Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: https://www.tandfonline.com/loi/ienz20

Alkaloid profiling and anticholinesterase activity of South American *Lycopodiaceae* species

Eduardo Luis Konrath, María Gabriela Ortega, Sérgio de Loreto Bordignon, Miriam Anders Apel, Amélia Teresinha Henriques & José Luis Cabrera

To cite this article: Eduardo Luis Konrath, María Gabriela Ortega, Sérgio de Loreto Bordignon, Miriam Anders Apel, Amélia Teresinha Henriques & José Luis Cabrera (2013) Alkaloid profiling and anticholinesterase activity of South American *Lycopodiaceae* species, Journal of Enzyme Inhibition and Medicinal Chemistry, 28:1, 218-222, DOI: 10.3109/14756366.2011.633908

To link to this article: https://doi.org/10.3109/14756366.2011.633908

Journal of Enzyme Inhibition and Medicinal Chemistry, 2013; 28(1): 218–222

ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.3109/14756366.2011.633908

informa
healthcare

SHORT COMMUNICATION

Alkaloid profiling and anticholinesterase activity of South American *Lycopodiaceae* species

Eduardo Luis Konrath[1], María Gabriela Ortega[2,3], Sérgio de Loreto Bordignon[4], Miriam Anders Apel[1], Amélia Teresinha Henriques[1], and José Luis Cabrera[2,3]

[1]Programa de Pós Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, [2]Farmacognosia, Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina, [3]Instituto Multidisciplinario de Biologia Vegetal (IMBIV-CONICET), Córdoba, Argentina, and [4]Centro Universitário La Salle, Canoas, RS, Brazil

Abstract

The alkaloid extracts of four *Huperzia* and one *Lycopodiella* species, from Brazilian habitats, were tested for their *in vitro* anticholinesterase activities. IC_{50} values showed a potent acetylcholinesterase inhibition for *H. reflexa* (0.11 ± 0.05 μg/mL), followed by *H. quadrifariata* (2.0 ± 0.3 μg/mL), *H. acerosa* (5.5 ± 0.9 μg/mL), *H. heterocarpon* (25.6 ± 2.7 μg/mL) and *L. cernua* (42.6 ± 1.5 μg/mL). A lower inhibition of butyrylcholinesterase was observed for all species with the exception of *H. heterocarpon* (8.3 ± 0.9 μg/mL), whose alkaloid extract presented a selectivity for pseudocholinesterase. Moreover, the chemical study of the bioactive extracts performed by GC-MS, revealed the presence of a number of *Lycopodium* alkaloids belonging to the lycopodane, flabellidane and cernuane groups. Surprisingly, the potent acetylcholinesterase inhibitors huperzines A and B were not detected in the extracts, suggesting that other alkaloids may be responsible for such an effect.

Keywords: *Huperzia acerosa*, *Huperzia heterocarpon*, *Huperzia quadrifariata*, *Huperzia reflexa*, AChE and BChE inhibition

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that has emerged as one of the major public health problems in the elderly, resulting in impaired memory and behavior, as well as loss of intellectual and social abilities[1,2]. The main treatment strategies available have been based on the cholinergic hypothesis, which postulates that memory impairment in AD patients' results from a deficit of acetylcholine (ACh) levels in the brain. As the cholinergic system has been shown to be the predominantly affected neurotransmitter system in this disease[3], the most effective available pharmacotherapy for AD is the use of acetylcholinesterase (AChE) inhibitors, which indirectly elevate the ACh concentrations in the brain[4]. There has been an extensive search for new AChE inhibitors with higher efficacy and fewer side effects, particularly in those derived from natural products[5]. In recent years, the Lycopodiaceae family has been well studied as a source of new and potent inhibitors of the AChE enzyme, with the isolation of huperzines A and B from the Chinese medicinal plant, *Huperzia serrata* (Thunb.) Trev[6]. However, huperzine A is still the most well known *Lycopodium* alkaloid, and is considered to be a highly potent, reversible and selective inhibitor for the AChE enzyme, when compared with other inhibitors that are currently used in therapeutics[7].

