Final scientific report

Project title: **Antiaggregation potential of 6-hydroxy-L-nicotine from *Paenarthrobacter nicotinovorans* pAO1 against amyloid peptide: in vitro and in vivo studies**
Contract no.: **PCE 49/2022**
Project code: **PN-III-P4-PCE-2021-1692**
Project manager: **Prof. univ. dr. habil. Lucian HRIȚCU**
Time frame: **02/06/2022 – 31/12/2024**

1. Planned/Achieved Objectives.

The research project aimed to achieve and accomplish 3 main objectives:

1. In vitro evaluation of the anti-aggregation potential of 6HLN against Aβ1-42. This objective was achieved, demonstrating that 6HLN exhibits a high anti-aggregation and disaggregation potential against mature Aβ1-42 fibrils in A549 (human lung carcinoma) and MCF7 (human breast carcinoma) cell lines. Furthermore, the docking results obtained during the project suggest that 6HLN could bind to Aβ1-42 peptides with an affinity similar to or stronger than that of nicotine, thus potentially preventing their aggregation. The production of 6HLN was carried out using biotechnology based on *Paenarthrobacter nicotinovorans*.

2. In vivo evaluation of the anti-aggregation potential of 6HLN against Aβ1-42. This objective was achieved, with 6HLN demonstrating anti-aggregation and anti-apoptotic effects in hippocampal homogenates from 5xFAD transgenic mice. Moreover, the activation of nicotinic cholinergic receptors by 6HLN, along with the reduction of Aβ1-42 levels, led to enhanced spatial memory processes, as evaluated by the Y-maze test (for short-term memory) and the radial arm-maze test (for working and reference memory). Additionally, a decrease in anxiety response (in the elevated plus-maze test) and depressive response (in the forced swimming test) was observed as a result of the reduced Aβ1-42 levels in the hippocampus of 5xFAD transgenic mice. Alongside cognitive enhancement, 6HLN administration was found to reduce neuroinflammation (decreased levels of TLR4, NF-κB, TNF-α, and IL6), restore antioxidant status (increased specific activities of antioxidant enzymes – SOD, GPX, and CAT), raise reduced GSH levels, and lower lipid peroxidation (MDA) and protein oxidation (carbonylated proteins) in hippocampal homogenates of 5xFAD transgenic mice. The improvement in memory processes following 6HLN administration in 5xFAD transgenic mice can be associated with the restoration of cholinergic system activity (reduced specific activity of AChE), decreased Aβ1-42 levels, reduced neuroinflammatory response, and oxidative stress in the hippocampus.

3. Dissemination of results at conferences and publication of articles. The objective was achieved. The group members participated in 17 conferences (13 international and 4 national), giving 17 oral presentations and 5 posters. The project results led to the publication of 3 articles indexed in the Web of Science in the red zone (Q1).

Details regarding the planned objectives, associated activities, as well as verifiable estimated results of the activities are presented in Table 1 below:

Planned activities	**Degree of completion**	**Verifiable Estimated Results of the Activity**
Stage 1 (02/06/2022 – 31/12/2022) – In vitro evaluation of the anti-aggregation potential of 6HLN against Aβ1-42; *Verifiable Estimated Results of the Activity*: Data on the in vitro anti-aggregation properties of 6HLN against Aβ1-42; project website; scientific report after the stage.		
Act 1.1. Purchases: reagents,	Total	The reagents, consumables, and cell lines

consumables, cell lines.		required for the project's activities have been purchased.
Act 1.2 - Evaluation of the 6HLN-nAChRs-Aβ1-42 interactions through molecular docking studies	Total	Results are presented in Fig. 1 A, B, and C.
Act 1.3 - Production of 6HLN using biotechnology based on *Paenarthrobacter nicotinovorans*.	Total	Results are presented in Fig. 2A and B.
Act 1.4 - Evaluation of the anti-aggregation and disaggregation potential of 6HLN against mature Aβ1-42 fibrils.	Total	Data on the *in vitro* anti-aggregation properties of 6HLN against Aβ1-42 are presented in Fig. 3A-F and Fig. 4A-F.
Act 1.5 - Evaluation of the Aβ1-42 levels in cell lines under the action of 6HLN.	Total	Results are presented in Fig. 3A-F and Fig. 4A-F.
Act 1.6 - Analysis of experimental data and evaluation of the scientific literature (part 1).	Total	The activity is carried out continuously to adapt protocols and assess the data quality.
Act 1.7 - Dissemination of the obtained results (part 1).	Total	Lucian Hritcu participated in 3 conferences (2 international and 1 national) with oral presentations. The project website has been created: http://cercetare.bio.uaic.ro/grupuri/bioactive/content/grants/PCE2022_hl.html
Stage 2 (01/01/2023 – 31/12/2023) – In vivo evaluation of the anti-aggregation potential of 6HLN against Aβ1-42. In vivo evaluation of 6HLN against Aβ1-42-induced cytotoxicity. In vivo evaluation of 6HLN against Aβ1-42-induced memory deficits; *Verifiable Estimated Results of the Activity*: Data on the aggregated forms of Aβ1-42 in the brain after exposure to 6HLN; data on Aβ1-42-induced cytotoxicity in the brain of 5xFAD transgenic mice after exposure to 6HLN; data on memory deficits induced by Aβ1-42 after exposure to 6HLN; 1 scientific article; participation in conferences (1 national and 1 international); involvement in 1 workshop; scientific report after the stage.		
Act 2.1. Purchases: reagents, consumables, 5xFAD transgenic mice, 4 months old, Jackson Laboratories, USA (part 1).	Total	The 5xFAD transgenic mice, reagents, and consumables required for the activities of Stage 2 have been purchased.
Act 2.2 – Quantification of Aβ1-42 levels in hippocampal homogenates from mice after exposure to 6HLN.	Total	Data on the aggregated forms of Aβ1-42 in the brain after exposure to 6HLN are presented in Fig. 5.
Act 2.3 – Evaluation of the anti-apoptotic activity of 6HLN in hippocampal homogenates from mice using the DNA fragmentation method.	Total	Data on the Aβ1-42-induced cytotoxicity in the brains of 5xFAD transgenic mice after exposure to 6HLN are presented in Fig. 6.
Act 2.4 - Evaluation of short-term memory in the Y-maze test.	Total	Data on the memory deficits induced by Aβ1-42 after exposure to 6HLN are presented in Fig. 7A-B.
Act 2.5 - Evaluation of working and reference memory in the radial arm-maze test.	Total	Data on the memory deficits induced by Aβ1-42 after exposure to 6HLN are presented in Fig. 8A-B.
Act 2.6 - Evaluation of anxious behavior in the elevated plus-maze test.	Total	Data on the memory deficits induced by Aβ1-42 after exposure to 6HLN are presented in Fig. 9 A-C.

