www.neuro-sys.com

NEUROSYS
CNS/PNS IN VITRO DRUG DEVELOPMENT

From traditional use to standardized neuroprotective green-extract of *Huperzia serrata*

30th International Symposium on the Chemistry of Natural Products

Elnur GARAYEV, Laurent BOYER, Patricia FRANCON, Philippe POINDRON, Maud COMBES, Noelle CALLIZOT
Neuro-Sys SAS, R&D Department, 410 Chemin Departemental 60, 13120 Gardanne, France
Corresponding author: elnur.garayev@neuro-sys.com

INTRODUCTION

Some plants belonging to the *Lycopodiaceae* species such as *Huperzia serrata* (Thunb.) Trevis (Fig. 1), *H. squarrosa* (Forst.) Trevis, *Lycopodium complanatum* L. and *L. cernua* (L.) Franco & Vasc have been used in Asiatic folk medicine for thousands of years to treat contusions, strains, schizophrenia and memory dysfunction [1, 2]. *H. serrata* and *H. saururus* are used respectively in Chinese and Argentinian folk medicine, via infusion, for their neuromuscular and memory-improving properties [3, 4].
Huperzine A, one of the constituents of *H. serrata*, was proven to be a reversible inhibitor of acetylcholinesterase with neuroprotective effects [5]. The results on more than 100,000 Chinese patients indicated that Huperzine A significantly improved cognitive functions [6] and had the potential to become an alternative treatment for Alzheimer's disease (AD). However, clinical trials with Huperzine A treatment also indicated that large doses of this molecule were needed to obtain significant effects in patients suffering from AD, thus increasing the occurrence of side effects [7].
Inspired by the traditional use (infusion/decoction), a standardized extract was obtained using a green extraction technique. This green extract has conserved original chemical profile of traditional preparation. The pharmacological activity was demonstrated with the synergistic effect of three compounds belonging to these extracts: Huperzine A (HA), Caffeic acid (CA) and Ferulic acid (FA) [8].

Fig. 1 *Huperzia serrata* (Thunb.) Trevis

METHODS

Plant material: whole plant of wild *Huperzia serrata* was harvested in Vietnam in 2013. The plant was certified by Pr. Tran Hop and voucher specimen (TH000) was deposited in the laboratory of Neuro-Sys.

Extraction: traditional extract (TE) was prepared by boiling 10 g of grinded plant in 150 mL of water for 30 minutes. After filtration through cotton the filtrate was freeze dried. A microwave extractor Ethos X (Milestone, Italy) was used in the preparation of NSP01 (batch 005-E003a).

Chromatographical analysis was realized using Hitachi Chromaster Ultra RS system coupled to DAD detector. 0.1 % of formic acid in water and 0.1 % of formic acid in methanol were used as mobile phase. The separation was achieved on the Thermo Acclaim RSLC 120 C18 column (150 × 2.1 mm, 2.2 µm).

Pharmacological evaluation: Culture of cortical neurons: Primary rat cortical neurons were cultured as described by Callizot *et al.*, 2013 [9]. Briefly, pregnant females at 15 days of gestation were used. Cortex was dissociated then cells were centrifuged and resuspended in a defined culture medium The cells were seeded at a density of 25,000 per well in poly-L-lysine pre-coated 96-well plates and cultured at 37 °C in an air (95 %)-CO2 (5 %) incubator. The medium was changed every 2 days. On day 13 of culture, extract or compound and/or mix of compounds were solved in culture medium and pre-incubated for 1 hour before glutamate exposure. The cortical neurons were intoxicated with glutamate solution (40 µmol/L) for 20 min. After 20 min exposure, glutamate was washed out and fresh culture medium with extract or compound and/or mix of compounds was added for additional 48 hours.
Robotized platform (HCS system): End point evaluation: Neuron survival: 48 hours after intoxication, cortical neurons were stained with anti-MAP-2 antibody and revealed with Alexa Fluor IgG. Analysis of total number of neurons was performed automatically (30 randomly selected pictures per well; 20 x magnification). All data were expressed in percentage of control conditions (no intoxication, no glutamate = 100 %). All values expressed as mean +/- SEM (standard error of the mean) (n = 6 wells per condition per culture). ANOVA followed by the Fisher's test when allowed.

RESULTS

A "Green" extract **NSP01**, obtained via microwave-assisted extraction, was compared to the TE. The superposition of chromatograms (Figure 2) showed comparable profiles for the two extracts. The content of Huperzine A was 0.21 % (m/m) in NSP01 vs 0.20 % (m/m) in the TE.