In our ongoing investigation for new bioactive *Lycopodium* alkaloids in habitats of South America, we herein studied five Brazilian species from the Lycopodiaceae family: *Huperzia acerosa* (Sw.) Holub, *H. heterocarpon* (Fée) Holub, *H. quadrifariata* (Bory) Rothm., *H. reflexa* (Lam.) Trevis. and *Lycopodiella cernua* (L.) Pic Serm., whose alkaloid extracts were evaluated regarding AChE and butyrylcholinesterase (BChE)

Address for Correspondence: Dr. José L. Cabrera, Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria 5000, Córdoba, Argentina. Tel: 54-0351-433-4163. Fax: 54-0351-433-4127. E-mail: jcabrera@dqo.fcq.unc.edu.ar
Eduardo Luis Konrath and María Gabriela Ortega contributed equally to this publication.

(Received 25 August 2011; revised 12 October 2011; accepted 13 October 2011)

inhibition, together with their chemical content. In addition, inedited biological activities (AChE and BChE) for the pure alkaloids cernuine, clavolonine, lycocernuine and lycopodine, isolated from different *Lycopodiaceae* species, were also assessed in a similar mode. Results of previous studies performed by our group with *Huperzia saururus* (Lam.) Trevis., a plant used in the Argentinean folk medicine as an aphrodisiac and memory improver, demonstrated a strong and selective inhibition of the AChE enzyme[8]. Recently, sauroine, the main alkaloid found in *H. saururus*, was also shown to display an improvement in memory retention and learning in rats[9], which may corroborate some previous findings related to the alkaloid extract, such as an increase in hippocampal plasticity[10] and in the memory retention in rats[11]. Among the species studied, *L. cernua* has been reported to have medicinal uses for spasms, rheumatism and as an anti-inflammatory agent[12,13]. A previous study conducted with *Lycopodiaceae* species from French Polynesia revealed that *L. cernua* has anticholinesterase activity, effect associated with the presence of huperzines and lycopodine[14]. To our knowledge, however, there are no publications regarding the species *H. acerosa*, *H. heterocarpon*, *H. quadrifariata* and *H. reflexa*.

Experimental

Plant material

Whole plants of *Huperzia acerosa* (Sw.) Holub (HAS 47477), *Huperzia heterocarpon* (Fée) Holub (HAS 47478) and *Huperzia quadrifariata* (Bory) Rothm. (HAS 47475) were collected in the city of Riozinho (2007); *Huperzia reflexa* (Lam.) Trevis. (HAS 45877) in Barra do Ouro (2008) and *Lycopodiella cernua* (L.) Pic Serm. (HAS 47473) in Santo Antônio da Patrulha (2008), all in the state of Rio Grande do Sul, Brazil. All samples were identified by Dr. Rosana M. Sena, and for each plant a voucher specimen has been deposited in the herbarium (HAS) of the Fundação Zoobotânica do Rio Grande do Sul, Brazil, as indicated.

Preparation of the alkaloid extracts and isolation

Dried and crushed aerial parts of *H. quadrifariata* (580 g), *L. cernua* (396 g), *H. reflexa* (92 g), *H. heterocarpon* (23 g) and *H. acerosa* (14 g) were separately defatted with *n*-hexane and then extracted with ethanol by using a Soxhlet extractor. The use of temperature did not alter the chemical composition of the plants, as checked by TLC profile before and after the procedure. After exhaustive extraction, the ethanolic extracts were filtered and concentrated under reduced pressure. Then the residues were solubilized with 5% aqueous HCl and partitioned three times with CH_2Cl_2. The acidic H_2O layer was basified with NH_4OH (pH 11) in order to produce free bases, and before being extracted with CH_2Cl_2. The resulting organic phases were evaporated to dryness and lyophilized, which yielded the powdered crude total alkaloid extracts (E): *H. quadrifariata* (E_{HQ}): 2.26 g, (0.40%); *L. cernua* (E_{LC}): 0.67 g, (0.17%); *H. reflexa* (E_{HR}): 0.64 g, (0.69%); *H. heterocarpon* (E_{HH}): 0.055 g, (0.24%); *H. acerosa* (E_{HA}): 0.054 g, (0.38%). The alkaloids used in the anticholinesterase assays were previously isolated and purified from different alkaloid extracts through successive chromatographic techniques[9], and the identification was developed by spectroscopic analyses, compared with data obtained from literature[8,15–19].