Act 2.7 - Evaluation of depressive behavior in the forced swimming test.	Total	Data on the memory deficits induced by Aβ1-42 after exposure to 6HLN are presented in Fig. 10 A-B.
Act 2.8 - Analysis of experimental data and evaluation of the scientific literature (part 2).	Total	The activity is carried out continuously to adapt working protocols and assess the data quality.
Act 2.9 - Dissemination of the obtained results (part 2).	Total	- 1 scientific article has been published in *Phytotherapy Research* (Q1, IF 7.2, AIS 0.841) (https://pubmed.ncbi.nlm.nih.gov/36760217/) - Lucian Hritcu, Razvan Ștefan Boiangiu, and Iasmina Honceriu participated in 6 international conferences (FEBS, IBRO, ICPNU, 7-ISPMF, TBS, FEBS-IUBMB) and 2 national conferences (ICON, CONFER). Lucian Hritcu and Iasmina Honceriu participated in 3 international workshops. The results and activities of the project are periodically posted on the project website:http://cercetare.bio.uaic.ro/grupuri/bioactive/content/grants/PCE2022_hl.html
Activities completed ahead of schedule in Stage 2 (2023)		
Act 3.1 - Purchases: 5xFAD transgenic mice, 4 months old, Jackson Laboratories, USA (part 2).	Total	The 5xFAD transgenic mice were purchased in Stage 2.
Stage 3 (01/01/2024 – 31/12/2024) – *In vivo* evaluation of 6HLN against Aβ1-42-induced neuroinflammation. In vivo evaluation of 6HLN against Aβ1-42-induced oxidative stress. *Verifiable Estimated Results of the Activity*: Data on Aβ1-42-induced neuroinflammation in the hippocampus of 5xFAD transgenic mice after exposure to 6HLN; data on Aβ1-42-induced oxidative stress in the hippocampus of 5xFAD transgenic mice after exposure to 6HLN; 1 scientific article; participation in conferences (1 national and 1 international); participation in 1 workshop; scientific report after the project.		
Act 3.2 - Immunostaining for GFAP and IBA1 for astrocytes and microglia.	Total	The results are presented in Fig. 11 A-D.
Act 3.3 - Evaluation of the levels of inflammatory markers: TLR4, NF-κβ, TNF-α, and IL6 in hippocampal homogenates from mice after exposure to 6HLN.	Total	Data on Aβ1-42-induced neuroinflammation in the hippocampus of 5xFAD transgenic mice after exposure to 6HLN are presented in Fig. 12 A-D.
Act 3.4 - Evaluation of the specific activities of acetylcholinesterase (AChE) and antioxidant enzymes (SOD, GPX, CAT), and the total reduced GSH content in hippocampal homogenates from mice after exposure to 6HLN.	Total	Data on Aβ1-42-induced oxidative stress in the hippocampus of 5xFAD transgenic mice after exposure to 6HLN are presented in Fig. 13 A-E.
Act 3.5 - Evaluation of MDA levels (lipid peroxidation) and carbonylated proteins (protein oxidation) in	Total	Data on Aβ1-42-induced oxidative stress in the hippocampus of 5xFAD transgenic mice after exposure to 6HLN are

hippocampal homogenates from mice after exposure to 6HLN.		presented in Fig. 14A-B.
Act 3.6 - Analysis of experimental data and evaluation of scientific literature (part 3).	Total	The activity is carried out continuously to adapt working protocols and assess the data quality.
Act 3.7 - Dissemination of the obtained results (part 3).	Total	- 1 scientific article has been published in *Biomolecules* (Q1, IF 4,8, AIS 1,047) (https://pubmed.ncbi.nlm.nih.gov/38254623/) - Lucian Hritcu, Răzvan Ștefan Boiangiu, Ion Brînza, Paula Alexandra Postu, and Marius Mihășan participated in 4 international conferences (FEBS, 8-ISPMF, TBS) and 2 national conferences (UAIC, CONFER). - Lucian Hritcu participated in 1 international workshop. The results and activities of the project are periodically posted on the project website."http://cercetare.bio.uaic.ro/grupuri/bioactive/content/grants/PCE2022_hl.html

Project Results Dissemination Approach

At the end of the project, the generated results were presented as follows:
- **Stage 1 (2022)** – 2 international conferences and 1 national conference with oral presentations delivered by the project director.
- **Stage 2 (2023)** – 6 international conferences and 2 national conferences with 9 oral presentations and 2 posters presented by team members; 1 article indexed in Web of Science in *Phytotherapy Research* (Q1, IF 7.2, AIS 0.841); participation of team members in 3 international workshops.
- **Stage 3 (2024)** – 4 international conferences and 2 national conferences with 5 oral presentations and 1 poster presented by team members; 1 article indexed in Web of Science in *Biomolecules* (Q1, IF 4.8, AIS 1.047); participation of the project director in 1 international workshop.

