Anticholinesterase activity and identification of huperzine A in three Mexican lycopods: *Huperzia cuernavacensis*, *Huperzia dichotoma* and *Huperzia linifolia* (Lycopodiaceae)

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Abstract: Huperzine A (Hup A), the alkaloid produced by the Chinese medicinal plant *Huperzia serrata*, has been documented to be a promising agent for the treatment of Alzheimer's disease due to its potent acetylcholinesterase inhibitory (AChEI) activity. The search for anticholinesterase natural products, as well as for alternative sources of Hup A in Mexican lycopods, prompted us to investigate these plants. The action of methanolic and alkaloidal extracts of three *Huperzia* species (*H. cuernavacensis, H. dichotoma*, and *H. linifolia*) was evaluated using an *in vitro* anticholinesterase activity assay. Also, chromatographic and spectroscopic analyses were employed to detect the presence of Hup A. Methanolic and alkaloidal extracts of *H. cuernavacensis* showed IC_{50} =5.32±0.8µg/mL and 0.74±0.05µg/mL; *H. dichotoma* displayed AChEI with IC_{50} values =14.11±2.1µg/mL and 0.64±0.09µg/mL; and *H. linifolia* presented IC_{50} =158.37±8.7µg/mL and 4.2±1.24µg/mL, respectively, compared to the control Hup A (IC_{50}= 0.16±0.03µg/mL). Hup A was identified in the extracts of *H. dichotoma*, but it was not detected in the extracts of *H. cuernavacensis* and *H. linifolia* by ^{1}H NMR techniques. This study reveals *H. dichotoma* as a new source of Hup A, and presents *H. linifolia* and *H. cuernavacensis* as potential candidates to obtain other anticholinesterase compounds useful in the Alzheimer's disease treatment.

Keywords: Huperzine A, *Huperzia cuernavacensis*, *H. dichotoma*, *H. linifolia*, acetylcholinesterase inhibition, ^{1}H NMR.

INTRODUCTION

About 47 million people worldwide currently suffer dementia (Alzheimer's Disease International, 2015), being Alzheimer's disease (AD) the most common condition. AD is a neurodegenerative disorder associated with the loss of neurons, which are essential for memory and other cognitive functions (Mattson, 2004; Barnes *et al*., 2006). This disease is characterized by the significant decrease in levels of the neurotransmitter acetylcholine; and the use of acetylcholinesterase inhibitors (AChEI) is the actual FDA approved pharmacotherapy approach to treat the symptoms of mild to moderately severe Alzheimer's disease, by improving the cholinergic deficit (Terry and Buccafusco, 2003). However, the drugs currently used in the treatment of AD (e.g. rivastigmine and donepezil) possesses some considerable side effects (Francis *et al.*, 1999). Natural products have shown potential benefits in the treatment of AD symptoms, especially acting in the cholinergic pathway (Mukherjee *et al.*, 2007). Therefore, the search for new AChE inhibitors exploring natural resources is of great interest.

Huperzine A, an alkaloid isolated from the Chinese medicinal herb *Huperzia serrata*, is a potent, selective and reversible AChE inhibitor (Liu *et al.*, 1986; Ayer, 1991). Moreover, Hup A improves memory and behavior in AD patients (Yang *et al.*, 2013) by acting in the beta-amyloid peptide processing, which reduces neuronal injury (Gao *et al.*, 2009). In addition, it promotes reduction of oxidative stress, neuroinflammation, apoptosis, nitric oxide signaling, and is a NMDA receptor antagonist (Gao and Tang, 2006; Xiao *et al.*, 2000; Tang *et al.*, 2005; Zhang and Tang, 2006). Because of all these beneficial properties, Hup A has been licensed as an anti-Alzheimer's disease drug in China since 1996 (Tang, 1996), and is nowadays available as a nutraceutical in the USA (Orhan *et al.*, 2011).

