the mean of three independent replicates ± SD. Different superscript letters (a–d) denote statistically significant difference ($p < 0.05$).

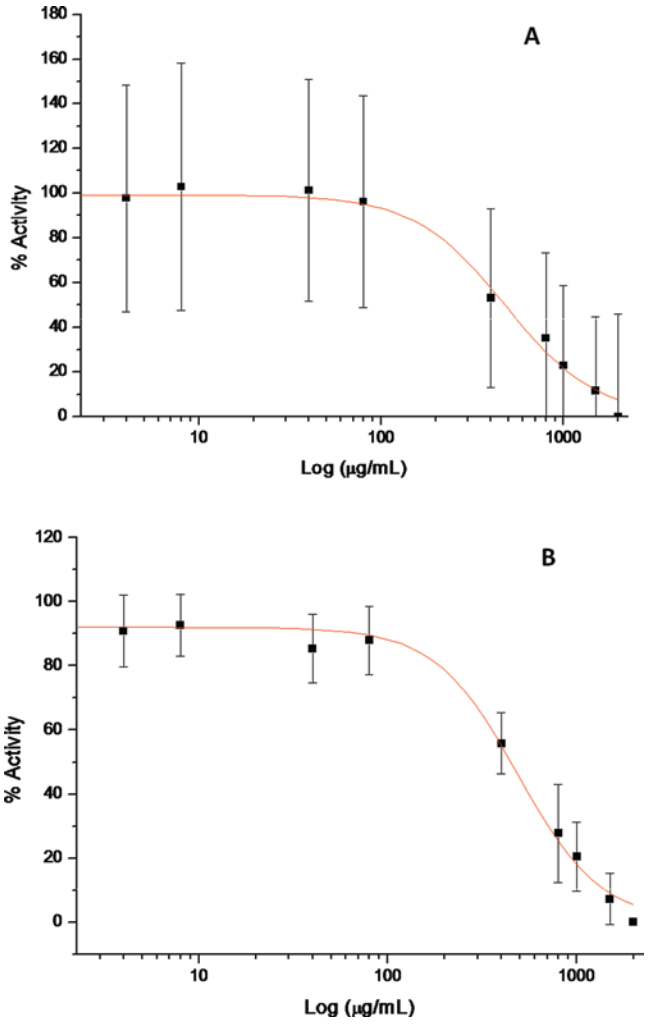

Figure 3. Nonlinear regression of acetylcholinesterase inhibition profile obtained with Origin Pro 6.1 software (OriginLab©, USA) for plant hydroethanolic extracts. A- *A. rouyana*, B- *T. capitellatus*. AChE inhibition was determined based on Ellman's reaction. Using AChE from electric eel as described in methods. Effect on AChE activity was calculated as an inhibition percentage (%) of the maximum activity (control without inhibitor).

levels reflecting greater antioxidant capacity. However, some points must be discussed.

S. impressa demonstrated low levels of phenolic compounds and flavonoids as well as limited peroxyl radical antioxidant capacity; however, its hydroxyl radical scavenging capacity was similar to that of *A. rouyana*, the extract with the highest value (Table 1). These results suggest either the existence of polyphenols displaying differential antioxidant potencies for the two radicals or a possible combination of compounds interfering with antioxidant capacity and/or the presence of additional antioxidant molecules other than polyphenols. Such has already been reported for other plant extracts (Tavares et al., 2010a). No data are yet available regarding the phenolic and flavonoid leaf content or antioxidant capacity of these species.

Analyses of the phytochemical composition of extracts (Figure 2A) reveal ED peaks, corresponding to identified compounds in HPLC-DAD. This suggests that detected compounds are associated with antioxidant properties. L-Ascorbic acid is already recognized as an antioxidant molecule (Padayatty et al., 2003) contributing greatly to the antioxidant capacity of *A. rouyana* (Figure 2B). *A. rouyana* and *T. capitellatus* were found to be the most efficient AChE inhibitors displaying higher rates of inhibition than those plants in the literature (Mukherjee et al., 2007a). They also belong to families of species known to be AChE inhibitors (Adewusi et al., 2010). *A. rouyana* demonstrated an AChE inhibition level of 72% using 1 mg mL^{-1} of extract, whereas that of *T. capitellatus* was 75.9%. These extracts were definitely stronger inhibitors than those described in the literature (Adewusi et al., 2010). Among others they surpass the inhibition levels of the following: *Albizia adianthifolia* (Schumach.) W.F. Wight. (Fabaceae; 51.00 ± 3.40%) (Eldeen et al., 2005); *Trichilia dregeana* Sond. (Meliaceae; 55.00 ± 4.40%; Eldeen et al., 2005); *Buxus sempervirens* L. (Buxaceae; 61.76 ± 0.76%; Orhan et al., 2004); and *Lavandula augustifolia* Miller (Lamiaceae; 64.30 ± 9.00%; Orhan et al., 2007).

Physostigmine, one of the AChE inhibitors used in Alzheimer's treatments (Mohs et al., 1985), was used in this work as a positive control. As expected, the plant extracts were less effective than this pure compound.

Although well-known AChE inhibitors include alkaloids such as physostigmine and galantamine other sources have been described, including ursolic acid (Chung et al., 2001), lignans (El-Hassan et al., 2003) flavonoids, terpenoids and coumarins (Williams et al., 2011). We performed a SPE fractionation on *A. rouyana* and *T. capitellatus*. The unbound fraction corresponding to sugars and organic acids (including ascorbic acid) and a bound fraction corresponding to the polyphenols were tested for AChE inhibition. Table 3 shows that polyphenols-enriched fractions demonstrated inhibitory capacity. However, these polyphenols-enriched fractions had a lower AChE inhibitory activity than the total extracts, suggesting an absence of compounds which potentiate the AChE inhibitory capacity of polyphenols.

Table 3. Acetylcholinesterase inhibitory activity of fractions obtained from SPE fractioning of hydroethanolic extracts of *A. rouyana* and *T. capitellatus*. Unbound fraction, that includes sugars and organic acids (including L-ascorbic acid), and Bound fraction, that includes the polyphenols enriched fraction. AChE inhibition is presented as percentage of inhibition using 2 mg mL^{-1} of fraction.

	AChE inhibition	
Species	Fraction	% (2 mg mL^{-1})
A. rouyana	Unbound	nd
	Bound	57.2 ± 6.8
T. capitellatus	Unbound	nd
	Bound	59.8 ± 4.1

Note: nd: not detected, values are below the detection limit.

Polyphenols were abundant in both extracts and associated with part of the antioxidant capacity and with the AChE inhibitory capacity. L-Ascorbic acid also seemed to make an important contribution to antioxidant capacity in *A. rouyana*, although irrespective of AChE inhibition. In summary, these results highlight the potential of these compounds as candidates for neuroprotective natural products and reinforce the need for their further study.

Conclusions

Due to their antioxidant and AChE inhibitory capacities, *A. rouyana* and *T. capitellatus* are considered promising sources of neuroprotective phytomolecules, either as alternative neuroprotective drugs or as leads for synthesizing more effective molecules. The extracts, therefore, deserve to be the targets of bioguided fractionation and further characterization.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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