The results and activities of the project were periodically posted on the project website: http://cercetare.bio.uaic.ro/grupuri/bioactive/content/grants/PCE2022_hl.html

2. Presentation of the results obtained, of the achieved result indicators; of the non-achievements recorded against the results estimated by the funding request (if applicable), with their justification.

A. Presentation of the obtained results

A.1. *In vitro* evaluation of the antiaggregating potential of 6HLN against Aβ1-42.

Evaluation of 6HLN-nAChRs-Aβ1-42 interactions by molecular docking studies. Within the project, a series of *in silico* molecular docking simulations were performed to analyze the interaction of 6HLN with different Aβ peptides and to define a possible mechanism that could explain the effect of 6HLN on Aβ peptide aggregation. Thus, the binding potential of 6HLN to the following Aβ peptides was evaluated: Aβ1-42, Aβ1-40 and Aβ25-35. Nicotine, the 6HLN precursor, and the reference compound

were also included in the study as several studies have shown that nicotine interacts with Aβ and prevents its aggregation (**Fig.1**).

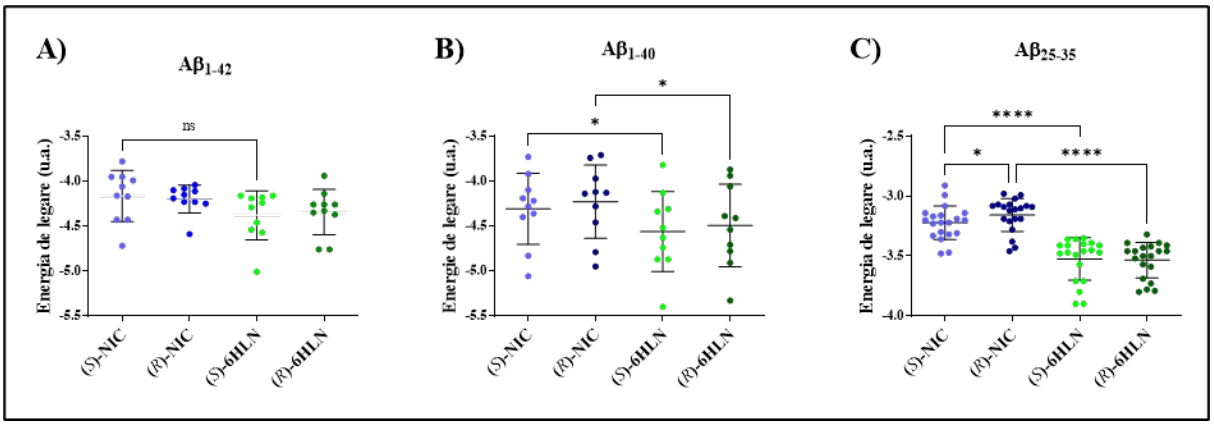

Fig. 1. Binding potential of nicotine (NIC) and 6-hydroxy-L-nicotine (6HLN) to Aβ1-42 **(A)**, Aβ1-40 **(B)**, and Aβ25-35 **(C)** receptors. Values are expressed as means ± S.E.M (n=10 conformations/receptor for Aβ1-42 and Aβ1-40 and n=20 conformations/receptor for Aβ25-35). ANOVA analysis identified overall significant differences for **B)** $F(1.693; 15.23)=10.44$; $p=0.002$ and **C)** $F(1.593; 30.26)=101.9$; $p<0.0001$. For Tukey post hoc analyses – **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns – non-significant.

The 3D structures of the receptors were elucidated by NMR and 10 conformations for the Aβ1-42 and Aβ1-40 peptides and 20 conformations for the Aβ25-35 receptor were downloaded from the PDB database. The ligands used were docked in all receptor conformations. Thus, the corresponding binding energy was calculated for each ligand-receptor complex. A low binding energy suggests a high affinity of the ligand for the receptor. The obtained results showed that (S)-6HLN presents binding energy similar to that of (S)-nicotine, thus suggesting a similar affinity of the two compounds to the Aβ1-42 peptide (**Fig. 1A**). Regarding the Aβ1-40 peptide, 6HLN showed a higher affinity for the receptor compared to its precursor, as the binding energies of (S)-6HLN were significantly lower ($p<0.05$) than those of (S)-NIC. This trend was also observed in the case of the (R) enantiomers of the two ligands (**Fig. 1B**). Eloquent results were also obtained when the ligands were docked to the Aβ25-35 peptide. As can be seen in **Fig. 1C**, the binding energies of (S)- and (R)-6HLN are significantly lower ($p<0.0001$) than those of (S)- and (R)-NIC, respectively. This denotes a stronger affinity of 6HLN for the Aβ25-35 peptide compared to its precursor. Finally, analyzing **Fig. 1**, we can see that 6HLN attaches to Aβ peptides with similar or higher affinity than NIC and therefore could prevent their aggregation.