In the last decade, some acetylcholinesterase activity studies conducted with South and Central America *Huperzia* species have been appeared (Konrath *et al.*, 2012, Konrath *et al.*, 2013), but just few of them reported Hup A identification (Lim *et al*., 2010). The purpose of the present study was to investigate three *Huperzia* species from Mexico for their *in vitro* anticholinesterase activity and to verify the presence of Hup A in the fern material.

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MATERIALS AND METHODS

Plant material

Huperzia cuernavacensis (Underw. & F.E. Lloyd) Holub, voucher AFM219, was collected in Huitzilac, Morelos, while, *H. dichotoma* (Jacq.) Trevis. (voucher 33589) and *H. linifolia* (L.) Trevis. (voucher 33590) were found in the area of Coatepec, Veracruz, Mexico (fig. 1). All of them were collected in July 2014. Samples were authenticated by Aniceto Mendoza and deposited at the HUMO Herbarium, CIBYC (Centro de Investigación en Biodiversidad y Conservación), UAEM.

Extracts preparation

Dried and crushed aerial parts (5g) of the three species were separately defatted with *n*-hexane by maceration. Afterwards, defatted plant material was macerated during 12h with methanol and then sonicated for 10 minutes. All extracts were concentrated under reduced pressure.

Each methanolic crude extract (1g) was solubilized in 100mL of 5% HCl and sonicated for 5 min. Then, it was partitioned with $CHCl_3$ (20mL) for 3 times. The organic phase was discarded and the remaining aqueous phase was basified with NH_4OH to pH 10. A last extraction using $CHCl_3$ was performed. The organic phase was neutralized with 5% HCl, and then evaporated to yield the total alkaloidal extract.

Anticholinesterase activity assay

The *in vitro* acetylcholinesterase inhibitory assay (AChEI) was performed using the enzyme acetylcholinesterase (EC 3.1.1.7 Type VI-S, Sigma, 0.02U/mL) obtained from electric eel. The enzymatic activity was measured by the colorimetric method of Ellman *et al.*, 1961, with some minor modifications.

The enzyme was incubated in phosphate buffer 1.0 M (pH 8) in the presence of 10 µL of 0.5mM of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Sigma-Aldrich) solution with different concentrations of each sample solubilized in 1% DMSO (Sigma-Aldrich). The enzyme reaction was initiated by the addition of 10µL of 20mM acetylthiocholine iodide after pre-incubation for 30 min at 25°C. Substrate hydrolysis was monitored by formation of a yellow anion of 5-thio-2-nitrobenzoic acid at 412nm, every 30 seconds during 3min, using a 96-well microplate reader spectrophotometer (Epoch™). Enzyme activity was calculated as a percentage compared to the buffer solution without any inhibitor. AChE inhibitors physostigmine salicylate (Sigma-Aldrich), and (-)-huperzine A (Sigma-Aldrich) were used in the assays as positive controls.

The percent inhibition was calculated as: % inhibition = (1 - S/E) x 100, where E and S were the enzyme activities with and without the test sample, respectively. The AChEI activity of each sample was expressed in terms of IC_{50} (concentration required to inhibit the hydrolysis of the substrate ACh by 50%).

Identification of huperzine A

Crude methanolic and alkaloidal extracts (5mg), as well as the standard Hup A were dissolved in CD_3OD (700µL) and registered in a NMR apparatus (500 MHz) in order to obtain the ^1H spectra. Identification of Hup A in the extracts was followed by the presence of characteristic chemical shift signals and multiplicity patterns of the deshielded olefinic protons H-2 and H-3.

STATISTICS ANALYSIS

All assays were independently performed in triplicate and results were expressed as means ± SEM (standard error of the mean). The IC_{50} values were estimated from a non-linear fitting of the concentration-response data, using the Sigmaplot 13.0 software.

RESULTS

Extracts yields

The yields of methanolic crude extracts were 19.4%, 17.33%, and 21.35% (plant DW) for *H. cuernavacensis, H. dichotoma*, and *H. linifolia*, respectively; while, the total alkaloidal extracts were obtained at 0.27%, 0.23%, and 0.09% (plant DW), respectively, for the species.