Alkaloid analysis by GC-MS

The identification of the components present in the extracts through GC-MS was performed using an Agilent 7890A directly coupled to a quadrupole mass spectrometer Agilent 5975C and a capillary column SE 30 (30 m in length). The injection volume was 0.2 mL with He as carrier, at a flow rate of 1 mL/min. The temperature program was: 230°C (2 min), 230–250°C at 20°C/min, 250°C (2 min), 250–280°C at 5°C/min, and 280°C (2 min). The temperatures of the injector, interface and ion source were 300, 280, and 280°C, respectively, and the ionization energy was 70 eV. The individual alkaloid identifications were made by examining a reference library of mass spectra obtained from authentic samples of our own *Lycopodium* alkaloid collection and also by comparing fragmentation patterns with those found in the literature[8,15–19]. See the gas chromatography profile of the alkaloid extracts and the MS fragmentation of the known alkaloids in Supplementary Material (Figures S1–S5 and Table S1).

Anticholinesterase activity assays

The anticholinesterase inhibitory assay was performed *in vitro* using human blood as sources of AChE and BChE, and the enzymatic activity was measured using the Ellman colorimetric method with some modifications introduced by our group[8,20]. Human blood, obtained by venipuncture from human healthy volunteers, was mixed with EDTA (1 mg/mL) and after centrifuged at 2500*g* for 10 min for serum separation and then used as a source of enzyme BChE. The red cells obtained were washed with physiological 0.9% (w/v) saline solution and lysed with distilled water. Erythrocyte membranes were then separated by centrifugation at 14,500*g* for 20 min. An aliquot (0.4 mL) was resuspended in phosphate buffer, pH 7, and used as an enzyme source of AChE.

A typical experiment performed in order to calculate the enzymatic activities consisted of 750 µL of 50 mM phosphate buffer of pH 7.2 containing 0.25 mM of 5,5-dithiobis-2-nitrobenzoic acid (buffer + DTNB), 100 µL of distilled water, 5 µL of enzyme (membrane suspension or plasma) obtained from human blood, and 25 µL of acetylthiocholine iodide 58 mM or butyrylthiocholine chloride 54.5 mM (V = 880 µL). The reactants were placed in a microcuvette and the absorbance measured using a spectrophotometer at 405 nm (Spectronic Genesys 5, Milton Roy Co., Rochester, NY), with readings being taken during 3 min at intervals of 30 s to verify the linearity of the reaction. The enzymatic activity was calculated using the absorption

coefficients of 23,460 U/L (for AChE) and 22,760 U/L (for BChE), and the resultant value was considered as 100% activity. All experiments were performed at 25°C.

Similarly, the different crude alkaloid extracts of each species and some previously isolated alkaloids were evaluated. In this assay, and for each determination, a quantity of sample solutions (1000 µg/mL, each prepared with 1% DMSO in buffer) was added to the buffer + DTNB solution, together with distilled water and the enzyme preparation. The quantity of the solution of extracts/compounds used was determined by taking into account that the final concentration corresponding to each one of the different assayed concentrations ranging between 100 and 0.1 µg/mL (lower concentrations for the most active extracts). Physostigmine salicylate was employed as a reference inhibitor (Merck, Germany).

Statistical 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 GraFit 6.0 software on a PC compatible computer.

Results and discussion

A preliminary analysis of the alkaloidal extracts by GC-MS indicated the presence of a number of *Lycopodium* alkaloids, which have already been described for other species (Figure 1). The chromatographic profile obtained for *H. reflexa*, indicated the presence of lycodine, anhydrolycodoline, lycodoline, 6-hydroxylycopodine, α-obscurine, des-*N*-methyl-α-obscurine, together with lycopodine and an unidentified compound of *m/z* 245, with the typical fragmentation pattern occurring for an alkaloid from the lycopodane group. The alkaloidal extract of *H. acerosa* showed three alkaloids, identified as lycopodine, lycodine and flabelline, and *H. quadrifariata* demonstrated the presence of lycopodine, 6-hydroxylycopodine, lycodoline, clavolonine, acetylclavolonine, sauroine and an as yet unidentified alkaloid (*m/z* 272), but recognized as belonging to the flabellidane group. To date, this is the first report for the identification of acetylclavolonine from a natural

