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Comparative Study on the Inhibition of Acetylcholinesterase Activity by *Hyptis marrubioides*, *Hyptis pectinata* and *Hyptis suaveolens* Methanolic Extracts †

Vanessa Magalhães [1,2,*], Rejaine Rios [3] and Alberto Dias [1,2,4]

[1] Centre for the Research and Technology of Agro-Environment and Biological Sciences (CITAB-UM), AgroBioPlant Group, Department of Biology, University of Minho, 4710-057 Braga, Portugal; albertocpdias66@gmail.com

[2] Centre of Molecular and Environmental Biology (CBMA), Biology Department, University of Minho, 4710-057 Braga, Portugal

[3] Biology Department, Federal Institute of Education, Science and Technology Goiano, Morrinhos, GO, Brazil; rejainerios@gmail.com

[4] Centre of Biological Engineering (CEB), University of Minho, 4710-057 Braga, Portugal

* Correspondence: vamagalhaes2012@hotmail.com

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Abstract: Inhibition of acetylcholinesterase (AChE), the key enzyme in the breakdown of acetylcholine, may be considered as one of the treatment approaches against several neurological disorders including Alzheimer's. The purpose of this study is to evaluate, compare and discuss the anti-acetylcholinesterase activity of three methanolics extracts from Hyptis leaves': *Hyptis marrubioides* (Hm), *Hyptis pectinate* (Hp) and *Hyptis suaveolens* (Hs). AChE activity was measured using a modified 96-well microplate assay based on Ellman's method. IC50 (half maximal inhibitory concentration) values were calculated for Hm, Hp and Hs methanolic extracts using physostigmine as a positive control. All the extracts exhibited a dose-dependent AChE percent inhibition with IC50 values lower for Hm, followed by Hp and Hs. Several polyphenols (such as flavonoids and phenolic acids) have been considered a prominent source of anti-Alzheimer disease compounds because of their potential AChE inhibitory activity allied to the well-known antioxidant activity and low toxicity. The results herein obtained are discussed under the light of the available literature regarding the phytochemical composition and antioxidant activity of Hyptis spp. extracts. Further studies are warranted regarding the role of these Hyptis extracts in neurological disorders progression.

Keywords: Acetylcholinesterase activity; Hyptis methanolics extracts

1. Introduction

It is known that increased oxidative stress is one of the factors involved in a number of diseases including age-related. ROS (reactive oxygen species) are well known in damaging all cellular biomolecules. In addition, the central nervous system, with high content of polyunsaturated lipids (the biomolecules most susceptible to oxidation), is particularly vulnerable to oxidative insult due to the high rate of O_2 utilization and relatively poor concentrations of classical antioxidants and related enzymes (1). The association between oxidative stress and neurodegeneration has been well

documented with increased levels of oxidative stress markers in tissues during disease progression (in Alzheimer and Parkinson diseases as examples) [1].

A few scientific studies have addressed the antioxidant activity of *Hyptis suaveolens* (Hs, [2–4]) and *Hyptis pectinata* (Hp, [5,6]) extracts. Hs ethanolic extract has been found to have a potent antioxidant ability, evaluated by a number of in vitro biochemical assays, being concluded that it could be a potential source of natural antioxidants [2,3]. Importantly, Ghaffari et al. have recently reported, not only the antioxidant activity, but also a neuroprotective activity of the methanolic extract of Hs, being referred that the bioactive compounds might be phenolic compounds due to their higher content (2.6-folds more than flavonoids) [4]. This study shows that pretreatment with Hs methanolic extract promotes the upregulation of tyrosine hydroxylase and brain-derived neurotrophic factor genes (both playing a major role in brain homeostasis by regulating the neurotransmitter metabolism) against oxidative-induced cytotoxicity in N2A cells [4]. The comparative antioxidant and neuroprotective effects of Hm, Hp and Hs methanolic extracts have also been recently reported in *Caenorhabditis elegans* models of tauopathy and polyglutamine disease [7]. They have been shown to enhance the antioxidant responses and demonstrated neurotherapeutic potential in transgenic models of genetically determined human neurodegenerative diseases [7]. To our knowledge, Ghaffari et al. [4] and Vilasboas-Campos et al. [7] are the only studies addressing the neuroprotective activity of Hyptis extracts.