Anticholinesterase activity

The AChEI IC_{50} values of the methanolic crude extracts from the studied species were: 5.32±0.8µg/mL (*H. cuernavacensis*), 14.11±2.1µg/mL (*H. dichotoma*), and 158.37±8.7µg/mL (*H. linifolia*). In contrast, the IC_{50} values of the alkaloidal extracts of the three species were: 0.74±0.05µg/mL (*H. cuernavacensis*), 0.64±0.09µg/mL (*H. dichotoma*), and 4.2±1.24µg/mL (*H. linifolia*). Physostigmine salicylate and Hup A displayed AChEI IC_{50} values of 0.072±0.01 and 0.16±0.03µg/mL, respectively. The concentration-response curves obtained to calculate the IC_{50} values of the methanolic and alkaloidal extracts can be observed in fig. 2.

Identification of huperzine A

Based on their ^1H NMR spectrum, the three Mexican species showed wide differences in their chemical content and Hup A was detected only in *H. dichotoma* methanolic extract. Due to the signal complexity in the ^1H NMR spectrum of the extracts, only the deshielded signals H-2 and H-3 were used for identification purposes of Hup A (fig. 3). We clearly identified the protons H-2 (δ 6.50, $^3J_{HH}$= 9.5 Hz) and H-3 (δ 7.75, $^3J_{HH}$= 9.5 Hz) in *H. dichotoma* methanolic extract, which signals correlated between them in the COSY experiment.

DISCUSSION

Nowadays the use of acetylcholinesterase inhibitors is the first line pharmacotherapy approach for symptomatic relief of mild-to-moderate AD dementia (Wong, 2016).

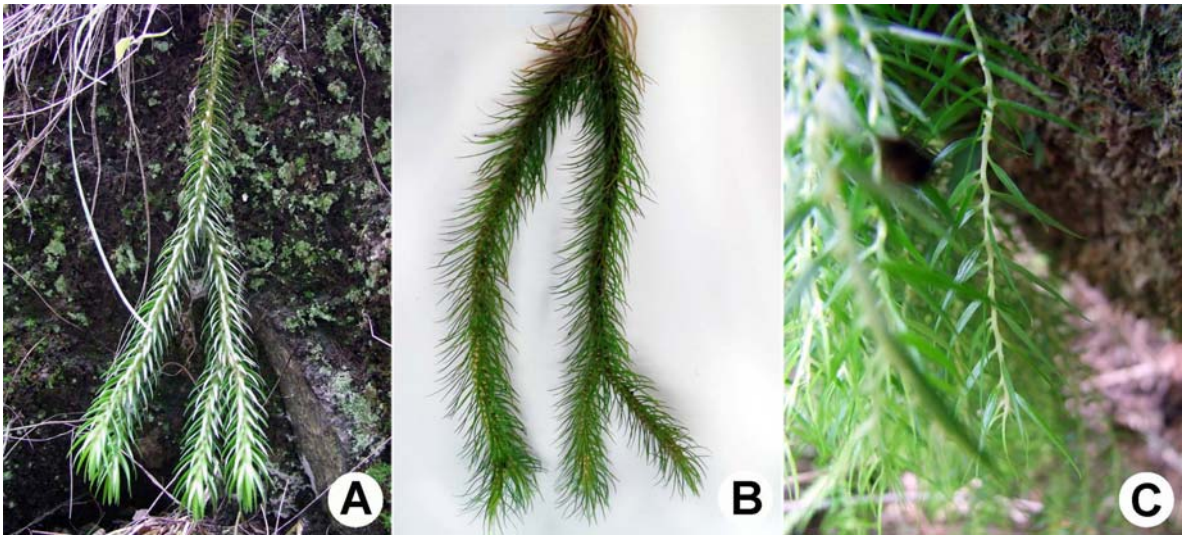

Fig. 1: Live plants. A) *Huperzia cuernavacensis*, B) *H. dichotoma*, and C) *H. linifolia*.