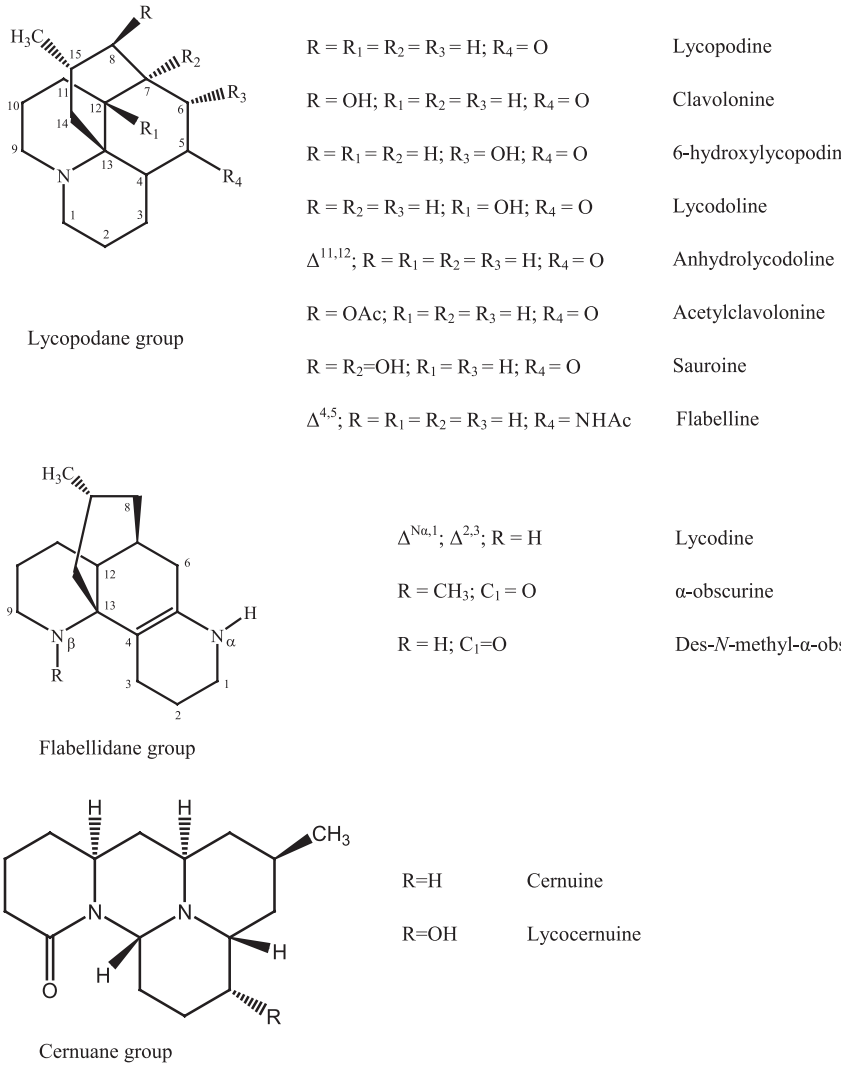

Figure 1. *Lycopodium* alkaloids identified in the species analyzed in the present work.

Table 1. The IC_{50} values by percent inhibition of cholinesterase activities to the alkaloid extracts and isolated compounds.