Production of 6HLN using biotechnology based on *Paenarthrobacter nicotinovorans*. After approximately 27 hours of culturing the *P. nicotinovorans* strain on a nicotine-supplemented citrate medium, a sample of the culture medium was collected and injected into the HPLC system for 6HLN identification. According to the obtained chromatogram (**Fig. 2**), 6HLN was identified by the absorption spectra at 2.5 min after elution and its amount in the culture medium was reasonable. Thus, the culture was stopped and used for the extraction of the 6HLN product. Bacterial cells were removed from the culture medium by centrifugation. The supernatant (culture medium) was concentrated by rotary evaporation under reduced pressure and the product 6HLN was extracted with equal volumes of dichloromethane. The organic solvent was subsequently removed by evaporation, and the purity and yield of the product were determined by HPLC. As can be seen in **Fig. 2**, 6HLN was extracted from the culture medium of the *P. nicotinovorans* strain, the peak corresponding to the product being detected after 2.4 min of elution and confirmed by absorption spectra. In terms of purity, the preparation is contaminated with another nicotinic derivative, namely the blue pigment. At 6 min after elution, a peak at 357 nm corresponding to the blue pigment

produced by the bacterium was identified. Initially, the peak corresponding to the blue pigment is identified after 10 min and at 585 nm. However, after correcting the pH of the culture medium in the extraction process, the blue pigment changes its color and therefore also changes its retention time and absorption spectrum. Using 6HLN standards, the product yield was calculated. Thus, an amount of ~30 mg of 6HLN was recovered from 2 L of culture medium supplemented with 1000 mg of nicotine resulting in a 3% yield.

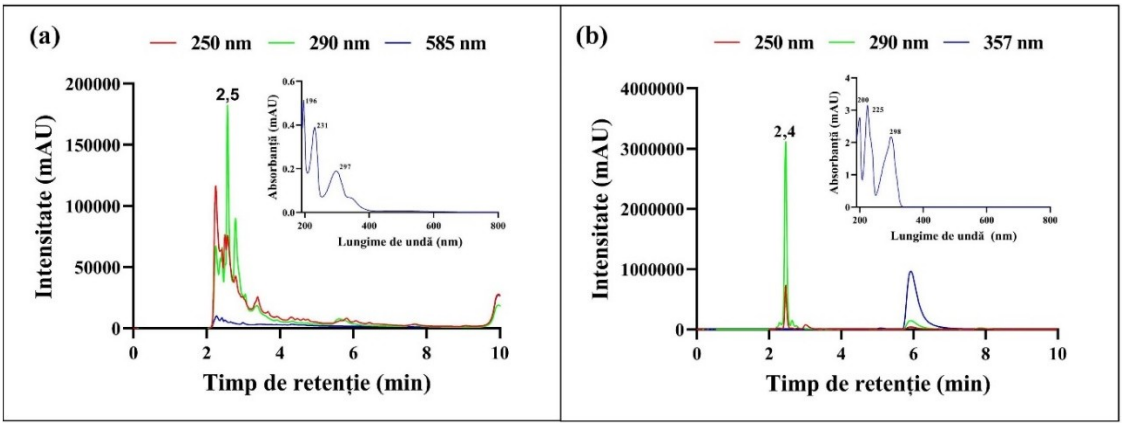

Fig. 2. HPLC analysis of the culture medium of the *P. nicotinovorans* strain after 27 h of cultivation (**a**) and 6HLN purified from the culture medium (**b**). The retention time of 6HLN is 2.5 ± 0.1 min. The absorption spectra of 6HLN are illustrated in the background of the graphs.

6HLN Evaluation of the antiaggregating and disaggregation potential of 6HLN against mature Aβ1-42 fibrils. 6HLN and Aβ1-42 were administered to A549 (human lung carcinoma) and MCF7 (human breast carcinoma) cell lines. Aβ1-42 can internalize these cell lines. 6HLN was administered at 3 concentrations (25 nM, 100 nM and 1000 nM), and Aβ1-42 was administered at 2 concentrations (1μM and 5μM). Aβ1-42 was used in both the monomeric and fibrillar states and subsequently incubated for 5 days at 37°C. Aβ1-42 aggregation was monitored using the thioflavin T assay. To monitor only intracellular Aβ1-42 aggregation, at the set monitoring intervals (24 h, 48 h, and 72 h), the incubation medium of cells containing Aβ1-42 was removed, a washing step was performed to ensure the effective removal of Aβ1-42 that was not internalized by the cells. Determination of thioflavin fluorescence intensity was performed in a cell growth medium without FBS and phenol red, and background noise caused by thioflavin was removed. Thus, at all monitoring intervals, a lower intensity of thioflavin was observed in cells incubated with both 6HLN and Aβ1-42 in monomeric form compared to the intensity of thioflavin corresponding to cells incubated only with Aβ1-42, an aspect correlated with the ability of 6HLN to inhibits the aggregation behavior of Aβ1-42.

At 24 h incubation, in A549 cells, all 3 concentrations of 6HLN significantly inhibited Aβ1-42 aggregation, but only when Aβ1-42 was used at 1 μM concentration (**Fig. 3A**). Similar effects were also observed in MCF7 cells, but only 6HLN at 100 nM and 1000 nM concentrations significantly inhibited Aβ1-42 aggregation at 1 μM concentration (**Fig. 3D**).

At 48 h of incubation, in A549 cells, 6HLN at concentrations of 100 nM and 1000 nM significantly inhibited the aggregation of 1 μM and 5 μM Aβ1-42, and 25 nM 6HLN significantly reduced only the aggregation ability of 1 μM Aβ1-42 (**Fig. 3B**). In MCF7 cells, only 6HLN 1000nM had a significant inhibitory effect on 1 μM and 5 μM Aβ1-42 aggregation, and 100 nM 6HLN significantly inhibited only 1μM Aβ1-42 aggregation (**Fig. 3E**).

At 72 h of incubation, in A549 cells, only 6HLN 1000 nM maintained its inhibitory effects on 1 μM and 5 μM Aβ1-42 aggregation, with 100 nM 6HLN inhibiting only 1μM Aβ1-42 aggregation (**Fig. 3C**). Alternatively, in MCF7 cells, the aggregation capacity of 1 μM Aβ1-42 is significantly reduced by 6HLN at 25 nM and 100 nM concentrations, whereas the aggregation capacity of 5μM Aβ1-42 is significantly diminished by 100 nM 6HLN (**Fig. 3F**).

