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

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Abstract: The 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 methanolic extracts from *Hyptis* leaves: *Hyptis marrubioides* (Hm), *Hyptis pectinata* (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 their well-known antioxidant activity and low toxicity. The results 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 the progression of neurological disorders.

Keywords: acetylcholinesterase activity; *Hyptis* methanolic extracts

1. Introduction

It is known that increased oxidative stress is one of the factors involved in a number of diseases, including age-related ones. ROS (reactive oxygen species) are known to damage all cellular biomolecules. In addition, the central nervous system, with a high content of polyunsaturated lipids (the biomolecules most susceptible to oxidation), is particularly vulnerable to oxidative insult due to the high rate of O2 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, as evaluated by a number of in vitro biochemical assays,
with it being concluded that it could be a potential source of natural antioxidants [2,3]. Importantly, Ghaffari et al. recently reported not only the antioxidant activity but also neuroprotective activity of the methanolic extract of Hs, with it being suggested that bioactive compounds might be phenolic compounds due to their higher content (2.6-fold 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 were also recently reported in Caenorhabditis elegans models of tauopathy and polyglutamine disease [7]. They were 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 few years, neuroprotective activity has been investigated through the search for acetylcholinesterase inhibitors due to the cholinergic hypothesis of Alzheimer’s 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 a 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. classified 54 plant extracts in accordance with their anti-AChE pharmacological activity, with no Hyptis species in the set of analyzed extracts [10]. Indeed, to the best of 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 three Hyptis species: Hm, Hp, and Hs. With the phytochemical profile and antioxidant activity of these Hyptis species having previously been reported, insights into 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), and 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-mercaptopthiocholine and 5-thio-2-ni-trobenzoate, which can be detected at 412 nm. A total of 50 mM of 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 to 1500 µg/mL, dissolved in buffer)

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were added to the wells in triplicate. 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 AChE (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 a 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 extract concentration via non-linear regression analysis (sigmoidal fitting with variableslope).

3. Results

The 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 (45.2 ± 1.7 µg/mL) than the other extracts: IC50 (Hp) = 66.3 ± 3.6 µg/mL and IC50 (Hs) = 68.0 ± 2.9 mg/mL (Figure 1B). IC50 (physostigmine) = 6.5 × 10^{−8} ± 7.6 × 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 (75 - 1500 µg/mL) were assayed for the inhibition of AChE activity, as described. IC50 values were obtained via non-linear regression analysis (sigmoidal fitting with variable slope). Physostigmine was used as a positive control. Asterisks represent significantly differences, obtained by one-way ANOVA followed by Tukey’s post-test for multiple comparisons. * p ≤ 0.05; ** p ≤ 0.01.

4. Discussion

The AChE inhibitory activity was recently evaluated for a huge number of plant extracts and isolated compounds [10] that are 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 were revealed to have a moderate potency, with Hm having the highest one (lower IC50).
A comparative phenolic analysis of Hm, Hp, and Hs extracts was 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 also being detected [7].

Chaowuttikul et al. also identified rosmarinic, caffeic, and chlorogenic acids and hydrocinnamic acid derivatives in Hs methanolic extracts [13]. In this work, those phenolic acids were quantified in 100 selected Thai plant methanolic extracts using RP-HPLC-DAD [13]. Rosmarinic acid was 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 were also evaluated for their AChE inhibitory activity [14]. In accordance with Gülçin et al., this study showed that plants’ rosmarinic acid contents seem to have a substantial influence on their AChE inhibitory and antioxidative properties [14]. However, a false-positive effect was 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]). A comparative quantification of phenolic compounds shows that Hm has a higher content of quercetin derivatives [7] that can be related to the significantly higher anti-AChE activity obtained for Hm methanolic extract.

The neuroprotective effect of chlorogenic acids against AD was 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 modulating the accumulation of ROS and regulating 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 to 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, was recently reviewed regarding its health-promoting effects, particularly its beneficial role in AD [20]. A number of studies from several animal models and human clinical trials on the therapeutic potential of apigenin are discussed in Salehi et al. review [20], including its antioxidant activity and its potential role as a neuroprotective agent. As an example, improvements in memory and learning deficits as well as a reduction in 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 the restoration of the ERK/CREB/BDNF pathway, which is involved in memory and typically affected in Alzheimer’s disease. 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 related to 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 showed a good antioxidant activity by their ability to scavenge DPPH free radicals [7]. In the second, Hm showed a significantly higher cytoprotective effect against the oxidative insult, following 24 h pre-incubation period with the extracts [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 suggested to have a part in neuroprotection. The anti-AChE activity studied herein can be likely be related to their flavonoid contents, 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 the extracts showed 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 the mild to moderate type of AD, provide only temporary symptomatic relief and possess some considerable side effects related to cholinergic stimulation in the 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 and 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).

References