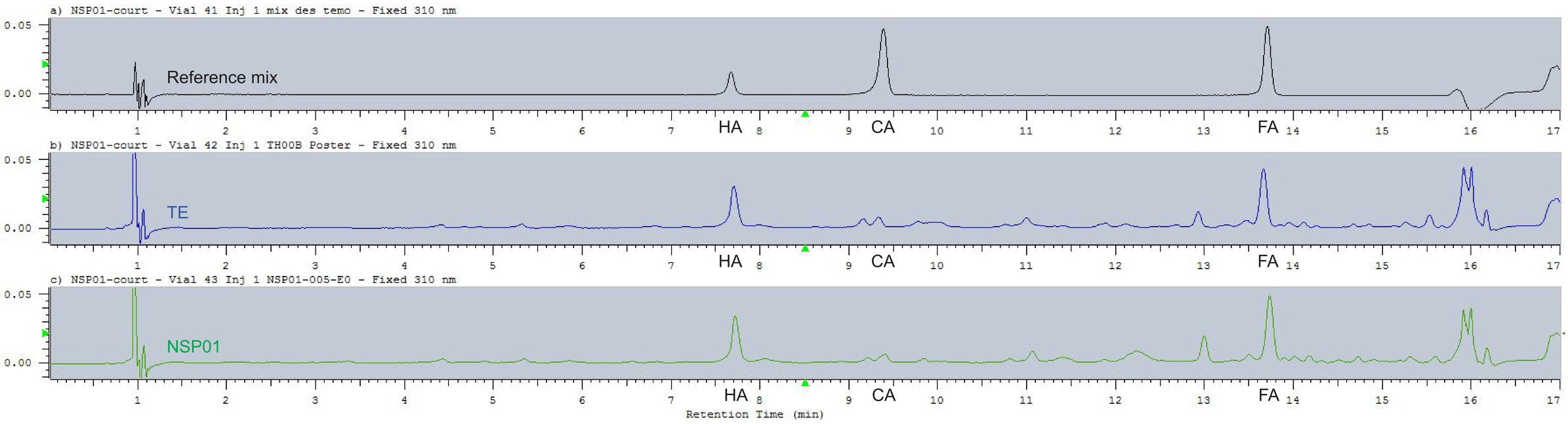
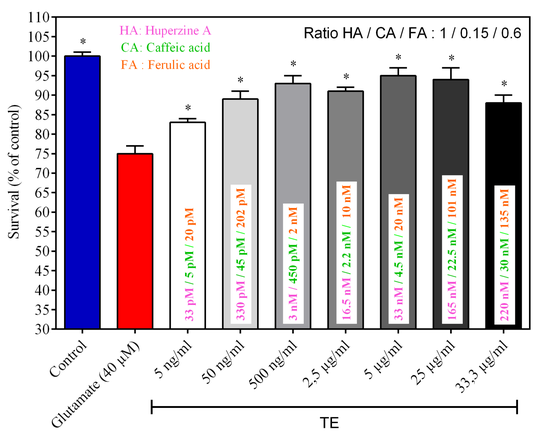


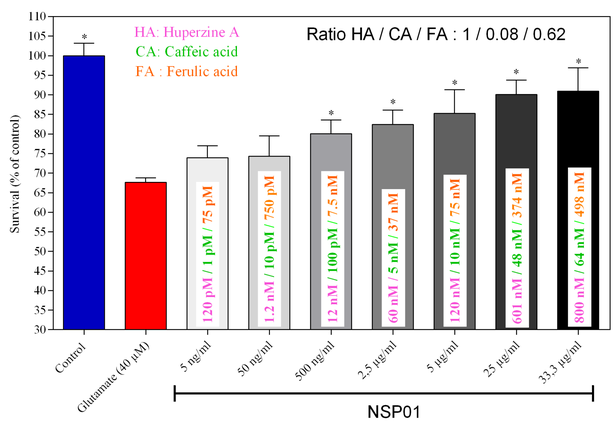
Fig. 2 Chromatograms (at 310 nm) of reference mix: Huperzine A (HA), Caffeic acid (CA) and Ferulic acid (FA) (black), traditional extract (blue) and NSP01 (green).

Pharmacological activity of TE and NSP01 extracts

Effect of TE (1 h pre-treatment) on neuron survival after glutamate injuries (40 µmol/L, 20 min)

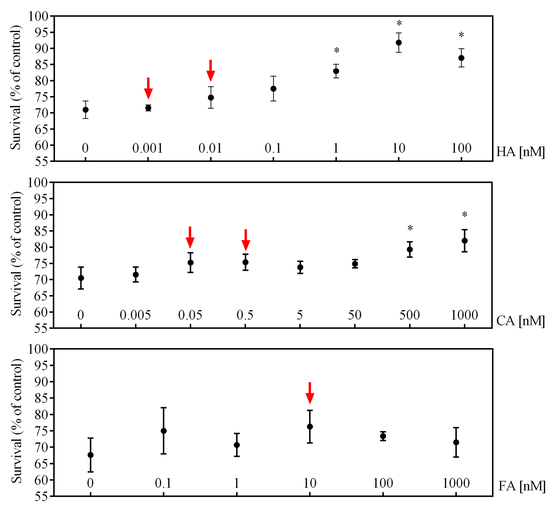


Effect of NSP01 (1 h pre-treatment) on neuron survival after glutamate injuries (40 µmol/L, 20 min)