Some potent AChEI compounds are derived from natural sources. *Lycopodium* alkaloids have been extensively investigated, being Hup A the most important and studied natural compound so far. After the discovery of Hup A from the club moss *H. serrata* in the 80´s, which is threatened in the wild in Chine, several efforts were developed to find new sources of this alkaloid; especially among the Huperziaceae family (Ma *et al.*, 2005; Ishiuchi *et al.*, 2013). This is the first chemical and pharmacological report regarding the study of the Mexican species *H. cuernavacensis*, *H. dichotoma*, and *H. linifolia*. In the present study Hup A was identified only in *H. dichotoma*. It has been reported that some Australasian *Huperzia* species do not present Hup A (Goodger *et al.*, 2008). In the same way some American *Huperzia* species, *H. aqualupiana* from Brazil and *H. taxifolia* from Mexico (Lim *et al.*, 2010) did not show the presence of the alkaloid.

Due that the H^1 NMR signals of Hup A were not detected in the *H. cuernavacensis* methanolic crude extract, other compounds could be involved in the observed AChEI action (IC_{50} = 0.74±0.05µg/mL). It has been reported that other *Lycopodium* alkaloids also possess AChEI activity, *e.g.* cryptadine B (IC_{50}=18.5µM), isolated from *L. cryptomerinum* (Koyama *et al*., 2007); carinatumin A (IC_{50} =5.6µM), isolated from *L. carinatum* (Choo *et al*., 2007); sieboldine A (IC_{50}=2.0µM), isolated from *Lycopodium sieboldii* (Hirasawa *et al.*, 2003). In addition, the AChEI values have been reported in *H. serrata* alkaloidal extract (IC_{50} =5.96µg/mL) (Ohba *et al*., 2015). Furthermore, some AChEI values reported in alkaloid extracts of *H. reflexa* (IC_{50}=0.11µg/mL), *H. quadrifariata* (IC_{50}=2.0µg/mL), *H. acerosa* (IC_{50}=5.5µg/mL), *H. heterocarpon* (IC_{50} =25.6µg/mL), and *L. cernua* (IC_{50}= 42.6µg/mL) (Konrath *et al*., 2013), as well as in *H. saururus* (IC_{50}=0.58µg/mL) (Ortega *et al*., 2004), showed similar activity as the ones found in the present study for the *H. cuernavacensis* methanolic crude extract. Our results regarding AChEI of alkaloid extracts from *H. dichotoma* and *H. cuernavacensis* (0.64±0.09 and 0.74±0.05µg/mL, respectively) showed the same order of magnitude compared to the most active species reported by Ortega *et al.* (2004) and Konrath *et al*. (2013).

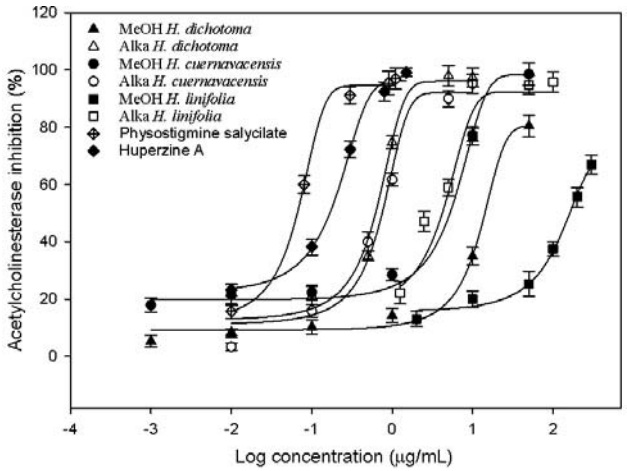

Fig. 2: Concentration-dependent AChEI of methanolic crude extracts (MeOH) and total alkaloidal extracts (Alka). Vertical bars represent the SEM.