In the past years, neuroprotective activity has been seeked through the search for acetylcholinesterase (AChE) inhibitors due to the cholinergic hypothesis of Alzheimer disease (AD) [8]. Acetylcholinesterase (AChE), the predominant cholinesterase in the brain, hydrolyzes acetylcholine to choline and acetate, terminating the effect of the neurotransmitter at cholinergic synapses. Indeed, the loss of acetylcholine is considered to play a vital role in the learning and memory deterioration of AD patients. Therefore, the rationale for this hypothesis is based in the inhibition of the referred hydrolysis and as a consequence, increased acetylcholine concentrations in the synaptic cleft and enhanced cholinergic transmission. Thereby, inhibitors of AChE have been proposed as treatment strategy for AD, senile dementia, ataxia, myasthenia gravis and Parkinson's disease [8–10]. Currently, the most prescribed AChE inhibitors are donepezil, galantamine and rivastigmine, used to treat patients with mild-to-moderate AD [11].

Several plant species producing diverse classes of alkaloids, coumarins, terpenes, and polyphenols have been assessed for their anti-AChE activity, becoming potential candidates for new anti-AD drugs [10]. Dos Santos et al. have classified 54 plants extracts in accordance to their anti-AChE pharmacological activity with no Hyptis species in the set of analyzed extracts [10]. Indeed, and to our knowledge, there are no studies addressing the anti-AchE activity of Hyptis extracts. Therefore, the aim of the present study is to evaluate, compare and discuss the anti-AchE activity of 3 Hyptis species: Hm, Hp and Hs. Being previously reported the phytochemical profile and antioxidant activity of these Hyptis species, insights on their anti-AchE activity can lead to a better understanding regarding the link between the phytochemical profile, antioxidant activity and AChE inhibitory activity to pinpoint assays addressing neuroprotection in age-related diseases including AD.

2. Materials and Methods

2.1. Chemicals

Acetylcholinesterase (AChE) type VI-S, from electric eel, 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (AChI) were bought from Sigma.

2.2. Determination of AchE Inhibitory Activity

AChE inhibitory activity was measured using a modified 96-well microplate assay based on Ellman's method [12]. AChE enzyme hydrolyses acetylthiocholine and the resulting thiocholine reacts with Ellman's reagent (DTNB) producing 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-ni- trobenzoate which can be detected at 412 nm. Fifty mM Tris–HCl pH 8.0 was used as a buffer

throughout the experiment. In 96-well plates, 100 μL of 3 mM DTNB (in buffer containing 0.1 M NaCl and 0.02 M MgCl2), 20 μl of 0.26 U/mL AChE (from electric eel, type VI-S, Sigma in 0.1% BSA), 40 μL of buffer and 20 μL of each extract in several concentrations (from 75–1500 μg/mL, dissolved in buffer) were added to the wells, in triplicates. After mixing, the plate was incubated for 15 min (25 °C). The enzymatic reaction was initiated by the addition of 20 μL of 15 mM AChI (in water) and the hydrolysis of acetylthiocholine was monitored at 412 nm every 5 min for 20 min, in a Tecan infinite 200 microplate reader. Physostigmine was used as positive control. % AChE inhibition = [(Ac − Abc) − (As − Abs)]/(Ac − Abc) × 100 (Ac = absorbance of the control; Abc = absorbance of the control blank; As = absorbance of the sample; Abs = absorbance of the sample blank). IC50 values were obtained from the graphical curves % AChE inhibition versus Hyptis extracts concentration, via non-linear regression analysis (sigmoidal fitting with variable slope).

3. Results

Inhibition of AChE, the key enzyme in the breakdown of acetylcholine, may be considered as one of the treatment approaches against several neurological disorders such as Alzheimer's disease, senile dementia, ataxia, and myasthenia gravis. IC50 values were calculated for Hm, Hp and Hs methanolic extracts using physostigmine as a positive control (Figure 1). Generally, the extracts exhibited a dose-dependent AChE percent inhibition (Figure 1A) with IC50 values lower for Hm (45,21 ± 1672 μg/mL) than the other extracts: IC50 (Hp) = 66,30 ± 3560 μg/mL) and IC50 (Hs) = 68,05 ± 2994 mg/mL (Figure 1B). IC50 (physostigmine) = 6480 × 10^{-8} ± 7586 × 10^{-9} μg/mL (Figure 1B, non-detected). One- way ANOVA statistics revealed a significant difference between Hm and the other extracts but no significant difference between Hp and Hs methanolic extracts.