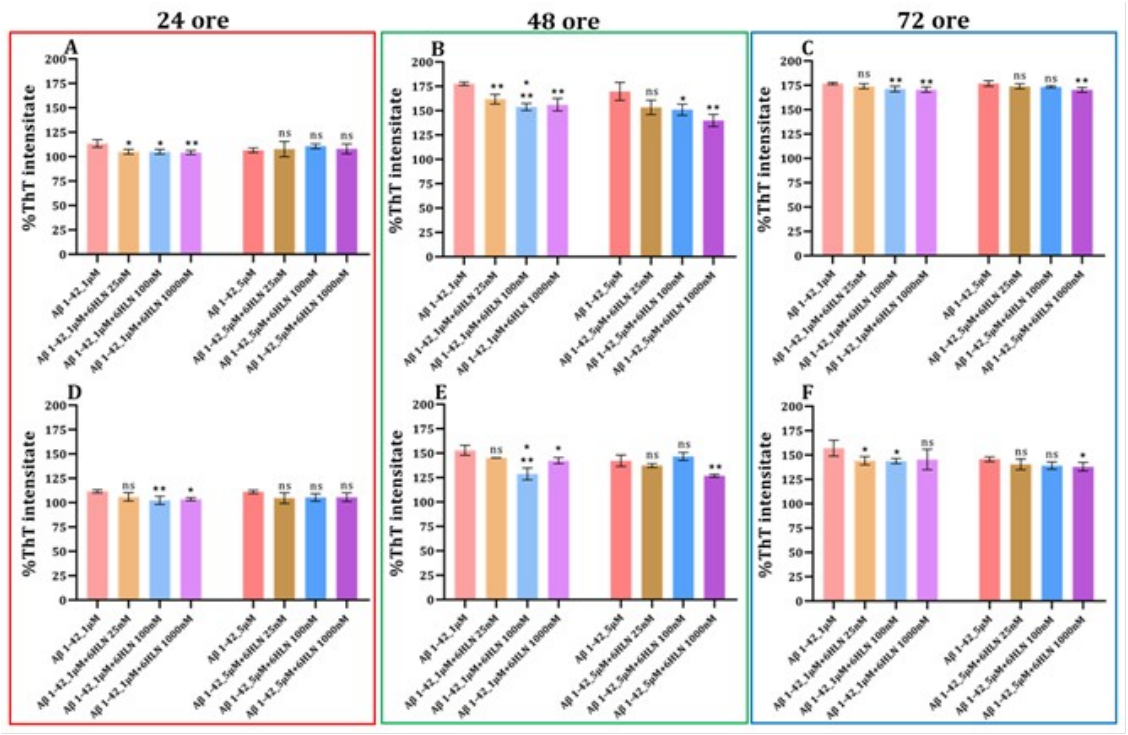

Fig. 3. Effects of 6HLN on the aggregation of Aβ1-42 used in the monomeric state in the A549 cell line (A, B, C) and the MCF7 cell line (D, E, F).

Although 6HLN can significantly influence the aggregation behavior of Aβ1-42 used in the monomeric state, it has little effect on Aβ1-42 used in the fibrillar state, with 6HLN causing weak disaggregation of Aβ1-42 fibrils (**Fig. 4**). Thus, only in A549 cells, significant disaggregation of Aβ1-42 was observed only at 24 h of incubation of 1 μM Aβ1-42 with 25 nM and 100 nM 6HLN (**Fig. 4A**).

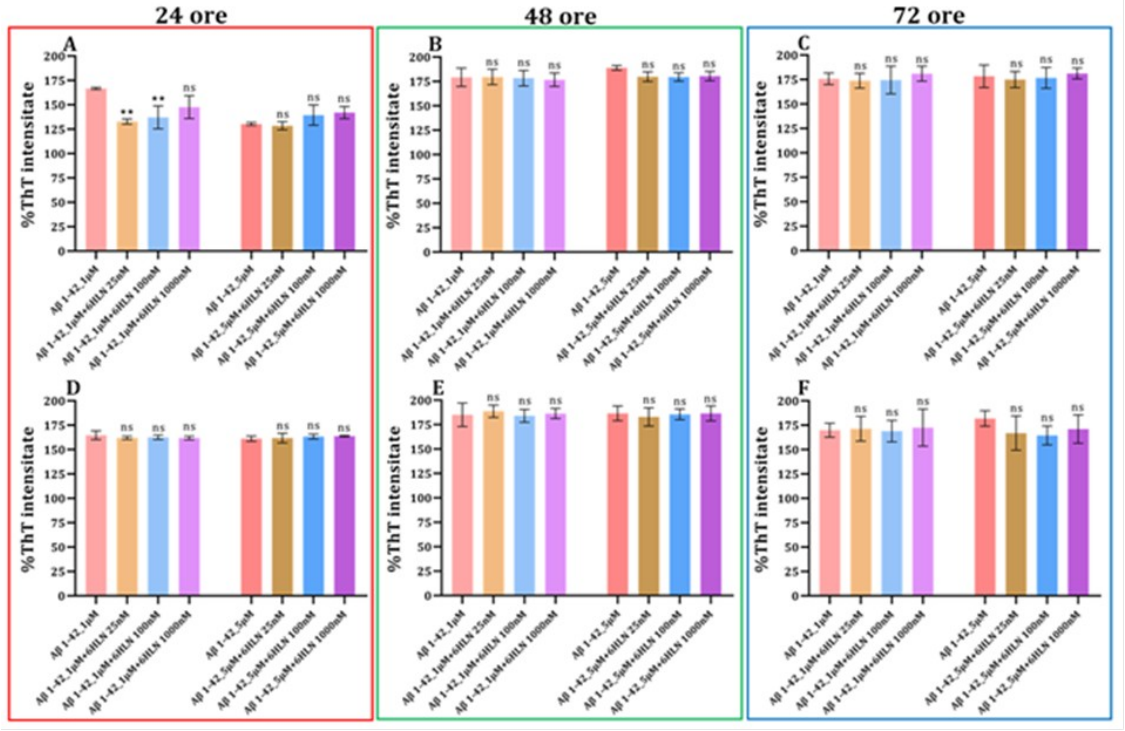

Fig. 4. Effects of 6HLN on the disaggregation process of Aβ1-42 used in the fibrillar state in the A549 cell line (A, B, C) and the MCF7 cell line (D, E, F).