In the present study, the methanolic crude extracts of *H. dichotoma* showed an important AChEI activity that can be attributed to the presence of Hup A. Nevertheless, this compound was not identified in the *H. cuernavacensis* methanolic crude extract, although the high AChEI value. A low AChEI activity was observed to *H. linifolia* methanolic crude extract (158.37±8.7µg/mL), but a high value of 4.2±1.24µg/mL was found to the corresponding total alkaloidal extract. Interestingly, this last extract did not show the presence of Hup A. We must take into account that other alkaloids and/or other non-alkaloidal compounds, *e.g.* terpenes, xanthones and coumarins, also

possess AChEI activity (Brühlmann *et al.*, 2004, Mukherjee *et al.*, 2007). In addition, an organic extracts of *L. clavatum* has been reported to possess the triterpenoid α-onocerin with AChE inhibitory activity (IC_{50}= 5.2mM), which was comparable to (±)-huperzine A (IC_{50} 1.6mM) (Hirasawa *et al*., 2003; Orhan *et al.*, 2003).

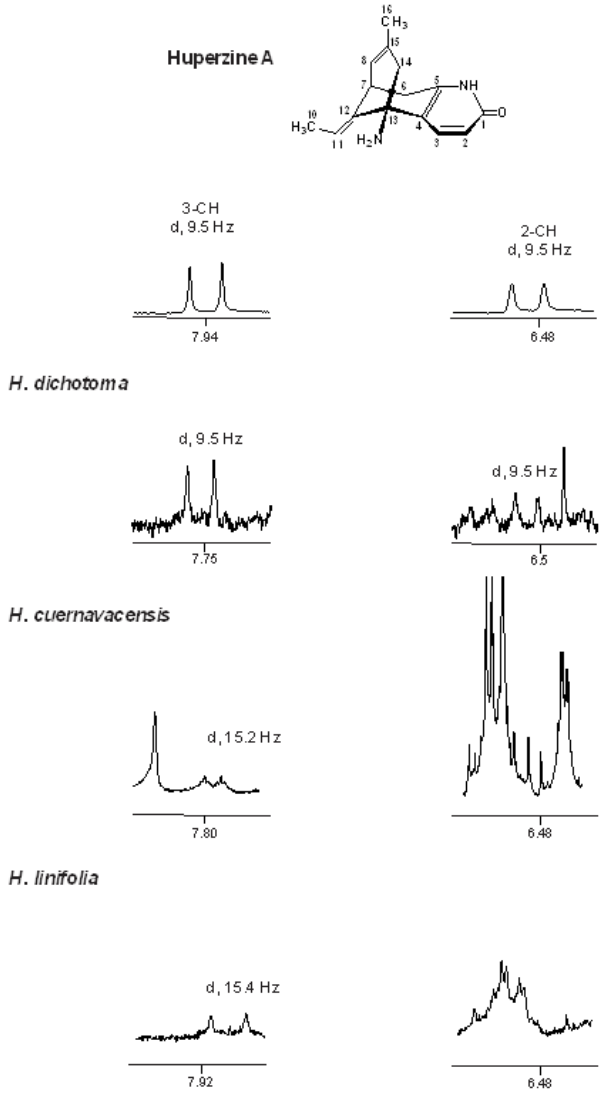

Fig. 3: ^{1}H NMR of the methanolic crude extract of *Huperzia* species, recorded in CD_3OD at 500 MHz. Diagnostic signal protons 2 and 3 were used for Hup A identification.

Since it is well known that ^{1}H NMR techniques are less sensitive than other identification analysis procedures *e.g.* GC-MS and LC-MS, the absence of Hup A traces in the studied extracts is not conclusive. Moreover, the synergistic effects on the AChEI activity should be considered, because it has been demonstrated that the presence of (-)-epigallocatechin-3-gallate in a methanolic extract may enhance the effect of Hup A and other active alkaloids over the AChEI (Xu *et al.*, 2008; Zhang *et al.*, 2009).