Sample	AChE inhibition IC_{50} [a]	BChE inhibition IC_{50} [a]
Huperzia acerosa	5.5 ± 0.9	>50.0
Huperzia heterocarpon	25.6 ± 2.7	8.3 ± 0.9
Huperzia quadrifariata	2.0 ± 0.3	5.5 ± 0.5
Huperzia reflexa	0.11 ± 0.05	5.6 ± 0.3
Lycopodiella cernua	42.6 ± 1.5	>100.0
Lycopodine	>250.0	>250.0
Clavolonine	>250.0	>250.0
Cernuine	32.7 ± 1.8	>250.0
Lycocernuine	>250.0	>250.0
Physostigmine salicylate[b]	0.08 ± 0.01	5.01 ± 0.2

[a]Results are the means of five independent experiments (μg/mL) ± SEM.
[b]Reference control.

source; since, it has only been described as a semisynthetic product obtained from clavolonine[21]. *L. cernua* indicated the presence of only two compounds, identified as cernuine and lycocernuine[17], and *H. heterocarpon* showed three alkaloids, but to date none of these have been identified. However, the fragmentation pattern corresponded to *Lycopodium* alkaloids belonging to lycopodane (*m/z* 245 and 261) and flabellidane (*m/z* 272) classes.

As shown in Table 1 and Figure 2, an important anticholinesterase activity in the extracts of *H. reflexa* (E_{HR}) and *H. quadrifariata* (E_{HQ}) was observed. The IC_{50} value for E_{HR} (0.11 μg/mL) was 1.5 times higher than the reference inhibitor (physostigmine salicylate, IC_{50} value = 0.08 μg/mL) and five times lower than the extract of *H. saururus* (IC_{50} value = 0.58 μg/mL), which also displayed a marked effect on AChE activity[8]. *H. acerosa* also showed an important effect on AChE inhibition, which was more potent than its effect on BChE. *H. reflexa* or *H. quadrifariata;* again, displayed an important inhibition of this enzyme when compared to the reference inhibitor. However, the cholinesterase specificity was related to true cholinesterase. *H. heterocarpon* showed a better inhibition for the BChE, compared to AChE inhibition; this effect is also of particular relevance since BChE has been targeted as a new approach to intercede the progression of AD in the latter stages, due to the fact that this enzyme represents the predominant cholinesterase in the brain[22].

The chemical examination of the five species studied did not reveal the presence of any *Lycopodium* alkaloids with a known inhibitory effect for AChE, such as huperzine A (M^+ 242), huperzine B (M^+ 256), *N*-demethylhuperzinine (M^+ 256), huperzine C (M^+ 242), huperzinine (M^+ 270), carinatumine A (M^+ 258) or sieboldine A (M^+ 293), with the exception of lycopodine[6,14,23–25]. To our knowledge, this is the first report demonstrating the biological properties of these *Huperzia* species, which are associated with the alkaloids present in the extracts. Phytochemical studies of *L. cernua* have been previously described[17,26]; but, in the current study, we only found two alkaloids that had already been reported