A.2. Evaluarea *in vivo* a potențialului de antiagregare al 6HLN împotriva Aβ1-42.

Quantification of Aβ1-42 level in hippocampal homogenates from 5xFAD transgenic mice after exposure to 6HLN. The 5xFAD transgenic mice used in this project were purchased from the NIH-supported Mutant Mouse Resource and Research Center (MMRRC), Jackson Laboratory, USA, and were donated to the MMRRC by Dr. Robert Vassar, Northwestern University, USA. 5xFAD transgenic mice overexpress human protein APP695 with three Swedish (K670N and M671L), Florida (I716V) and London (V717I) mutations and human PS1 protein with two mutations M146L and L286V. These mice significantly accumulate Aβ plaques in the brain at 2 months of age and their cognitive function is impaired at 4 months of age. Mouse Aβ42 ELISA Kit obtained from Antibodies-online GmbH (Aachen, Germany) was used to quantify the level of Aβ1-42 in the hippocampus. All experimental procedures were carried out by the manufacturer's instructions and a method described in the work reported in stage 2 of the project (El Sayed..(**Hritcu L**.).. et al., 2023, *Phytother Res*., 37(6):2437-2453, Q1, IF 7.2). Analyzes performed on homogenates from the hippocampi of 5xFAD animals revealed a significant increase in the level of Aβ1-42 ($p<0.0001$) (Fig. 5) compared to control animals [$F(3,16)=26.13$, $p<0 .0001$]. Administration of 6HLN (0.3 and 0.6 mg/kg, g.c., i.p.) in 5xFAD animals revealed significant decreases in Aβ1-42 level ($p<0.0001$ for 0.3 mg/kg and 0.6 mg/ kg) thus suggesting neuroprotective effects.

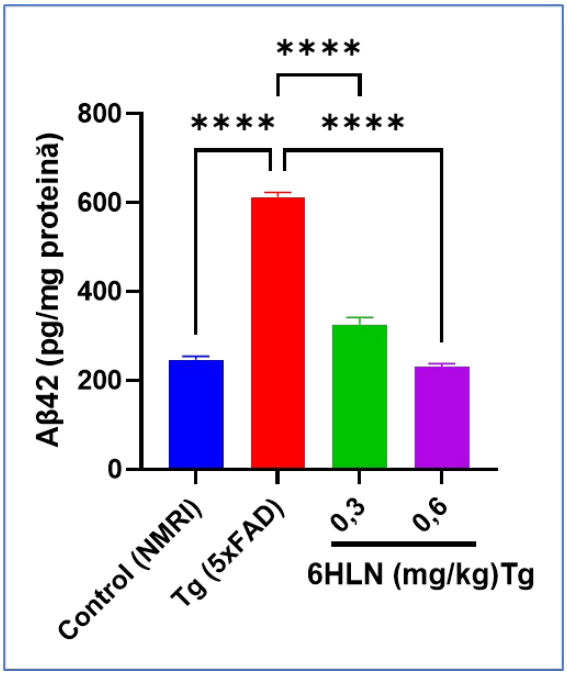

Fig. 5. Effects of 6-hydroxy-L-nicotine administration (6HLN, 0.3 and 0.6 mg/kg, bw, i.p.) on the level of Aβ1-42 in the hippocampi of NMRI mice and 5xFAD transgenic mice. Results are expressed as means ± E.S.M. (n=5), using one-way ANOVA followed by Tukey's post hoc test ($p<0.0001$ for 0.3 mg/kg and 0.6 mg/kg).administrării 6-hidroxi-L-nicotinei (6HLN, 0,3 și 0,6 mg/kg, bw, i.p.).

Evaluation of the antiapoptotic activity of 6HLN in mouse hippocampal homogenates by the DNA fragmentation method. Determination of histone-associated DNA fragments as an indicator of apoptosis was performed using the Cell Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions and a procedure previously described by Postu et al., 2018, *J. Cell . Mol. Med*. 22, 111–122. By using the one-way ANOVA test, significant differences were found between groups [$F(3,16)=37.29$, $p<0.0001$] demonstrated by the enrichment factor (EF) value. The determination of DNA fragmentation as an indicator of apoptosis demonstrated a significant increase in the enrichment factor value in the Tg group (5xFAD) compared

to the control group (p<0.00001). In addition, administration of 6HLN (0.3 and 0.6 mg/kg, g.c., i.p.) in 5xFAD animals demonstrated a significant reduction in the enrichment factor value compared to the Tg group (5xFAD), in a dose-dependent manner, suggesting - thus 6HLN exhibits antiapoptotic and anticytotoxic effects (**Fig. 6**).