CONCLUSION

This study reveals that *H. dichotoma* is a new source of Hup A. It opens new perspectives about this species as an interesting candidate to produce Hup A by biotechnological approaches. *H. cuernavacensis* and *H. linifolia* extracts displayed high AChEI activities despite Hup A was not detected in these plants, which could be linked to the presence of other bioactive compounds.

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REFERENCES

Alzheimer's Disease International (2015). World Alzheimer Report 2015. Available: https://www.alz.co.uk/research/world-report-2015

Ayer WA (1991). *Lycopodium* alkaloids. *Nat. Prod. Rep.*, **8**: 455-463.

Barnes LL, Schneider JA, Boyle PA, Bienias JL and Bennett DA (2006). Memory complaints are related to Alzheimer disease pathology in older persons. *Neurology,* **67**: 1581-1585.

Brühlmann C, Marston A, Hostettmann K, Carrupt PA and Testa B (2004). Screening of non-alkaloidal natural compounds as acetylcholinesterase inhibitors. *Chem. Biodivers.*, **1**: 819-829.

Choo CY, Hirasawa Y, Karimata C, Koyama K, Sekiguchi M, Kobayashi J and Morita H (2007). Carinatumins A-C, new alkaloids from *Lycopodium carinatum* inhibiting acetyl cholinesterase. *Bioorg. Med. Chem.*, **15**: 1703-1707.

Ellman G, Courtney D, Andres V and Featherstone R (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7**: 88-95.

Francis P, Palmer A, Snape M and Wilcock G (1999). The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J. Neurol. Neurosurg. Psychiatry*, **66**: 137-147.

Gao X and Tang X (2006). Huperzine A attenuates mitochondrial dysfunction in beta-amyloid-treated PC12 cells by reducing oxygen free radicals accumulation and improving mitochondrial energy metabolism. *J. Neurosci. Res.,* **83**: 1048-1057.

Gao X, Zheng CY, Yang L, Tang XC and Zhang HY (2009). Huperzine A protects isolated rat brain mitochondria against β-amyloid peptide. *Free Radic. Biol. Med.*, **46**: 1454-1462.

Goodger J, Whincup A, Field A, Holtum J and Woodrow I (2008). Variation in huperzine A and B in Australasian *Huperzia* species. *Biochem. Syst. Ecol.,* **36**: 612-618.

Hirasawa Y, Morita H, Shiro M and Kobayashi J (2003). Sieboldine A, a novel tetracyclic alkaloid from *Lycopodium sieboldii*, inhibiting acetylcholinesterase. *Org Lett.*, **5**: 3991-3993.

Ishiuchi K, Park J, Long R and Gang D (2013). Production of huperzine A and other *Lycopodium* alkaloids in *Huperzia* species grown under controlled conditions and *in vitro*. *Phytochemistry*, **91**: 208-219.

Konrath EL, Neves BM, Passos CS, Lunardi PS, Ortega MG, Cabrera JL, Gonçalves CA and Henriques AT (2012). *Huperzia quadrifariata* and *Huperzia reflexa* alkaloids inhibit acetylcholinesterase activity *in vivo* in mice brain. *Phytomedicine.*, **19**: 1321- 1324.

Konrath EL, Ortega MG, Bordignon SL. Apel MA, Henriques AT and Cabrera JL (2013). Alkaloid profiling and anticholinesterase activity of South American *Lycopodiaceae* species. *J. Enzyme Inhib. Med. Chem*., **28**: 218-222.

Koyama K, Hirasawa Y, Kobayashi J and Morita H (2007). Cryptadines A and B, novel $C_{27}N_3$-type pentacyclic alkaloids from *Lycopodium cryptomerinum*. *Bioorg. Med. Chem.*, **15**: 7803-7808.

