Introduction

Alzheimer’s disease (AD), a progressive neurodegenerative disorder that affects the elderly, is clinically characterized by loss of memory, progressive deficits in other cognitive functions, and alterations in behaviour, such as apathy, agitation and psychosis, and mortality. The prevalence of the disease increases exponentially with age, beginning at approx. 10% at the age of 65 years and reaching nearly 50% at 85 years (Francotte et al., 2006; Racchi et al., 2004).

In addition to the neuropathological hallmarks of the disease, namely the appearance of neurofibrillary tangles and neuritic plaques, AD is also characterized neurochemically by a decrease of nearly 90% of the neurotransmitter acetylcholine (ACh) in the hippocampus and cortex, which are the areas related to memory and learning (Cummings, 2004; Lahiri et al., 2002). Based on the cholinergic hypothesis (Whitehouse et al., 1982), that the memory impairment in AD patients is associated with a defect in the cholinergic system, enhancement of ACh levels in the brain by inhibiting the enzyme acetylcholinesterase (AChE) was proposed as an important therapeutic approach. Improving the cholinergic function in AD patients thus became the neurobiological aim for treatment (Perry, 1986).

The therapeutic potential of compounds from natural origin has been successfully demonstrated in the field of AD; e.g., galanthamine, a selective, reversible competitive AChE inhibitor, is a natural product occurring in the Amaryllidaceae family (Marco and Carreiras, 2006); huperzine A, a novel sesquiterpene alkaloid isolated from the Chinese herb *Huperzia serrata*, Lycopodiaceae, is a potent, highly selective, reversible AChE inhibitor (Wang et al., 2006). *Himatanthus lancifolius* (Muell. Arg.) Woodson, a Brazilian species of Apocynaceae, is a shrub that contains several indole alkaloids with a number of activities reported. First, a broad spectrum of *in vitro* antimicrobial activities against pathogenic microorganisms has been demonstrated (Souza et al., 2004). A gastroprotective effect of this fraction, in which uleine displayed an IC₅₀ value of 0.45 μM.

**Key words:** Acetylcholinesterase Inhibitors, Apocynaceae, *Himatanthus lancifolius*, Uleine

Acetylcholinesterase Inhibitory Activity of Uleine from *Himatanthus lancifolius*

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Application of acetylcholinesterase (AChE) inhibitors is the primary treatment for Alzheimer’s disease. Alkaloids, such as physostigmine, galanthamine, and huperzine A, play an important role as AChE inhibitors. The aim of this work was to evaluate *Himatanthus lancifolius* (Muell. Arg.) Woodson, a Brazilian species of Apocynaceae, and its main indole alkaloid uleine, in order to identify new AChE inhibitors. The plant fluid extract, fractions, and uleine were tested for AChE inhibitory activity using Ellman’s colorimetric method for thin-layer chromatography (TLC), 96-well microplates, and also Marston’s TLC colorimetric method. Both TLC assays showed similar results. At 5 mg/mL, the fluid extract inhibited the AChE enzyme by (50.71 ± 8.2)%). The ethyl acetate fraction exhibited the highest level of AChE inhibition, followed by the dichloromethane fraction. The isolated alkaloid uleine displayed an IC₅₀ value of 0.45 μM.

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(Fig. 1) was the main constituent, has been described (Baggio et al., 2005). In addition, this fraction was able to alter vascular and non-vascular smooth muscle responsiveness (Rattmann et al., 2005), and uleine purified from H. lancifolius bark was shown to influence the production of nitric oxide (Souza et al., 2007). Recently, it was shown that H. lancifolius has the potential to interfere with the inflammatory response acting on leukocyte traffic (Nardin et al., 2008) and regulating the immune system (Nardin et al., 2010). The aim of present study was to evaluate the potential of H. lancifolius extract and uleine, its major alkaloid, as anticholinesterasic agents as there are no reports regarding its contribution to neurological degenerative disorders such as AD.

Material and Methods

Plant material

H. lancifolius stem bark was commercially acquired in São Paulo (SP, Brazil). It was identified according to the Brazilian Pharmacopoeia (first edition) and by macroscopic and microscopic comparison with authentic samples from the Laboratory of Pharmacognosy, Department of Pharmacy, Federal University of Paraná, Curitiba, Brazil, where a voucher sample (number HL-9) has been deposited.

General experimental procedures

Thin-layer chromatography (TLC) was carried out on silica gel GF 254 plates (Merck). A Tecan Sunrise microplate reader was used to measure the absorbance of the enzyme reaction in the microplate assay at 405 nm.