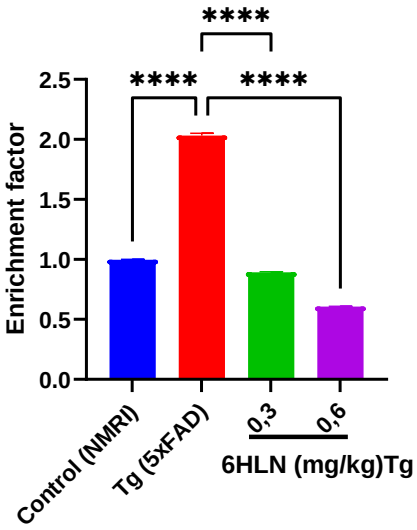

Fig. 6. Effects of 6-hydroxy-L-nicotine administration (6HLN, 0.3 and 0.6 mg/kg, bw, i.p.) on apoptosis in the hippocampus of NMRI mice and 5xFAD transgenic mice assessed by factor enrichment. Results are expressed as means ± E.S.M. (n=5), using one-way ANOVA followed by Tukey's post hoc test (p<0.00001 for 0.3 mg/kg and 0.6 mg/kg).

Assessment of spatial memory in the Y-maze and radial arm-maze tests. The Y-maze test was performed to assess the effects of 6HLN on short-term memory. In this study, administration of 6HLN stimulated short-term memory, assessed by significantly increasing the percentage of spontaneous alternation, especially at the dose of 0.6 mg/kg compared to the control group (p<0.001). Moreover, the percentage of spontaneous alternation showed significant decreases in the Tg (5xFAD) group compared to the NMRI control group (p<0.0001). Administration of 6HLN in Tg (5xFAD) groups resulted in significant stimulation of short-term memory, evidenced by a significant increase in the percentage of spontaneous alternation in both administered doses (**Fig. 7A**). Regarding locomotor activity, assessed by the number of arm entries (**Fig. 7B**), no significant differences were reported between groups, suggesting that the improvement in cognitive performance in the Y-maze test is not the result of increased locomotor activity (result shame-positive).

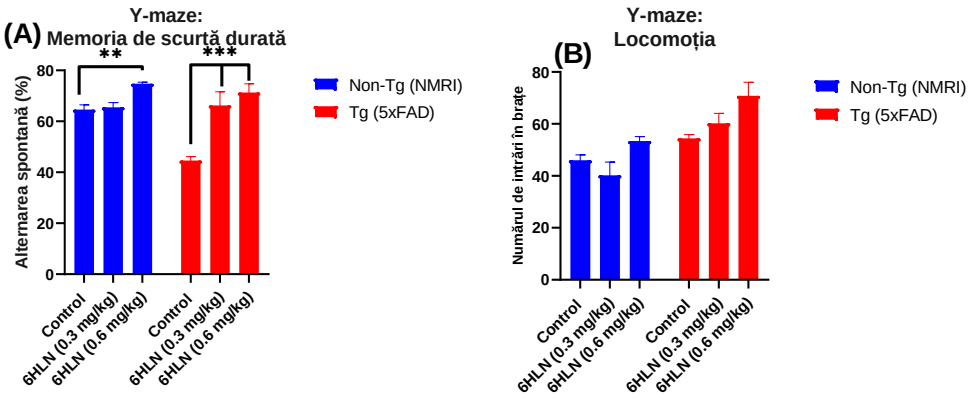

Fig. 7. Effects of 6-hydroxy-L-nicotine administration (6HLN, 0.3 and 0.6 mg/kg, bw, i.p.) on short-term memory (A. Spontaneous alternation%) and locomotor activity (B. Number of arm entries) in the Y-maze test. NMRI mice and transgenic Tg (5xFAD) mice were used. Results are expressed as means ± E.S.M. (n=5), using the two-way ANOVA test followed by Tukey's post hoc test (**p<0.001 and ***p<0.0001).

In the radial arm-maze test, a decrease in the number of working memory errors was found, especially in the group treated with 6HLN 0.6 mg/kg compared to the control group, thus indicating an improvement in working memory (a form of short-term memory) (**Fig. 8A**). Also, the number of working memory errors decreased in the Tg (5xFAD) group compared to the NMRI control group. The administration of 6HLN in the Tg groups (5xFAD) determined the improvement of working memory, evidenced by the significant decrease in the number of working memory errors at the doses of 6HLN used, but especially at the dose of 0.6 mg/kg (p<0.001) compared to the Tg group (5xFAD). The number of reference memory errors decreased slightly in the 6HLN 0.6 mg/kg group, suggesting an improvement in reference memory (a form of long-term memory) (**Fig. 8B**). A significant decrease (p<0.001) in the number of reference memory errors was revealed in the Tg (5xFAD) group pretreated with 6HLN 0.6 mg/kg compared to the Tg (5xFAD) group.