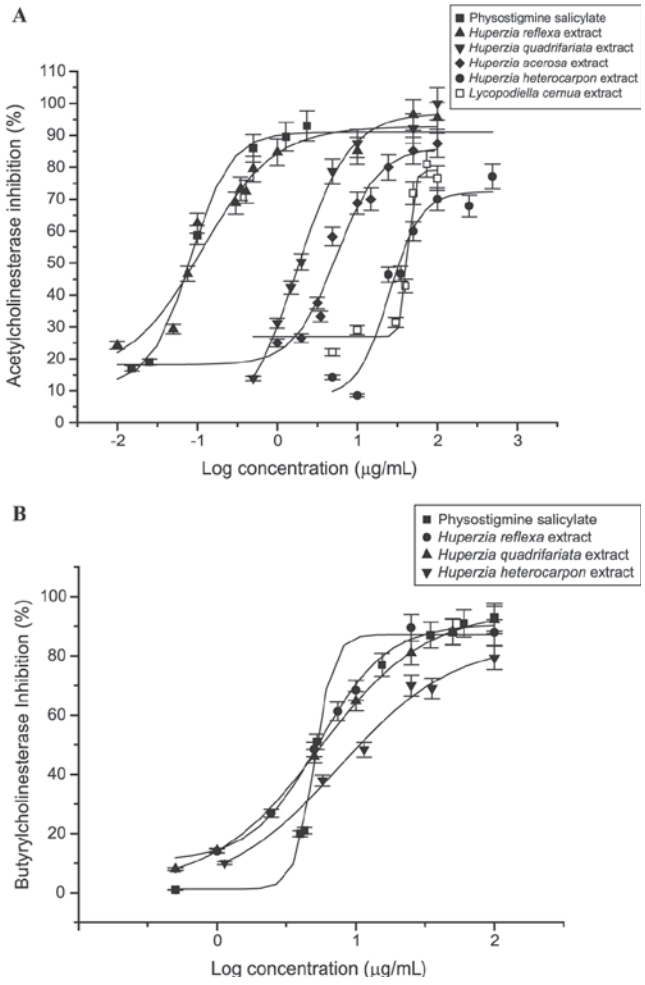

Figure 2. Concentration-dependent inhibition of AChE (A) and BChE (B) by alkaloidal extracts and physostigmine salicylate ($n = 5$). Vertical bars represent the SEM. The inhibition efficacy was expressed as a percentage of inhibited enzyme activity compared to the control value (100%).

for this species (cernuine and lycocernuine). However, to date, observations concerning anticholinesterase activities have been only described in the literature in a form of bioautographic detection assays[14], being quantitatively described here for the first time. Among the different alkaloids tested, only cernuine was considered to be active against AChE (IC_{50} value = 32.7 μg/mL), whereas clavolonine, lycocernuine and lycopodine until a concentration of 250 μg/mL were not active, in comparison with the alkaloid extracts.

In view of the significant inhibitory effect exhibited by *H. reflexa*, *H. quadrifariata* and *H. acerosa*, associated with the absence of the known anticholinesterasic inhibitors present in other species from the same family, the fractionation of the extracts is being conducted in our laboratory in order to obtain their active principles. Although the unidentified alkaloids from *H. quadrifariata* and *H. heterocarpon* showed a typical fragmentation pattern for *Lycopodium* alkaloids[15], they did not correspond conclusively with the reported spectra of any known alkaloid from this group. Further investigations of

the chemical structures of the active principles and their pharmacological evaluation are necessary to determine which alkaloids are responsible for the above-demonstrated biological effects.

Conclusion

From this study, it may be concluded that *H. acerosa*, *H. heterocarpon*, *H. quadrifariata*, *H. reflexa* and *L. cernua*, all *Lycopodiaceae* species with habitats in Brazilian montane forests, hold potential AChE and BChE inhibitory activity. The absence of the activity of known *Lycopodium* alkaloids as cholinesterase inhibitors, such as huperzines, was demonstrated, indicating that other alkaloids present in the extracts may be the bioactive constituents. Therefore, these plants representing the rich biodiversity of Pteridophyta for South America should be explored further as potential leads in the development of agents for the management of Alzheimer's disease.

Acknowledgments

The authors wish to thank Dr. Rosana M. Sena (Fundação Zoobotânica do Rio Grande do Sul) for identification of the species under study.

Declaration of interest

This work was supported in part by the Brazilian funding agencies Coordenação de Apefeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and by the Argentinean agencies SECyT-UNC N° 069/08 and FONCyT: Préstamo BID PICT N° 06-0120. E.L.K. is a doctoral fellow of CAPES, M.G.O. and J.L.C. are members of the Research Career of CONICET..