Extraction

The dried pulverized stem bark of H. lancifolius (100 g) was first percolated with 200 mL of 56% ethanol. The first 85 mL of the extract were removed and the remaining solution was exhaustively washed with 56% ethanol (approx. 1 L), concentrated to a syrup-like consistency, and then combined with the first 85 mL of extract. Finally, 56% ethanol was added to the extract to yield the fluid extract with a final volume of 100 mL and a final concentration of 1 g/mL. The fluid extract of H. lancifolius was evaporated in a water bath (60 °C) until the alcohol had evaporated. The resulting material was fractionated by liquid-liquid partitioning with n-hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and n-butanol. Each fraction was concentrated under reduced pressure to dryness to yield 2.89, 1.85, 0.43, 4.53, and 12.88 g, respectively, from the n-hexane, DCM, EtOAc, n-butanol, and water-soluble portions.

Preparation of the alkaloid-rich fraction

The preparation of the alkaloid-rich fraction has been fully described previously by Baggio et al. (2005).

Isolation and identification of uleine

Uleine was isolated by column chromatography as described previously (Baggio et al., 2005). Comparison of its spectral data with those found for uleine in the literature confirmed that the isolated compound was uleine (C_{18}H_{22}N_{2}) (França et al., 2000; Gaskell and Joule, 1967; Joule and Djerassi, 1964).

Acetylcholinesterase inhibitory activity

TLC assay

Two different autobiographic TLC assays were performed according to Rhee et al. (2001) and Marston et al. (2002). Briefly, 20 μL (10 mg/mL) of the fluid extract, liquid-liquid fractions, and 100 μL (2 mg/mL, 1.75 mg/mL, 1.26 mg/mL, 0.75 mg/mL, 0.5 mg/mL, 0.25 mg/mL) of the alkaloid-rich fraction and uleine were chromatographed on a silica gel plate by using the ethyl acetate/n-hexane/methanol/diethylamine solvent
system (4:5:0.8:0.2, v/v). The plates were subjected to each AChE inhibition assay based on the method of Ellman et al. (1961) and Marston et al. (2002). Physostigmine and uleine (both at 1 mg/mL) were also spotted as references. To control false-positive results, a negative control based on Rhee et al. (2003) was performed for all Ellman’s TLC assays.

Microplate assay
AChE inhibitory activities were measured according to the microplate assay by Rhee et al. (2001). The rate of acetylcholinesterase-mediated hydrolysis of acetylthiocholine was determined in the fluid extract, in all liquid-liquid fractions, and also for uleine by measuring the production rate of free sulfur groups produced as acetylthiocholine is hydrolyzed to thiococholine (Ellman et al., 1961). A flat-bottomed 96-well polystyrene cluster plate (300 μL/well; Techno Plastic Products, Switzerland) was used for the enzymatic reactions. Electric eel AChE (Type VI-S, Sigma, St. Louis, MO, USA) and acetylthiocholine iodide (ACTI; Sigma) were used as substrate of the reaction. 5,5'-Dithio-bis(2-nitrobenzoic)acid (DTNB; Sigma) was used for the measurement of the AChE activity. All other reagents and conditions were the same as described by Rhee et al. (2001). Briefly, to each well the following solutions were added: 3 μL DTNB (125 μL), 15 μM ACTI (25 μL), 25 μL of fluid extract (1 mg/mL) or liquid-liquid fractions (5 mg/mL) dissolved in methanol and 50 mM Tris [tris(hydroxymethyl)- aminomethane] buffer (50 μL, pH 8.0) containing 0.1% bovine serum albumin (BSA, Sigma). The absorbance was read every 10 s for 230 s and the reaction was then initiated adding 0.22 U/mL AChE (25 μL). The absorbance was again read every 10 s for 230 s. The rates of reaction were calculated using appropriate software. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the rate of reaction before addition of the enzyme from the rate after addition. The percentage of inhibition was calculated by comparing the rates for the sample and the blank (MeOH). A stock solution of uleine (1.0 mg/mL) was prepared in methanol and further diluted to give final concentrations ranging from 0.015 to 1.0 mg/mL, and the same procedure was performed. An inhibition curve was obtained by plotting the percentage of inhibition versus the logarithm of inhibitor concentration in the assay solution. IC₅₀ values were determined from the inhibition curve by linear regression analysis. All tests were done in triplicate, and the results were statistically analysed by one way ANOVA using the Prism software, version 5.0, and are expressed as mean percentage ± standard deviation (SD). P values < 0.05 were considered significant.