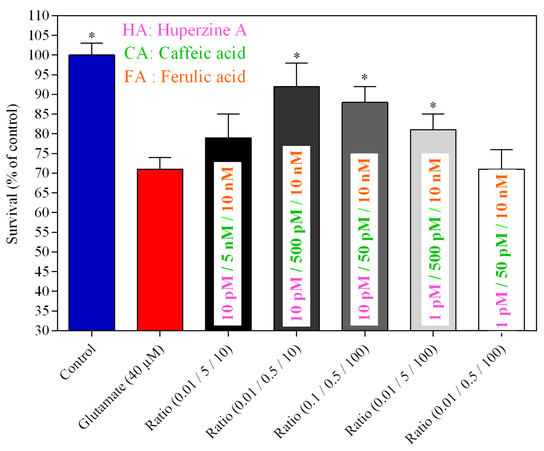


Discovery of synergistic effect of three compounds identified in TE and NSP01 extracts

Effect of HA/CA/FA (1 h pre-treatment) on neuron survival after glutamate injuries (40 µmol/L, 20 min)



Effect of mix of compounds (HA+CA+FA, 1 h pre-treatment) on neuron survival after glutamate injuries (40 µmol/L, 20 min)



Mix compounds (at single compound at non active concentrations) displayed a significant neuronal protection.

M stands for mol/L
* $p < 0.05$ *vs.* glutamate (one way ANOVA followed by Fisher's test)

↓ Non active concentrations of single compound on neuron survival

CONCLUSIONS

These results showed the similarity of chromatographical profile and of the content of active compound (HA) of NSP01 *vs.* TE.
The pharmacological activity of NSP01 lead to the discovery of synergistic activity between three compounds: Huperzine A, Caffeic acid and Ferulic acid. The active dose of Huperzine A was decreased from 1 nmol/L for a single compound to 10 pmol/L in a mix of three compounds. This synergistic activity should allow to decrease cholinergic side effects of high doses of Huperzine A.

Neuro-Sys is currently developing NSP01 as a food supplement for 2019.

References
[1] X. Ma, C. Tan, D. Zhu, et D. R. Gang, « A survey of potential huperzine A natural resources in China: The Huperziaceae », *J. Ethnopharmacol.*, vol. 104, no 1-2, p. 54-67, March 2006.
[2] N. N. Chuong, B. H. Trung, T. C. Luan, T. M. Hung, N. H. Dang, et N. T. Dat, « Anti-amnesic effect of alkaloid fraction from Lycopodiella cernua (L.) Pic. Serm. on scopolamine-induced memory impairment in mice », *Neurosci.* Lett., vol. 575, p. 42-46, July. 2014.
[3] D. Cuthbertson, J. Piljac-Žegarac, et B. M. Lange, « Validation of a microscale extraction and high-throughput UHPLC-QTOF-MS analysis method for huperzine A in Huperzia: Microscale UHPLC-MS analysis of huperzine A », *Biomed. Chromatogr.*, vol. 26, no 10, p. 1191-1195, Oct. 2012.
[4] M. G. Vallejo, J. A. Dimmer, M. G. Ortega, J. L. Cabrera, et A. M. Agnese, « Amino acid content and acetylcholinesterase inhibition of *Huperzia saururus* infusion and decoction », *Pharm. Biol.*, vol. 51, no 10, p. 1341-1345, Oct. 2013.
[5] Liu, J.S., Zhu, Y.L., Yu, C.M., Zhou, Y.Z., Han, Y.Y., Wu, F.W., Qi, B.F., « The structures of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity », *Canadian Journal of Chemistry 64*, 1986.
[6] X. Ma, C. Tan, D. Zhu, et D. R. Gang, « A survey of potential huperzine A natural resources in China: The Huperziaceae », *J. Ethnopharmacol.*, vol. 104, no 1-2, p. 54-67, March 2006.
[7] B. Wang, H. Wang, Z. Wei, Y. Song, L. Zhang, et H. Chen, « Efficacy and safety of natural acetylcholinesterase inhibitor huperzine A in the treatment of Alzheimer's disease: an updated meta-analysis », *J. Neural Transm.*, vol. 116, no 4, p. 457-465, Apr. 2009.
[8] N. Callizot, « Combination composition comprising Huperzine », EP3106160A1, 15-June-2015.
[9] N. Callizot, M. Combes, R. Steinschneider, et P. Poindron, « Operational dissection of β-amyloid cytopathic effects on cultured neurons », J. Neurosci. Res., vol. 91, no 5, p. 706-716, March 2013.