References

1. Cummings JL. Treatment of Alzheimer's disease: Current and future therapeutic approaches. Rev Neurol Dis 2004;1:60–69.
2. Mattson MP. Pathways towards and away from Alzheimer's disease. Nature 2004;430:631–639.
3. Giacobini E. Cholinesterases: New roles in brain function and in Alzheimer's disease. Neurochem Res 2003;28:512–522.
4. Martinez A, Castro A. Novel cholinesterase inhibitors as future effective drugs for the treatment of Alzheimer's disease. Expert Opin Investig Drugs 2006;15:1–12.
5. Hsieh MT, Peng WH, Wu CR, Ng KY, Cheng CL, Xu HX. Review on experimental research of herbal medicines with anti-amnesic activity. Planta Med 2010;76:203–217.
6. Liu JS, Zhu YL, Yu CM, Zhou YZ, Han YY, Wu FW, et al. The structure of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. Can J Chem 1986;64:837–839.
7. Ma X, Tan C, Zhu D, Gang DR, Xiao P. Huperzine A from *Huperzia* species–an ethnopharmacolgical review. J Ethnopharmacol 2007;113:15–34.
8. Ortega MG, Agnese AM, Cabrera JL. Anticholinesterase activity in an alkaloid extract of Huperzia saururus. Phytomedicine 2004;11:539–543.
9. Vallejo MG, Ortega MG, Cabrera JL, Carlini VP, Rubiales de Barioglio S, Almirón RS et al. Sauroine, an alkaloid from *Huperzia saururus* with activity in wistar rats in electrophysiological and behavioral assays related to memory retention. J Nat Prod 2009;72:156–158.
10. Ortega MG, Vallejo MG, Cabrera JL, Pérez MF, Almirón RS, Ramírez OA et al. *Huperzia saururus*, activity on synaptic transmission in the hippocampus. J Ethnopharmacol 2006;104:374–378.
11. Vallejo MG, Ortega MG, Cabrera JL, Carlini VP, Rubiales de Barioglio S, Agnese AM. *Huperzia saururus* increases memory retention in rats. J Ethnopharmacol 2007;111:685–687.
12. Duke JA, Ayensu ES. Medicinal plants of China, vol II. Michigan: Reference Publications Inc., 1985:112–115.
13. Barboza GE, Cantero JJ, Núñez C, Pacciaroni A, Espinar LA. Medicinal plants: A general review and a phytochemical and ethnopharmacological screening of the native Argentine flora. Kurtziana 2009;34:7–365.
14. Ho R, Marsousi N, Eugster P, Bianchini JP, Raharivelomanana P. Detection by UPLC/ESI-TOF-MS of alkaloids in three *Lycopodiaceae* species from French Polynesia and their anticholinesterase activity. Nat Prod Commun 2009;4:1–4.
15. MacLean DB. *Lycopodium* alkaloids XIII. Mass spectra of representative alkaloids. Can J Chem 1963;41:2654–2670.
16. Alam SN, Adams AH, MacLean DB. *Lycopodium* alkaloids XV Structure and mass spectra of some minor alkaloids of *Lycopodium flabelliforme*. Can J Chem 1964;42:2456–2466.
17. Ayer W, Jenkins JK, Piers K, Valverde-Lopez S. The alkaloids of *Lycopodium cernuum* L. II. The stereochemistry of cernuine and lycocernuine. Can J Chem 1967;45:445–450.
18. Sun CM, Ho LK, Sun ML. Revised Stereochemistry of So-Called Isoselagine and Spectroscopic Analysis of 6α-Hydroxylycopodine from *Lycopodium serratum* var. longipetiolatum. Planta Med 1993;59:467–470.
19. Ortega MG, Agnese AM, Cabrera JL. Sauroine – a novel *Lycopodium* alkaloid from *Huperzia saururus*. Tetrahedron Lett 2004;45:7003–7005.
20. Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
21. MacLean DB. Lycopodium alkaloids. In: Brossi A, (ed). The Alkaloids, New York: Academic Press;1985. pp 241–298.
22. Ballard CG, Greig NH, Guillozet-Bongaarts AL, Enz A, Darvesh S. Cholinesterases: Roles in the brain during health and disease. Curr Alzheimer Res 2005;2:307–318.
23. Shen YC, Chen CH. Alkaloids from *Lycopodium casuarinoides*. J Nat Prod 1994;57:824–826.
24. Hirasawa Y, Morita H, Shiro M, Kobayashi J. Sieboldine A, a novel tetracyclic alkaloid from *Lycopodium sieboldii*, inhibiting acetylcholinesterase. Org Lett 2003;5:3991–3993.
25. Choo CY, Hirasawa Y, Karimata C, Koyama K, Sekiguchi M, Kobayashi J et al. Carinatumins A-C, new alkaloids from *Lycopodium carinatum* inhibiting acetylcholinesterase. Bioorg Med Chem 2007;15:1703–1707.
26. Morita H, Hirasawa Y, Shinzato T, Kobayashi J. New phlegmarane-type, cernuane-type and quinolizidine alkaloids from two species of *Lycopodium*. Tetrahedron 2004;60:7015–7023.