Results

TLC assay
The fluid extract and all fractions of H. lancifolius obtained by liquid-liquid partitioning were tested for their ability to inhibit AChE activity using Ellman’s and Marston’s colorimetric methods on TLC silica gel plates. Their inhibitory activity was easily detected by the clear appearance of characteristic white spots against, respectively, yellow (Ellman et al., 1961) or purple backgrounds (Marston et al., 2002) after revelation with the proper colorimetric agent. Among them, the DCM and EtOAc fractions showed the strongest reactions. Also, a strong inhibitory activity was showed by the alkaloid-rich fraction and its purified indole alkaloid uleine, which was concentration-dependent.

Microplate assay
To confirm these preliminary results, inhibition of the AChE activity was repeated for the fluid extract, the liquid-liquid fractions, and purified uleine using the microplate quantitative assay. As expected, the fluid extract at 1 mg/mL showed a significant [(50.71 ± 8.2)%] enzyme inhibition. Moreover, at 5 mg/mL, the DCM and EtOAc fractions were also significant for the effect, inhibiting the AChE activity by (54.73 ± 0.6) and (74.20 ± 2.3)%, respectively. The n-butanol, water-soluble, and n-hexane portions, although presenting lower values, were also active, ranging from (34.36 ± 3.5) to (9.80 ± 2.5) and (4.06 ± 1.2)%, respectively. For the purified uleine, a decay in enzyme activity was observed as the concentration increased (Fig. 2), with significant effects >50% starting at 0.12 mg/mL, and the IC₅₀ value of 0.45 μM was obtained by linear regression. It is important to note that in our experiments, physostigmine, a well known enzyme inhibitor used as a positive control, and uleine, both at 1 mg/mL, inhibited the AChE activity by (98.82 ± 0.3)% and (88.93 ± 0.1)%, respectively.
Discussion

The two different TLC methods used in this study showed that one or more groups of substances present in the *H. lancifolius* fluid extract and its fractions, particularly the DCM and EtOAc ones, were capable of inhibiting the AChE activity. Moreover, significantly high AChE activity was observed for both the alkaloid-rich fraction and uleine. It is relevant to say that uleine is the main compound present in the alkaloid-rich fraction (França et al., 2000). In addition, strong positive reaction was observed when DCM and EtOAc fractions were assessed by general reagents for alkaloids detection. Within this context, it is possible that uleine may be the compound responsible for the detected activity not only in these liquid-liquid fractions but also in the fluid extract.

The relationship of this effect between the alkaloid-rich fraction and uleine could also be established not only by the appearance of a stronger white spot amongst all others developed, but especially because of the $R_f$ value, which was the same as the one found for uleine used as reference.

If complex matrices such as plant extracts present inhibitory results around 50% or more, further studies should be undertaken to clarify whether one or more compounds can be considered as an AChE inhibitor candidate. Therefore, we have used the microplate assay to confirm the results obtained with the TLC assays, and a relevant activity was showed by the fluid extract from *H. lancifolius*, providing subsidies to test further the sub-fractions. As expected, the results were confirmed, and again, the fractions that showed the highest AChE inhibition capacity (DCM and EtOAc) were also those with the highest alkaloid content. The concentration-dependent activity of uleine was also confirmed by this assay, and the results enabled to determine its IC$_{50}$ value (0.45 $\mu$M), which laid in the range of 0.39 to 1.5 $\mu$M reported for galanthamine (Adersen et al., 2006; Hillhouse et al., 2004; Kissling et al., 2005).

Several indole alkaloids have already been reported as cholinesterase inhibitors (Mroue et al., 1996; Andrade et al., 2005; Orhan et al., 2007). In the present work, we used a combination of two simple tests such as TLC and microplate assays for a rapid assessment of the *H. lancifolius* AChE inhibitory activity. Although Ellman’s TLC method is widely used for this purpose, in our experience, Marston’s method facilitated the results interpretation as it provides a better background contrast. In addition, as no false-positive control test is necessary, it is less time-consuming.

In conclusion, to our knowledge, this is the first time that an uleine-type AChE inhibitory activity concerning the Apocynaceae family has been described, widening the pharmacological spectrum of this indole alkaloid as a potential new AChE inhibitor.

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Fig. 2. Uleine from *H. lancifolius* inhibits acetylcholinesterase. The indole alkaloid uleine, at the indicated concentrations, was tested for its acetylcholinesterase (AChE) inhibitory activity using the Ellman’s microplate assay. Each bar represents the mean percentage of inhibitory activity ± SD. (* $P < 0.01$; ** $P < 0.001$; $n = 6$). C, physostigmine at 1 mg/mL was used as control.