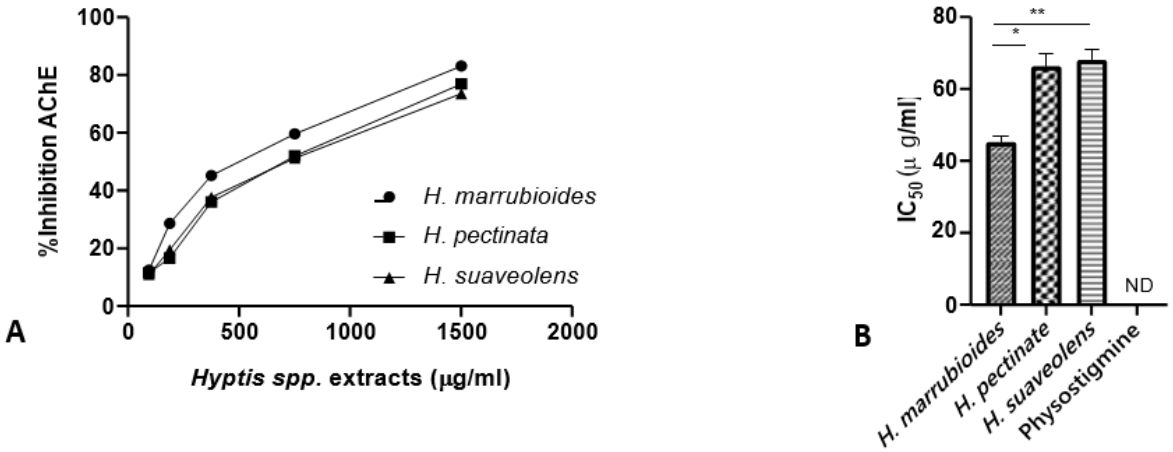

Figure 1. Graphical curves of % AChE inhibition versus Hyptis extracts concentration (**A**) and IC50 values of Hyptis spp. extracts (**B**). Twenty microliters of each extract in several concentrations (7,5–1500 μg/mL, final well concentrations) were assayed for inhibition of AChE activity as described. IC50 values were obtained via non-linear regression analysis (sigmoidal fitting with variable slope). Physostigmine was used as positive control. Asterisks mean significantly differences, obtained by one-way ANOVA followed by Tukey post-tests for multiple comparisons. * $p \leq 0.05$; ** $p \leq 0.01$.

4. Discussion

The AChE inhibitory activity of a huge number of plant extracts and isolated compounds has been recently evaluated [10] as promising candidates for new anti-AD drugs. Although antioxidant activity has been already reported for some Hyptis species (2–7), no studies have been carried out regarding their anti-AChE activity. In this study, we evaluated and compared the anti-AChE activity for Hm, Hp and Hs methanolic extracts. Hm was found to have significantly higher anti-AChE activity than Hp and Hs, with no significant difference between them (Hp and Hs). According to Dos Santos et al. [10], plant extracts were classified with high (IC50 < 20 μg/mL), moderate (20 < IC50 < 200 μg/mL) and low (200 < IC50 < 1000 μg/mL) potencies, regarding anti-AChE activity. The Hyptis

methanolic extracts, tested in this study, have revealed moderate potency, with Hm having the highest one (lower IC50).

The comparative phenolic analysis of Hm, Hp and Hs extracts has been recently reported (HPLC-DAD) revealing that rosmarinic acid derivatives, along with quercetin-3-glucoside are the most predominant compounds, with chlorogenic acid and apigenin derivatives being also detected [7].

Chaowuttikul et al. have also identified rosmarinic, caffeic and chlorogenic acids, hydrocycinnamic acid derivatives in Hs methanolic extracts [13]. In this work, those phenolic acids, have been quantified in 100 selected Thai plants methanolic extracts using RP-HPLC-DAD [13]. Rosmarinic acid has been recently reported to inhibit AChE, very effectively, with a Ki value of 42.52 pM [11]. In this study, Gülçin et al. also refer to donepezil hydrochloride (used for the treatment of mild-to-moderate AD and other memory impairments), with a lower AChE inhibitory activity (IC50 = 55.0 nM) [11], showing the potential of rosmarinic acid in inhibiting AChE. Other Lamiaceae ethanolic extracts growing wild in Croatia, found to be rich in rosmarinic acid, have been also evaluated for AChE inhibitory activity [14]. In accordance with Gülçin et al., this study showed that rosmarinic acid contents seems to have a substantial influence on their AChE inhibitory and antioxidant properties [14]. However, a false-positive effect has been reported for rosmarinic acid, due to the inhibition of the reaction between thiocholine and DTNB [15]. Given this information, the conclusions from Gülçin et al. [11] and Vladimir-Knežević et al. [14] should be interpreted with caution.