Lim WH, Goodger JQD, Field AR, Holtum JAM and Woodrow IE (2010). Huperzine alkaloids from Australasian and southeast Asian *Huperzia*. *Pharm. Biol.*, **48**: 1073-1078.

Liu JS, Zhu YL, Yu CM, Zhou YZ, Han YY, Wu FW and Qi BF (1986). The structures of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. *Can. J. Chem.* **64**: 837-839.

Ma X, Tan C, Zhu D and Gang D (2005). Is there a better source of huperzine A than *Huperzia serrata*? Huperzine A content of huperziaceae species in China. *J. Agric. Food Chem.*, **53**: 1393-1398.

Mattson MP (2004). Pathways towards and away from Alzheimer's disease. *Nature,* **430**:631-639.

Mukherjee PK, Kumar V, Mal M and Houghton PJ (2007). Acetyl cholinesterase inhibitors from plants. *Phytomedicine,* **14**: 289-300.

Ohba T, Yoshino Y, Ishisaka M, Abe N, Tsuruma K, Shimazawa M, Oyama M, Tabira T and Hara H (2015). Japanese *Huperzia serrata* extract and the constituent, huperzine A, ameliorate the scopolamine-induced cognitive impairment in mice. *Biosci. Biotechnol. Biochem*., **79**: 1838-1844.

Ortega MG, Agnese AM and Cabrera JL (2004). Anticholinesterase activity in an alkaloid extract of *Huperzia saururus*. *Phytomedicine.*, **11**: 539-543.

Orhan I, Orhan G and Gurkas E (2011). An overview on natural cholinesterase inhibitors–a multi-targeted drug class–and their mass production. *Mini-Rev. Med. Chem*., **11**: 836-842.

Orhan I, Terzioglu S and Sener B (2003). Alpha-onocerin: an acetylcholinesterase inhibitor from *Lycopodium clavatum*. *Planta Med*., **69**: 265-167.

Tang LL, Wang R and Tang XC (2005). Huperzine A protects SHSY5Y neuroblastoma cells against oxidative stress damage via nerve growth factor production. *Eur. J. Pharmacol.,* **519**: 9-15.

Tang XC 1996. Huperzine A shuangyiping: A promising drug for Alzheimer's disease. *Zhongguo. Yao. Li. Xue. Bao.*, **17**: 481-484.

Terry VJR and Buccafusco JJ (2003). The Cholinergic Hypothesis of Age and Alzheimer's Disease-Related Cognitive Deficits: Recent Challenges and Their Implications for Novel Drug Development. *J. Pharmacol. Exp. Ther.*, **306**: 821-827.

Wong CW (2016). Pharmacotherapy for Dementia: A Practical Approach to the Use of Cholinesterase Inhibitors and Memantine. *Drugs Aging*, pp.1-10.

Xiao XQ, Wang R and Tang XC (2000). Huperzine A and tacrine attenuate beta-amyloid peptide-induced oxidative injury. *J. Neurosci. Res.*, **61**: 564-569.

Xu M, Xiao J, Chen X, Zhang L, Talbot S and Li G (2008). Investigation of the mechanism of enhanced effect of EGCG on Huperzine A's inhibition of acetylcholinesterase activity in rats by a multispectroscopic method. *J. Agric. Food Chem.*, **56**: 910-915.

Yang G, Wang Y, Tian J and Liu JP (2013). Huperzine A for Alzheimer's Disease: A Systematic Review and Meta-Analysis of Randomized Clinical Trials. *PLoS ONE*, **8**: e74916.

Zhang HY and Tang XC (2006). Neuroprotective effects of huperzine A: new therapeutic targets for neurodegenerative disease. *Trends. Pharmacol. Sci*., **27**: 619-625.

Zhang L, Cao H, Wen J and Xu M (2009). Green tea polyphenol (-)-epigallocatechin-3- gallate enhances the inhibitory effect of huperzine A on acetylcholinesterase by increasing the affinity with serum albumin. *Nutr. Neurosci.*, **12**: 142-148.