Flavonoids, a heterogeneous group of polyphenols, are currently considered a prominent source of anti-AD compounds because of their potential AChE inhibitory activity allied to the well-known antioxidant activity and low toxicity [16,17]. They have been implicated in (i) neuronal proliferation and survival, by acting on a variety of cellular signaling cascades, (ii) oxidative stress reduction and (iii)) relief from Alzheimer's disease-type symptoms [17]. In addition, from an electrophysiological aspect, they have reported to promote long term potentiation in the hippocampus, supporting the hypothesis of synaptic plasticity mediation [18]. As an example, quercetin, a flavonol found in foods and coffee, seems to be a potential learning and memory enhancer, as shown in several mouse models of AD (reviewed in [18]). The comparative quantification of phenolics compounds shows that Hm has a higher content of quercetin derivatives [7] that can be related with the significantly higher anti-AChE activity obtained for Hm methanolic extract.

The neuroprotective effect of chlorogenic acids against AD have been recently reviewed [19], with supporting evidence of their neuroprotective effects in either epidemiological studies or in vitro and in vivo studies. In this review, chlorogenic acids are reported to be capable of modulate the accumulation of ROS and regulate the expression of key proteins and enzymes involved in cell apoptosis [19]. The comparative quantification of phenolic compounds in Hm, Hp and Hs methanolic extracts shows that Hp and Hs have a higher content of chlorogenic acid derivatives [7] which can be likely related with an indirect neuroprotective effect based on their antioxidant potential.

Apigenin, one of the most widely distributed flavonoids in the plant kingdom and present mainly in a glycosylated form, has been recently reviewed regarding its health-promoting effects,particularly its beneficial role in AD [20]. A number of evidence from the several animal models and human clinical trials on the therapeutic potential of apigenin is provided in this review, including its antioxidant activity and its potential role as a neuroprotective agent [20]. As an example, improvements in memory and learning deficits as well as a reduction of fibrillar amyloid deposits with lowered insoluble concentrations of β-amyloid peptide, were observed in apigenin-treated mice [20]. In addition, it was shown that apigenin caused restoration of the ERK/CREB/BDNF pathway, involved in memory and typically affected in Alzheimer's disease. The comparative quantification of phenolics compounds in the Hm, Hp and Hs methanolic extracts shows that Hm has a higher content of apigenin glucoside [7] that can be related with the significantly higher anti-AChE activity obtained for Hm methanolic extract.

Moreover, Hyptis extracts have been demonstrated to possess antioxidant activity [2–6]. Particularly, antioxidant activity has been comparatively evaluated in Hm, Hp and Hs extracts, in

vitro, either using the antiradicalar activity - DPPH scavenging assay [7] or assessing their cytoprotective effect against tert-butyl hydroperoxide oxidative insult using a cell culture model of human hepatocytes [21]. In the first study, all the extracts have shown a good antioxidant activity by their ability to scavenge DPPH free radical [7]. In the second, Hm has showed a significantly higher cytoprotective effect against the oxidative insult following [21].

5. Conclusions

This study shows that Hm, Hp and Hs methanolic extracts can be used as a source of compounds with pharmacological properties which could be helpful in age-related diseases. Indeed, all the identified phenolic compounds [7] have been previously referred to have a part in neuroprotection. The anti-AChE activity, herein studied, can be likely be related to their flavonoid content since the identified flavonoids (quercetin derivatives and apigenin glucoside) are present in higher contents in Hm methanolic extract which achieved the highest AChE inhibitory activity. Moreover, all extracts have shown a good antioxidant activity, either based on their DPPH index or on their cytoprotective effects against oxidative insult using a cell culture model of human hepatocytes. The current drugs with AChE inhibitory activity, only effective against mild to moderate type of AD, provide only temporary symptomatic relief, possess some considerable side effects related to cholinergic stimulation in brain and peripheral tissues [22]. Thereby, since Hyptis methanolic extracts have revealed both antioxidant and anti-AChE activities, and their phenolic compounds have been described to have a part in neuroprotection, they can be considered promising alternatives to current therapies for neurodegenerative disorders. However, further evaluation is warranted, either to unveil the neuroprotective mechanism underlying these activities, or to identify the active ingredients, assess their safety and bioavailability in in vivo animal models.

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Abbreviations

The following abbreviations are used in this manuscript: AChE (acetylcholinesterase); Acetylthiocholine iodide (AChI); Hm (*Hyptis marrubioides*); Hp (*Hyptis pectinata*); Hs (*Hyptis suaveolens*); IC50 (half maximal inhibitory concentration); AD (Alzheimer disease); ROS (reactive oxygen species).

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