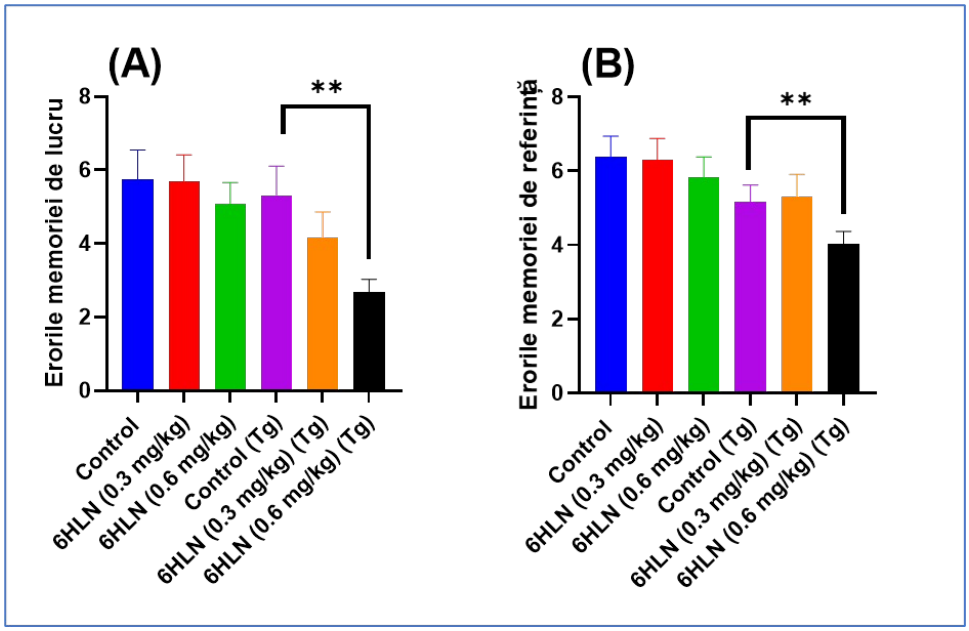

Fig. 8. Effects of 6-hydroxy-L-nicotine (6HLN, 0.3 and 0.6 mg/kg, bw, i.p.) administration on working memory and reference memory performance in the radial arm-maze test. A. Working memory errors; B. Reference memory errors. NMRI mice and transgenic Tg (5xFAD) mice were used. Results are expressed as means ± E.S.M. (n=5), using the two-way ANOVA test followed by the post hoc Tukey test (**p<0.001).

Evaluation of the anxious (elevated plus-maze test) and depressive (forced swimming test) response. In Fig. 9A, administration of 6HLN at both doses resulted in a significant increase in time spent in the open arms (p<0.0001) compared to the control group. Furthermore, the 5xFAD group of mice spent less time in the open arms, suggesting anxious behavior. Administration of 6HLN at both doses in 5xFAD mice significantly increased the time spent in the open arms (p<0.0001) compared to the 5xFAD group. The number of entries into the open arms (**Fig. 9B**) was significantly increased in the 6HLN-treated groups compared to the control group (p<0.0001). 5xFAD mice exhibited anxious behavior manifested by a decrease in the number of entries into the open arms, while administration of 6HLN at both doses significantly increased the number of entries into the open arms (p<0.0001) compared to the 5xFAD group. Administration of 6HLN caused a hyperlocomotor effect characterized

by a significant increase in the number of arm transitions (Fig. 9C) compared to the control group, especially at the dose of 0.6 mg/kg ($p<0.01$). In contrast, 5xFAD animals showed a hypolocomotor effect, which was attenuated by the administration of 6HLN, especially at the dose of 0.6 mg/kg ($p<0.01$), thus suggesting anxiolytic effects.

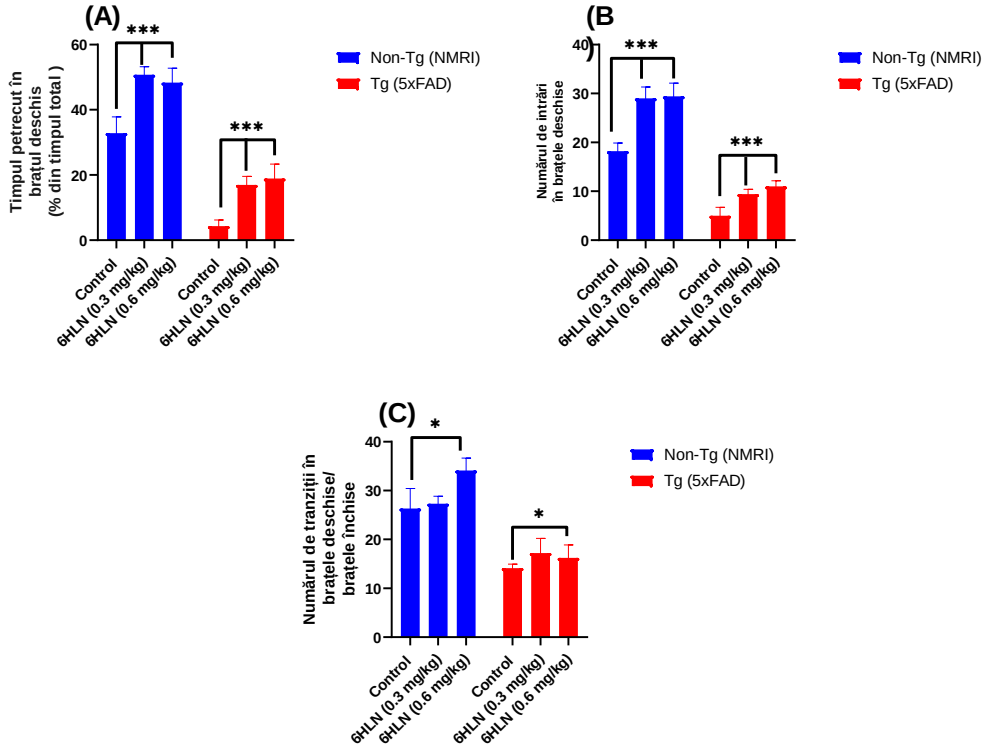

Fig. 9. Effects of 6-hydroxy-L-nicotine (6HLN, 0.3 and 0.6 mg/kg, bw, i.p.) administration on anxious responding in the elevated plus-maze test. (A) Time spent in open arms; (B) Number of entries into open arms; (C) Number of open arm/closed arm transitions. NMRI mice and transgenic Tg (5xFAD) mice were used. Results are expressed as means ± E.S.M. (n=5), using two-way ANOVA followed by Tukey's post hoc test (*$p<0.01$ and ***$p<0.0001$).

The results of the forced swimming test are presented in **Fig. 10**. According to Fig. **10A**, 6HLN induced antidepressant behavior, particularly at 0.6 mg/kg, as evidenced by significantly increased swimming time in both NMRI ($p<0.0001$ vs Control) and 5xFAD mice ($p<0.01$ vs 5xFAD). This effect of 6HLN paralleled the decrease in immobility time (**Fig. 10B**) in both NMRI mice ($p<0.0001$ vs Control) and 5xFAD mice ($p<0.00001$ vs 5xFAD), suggesting - it has antidepressant effects.

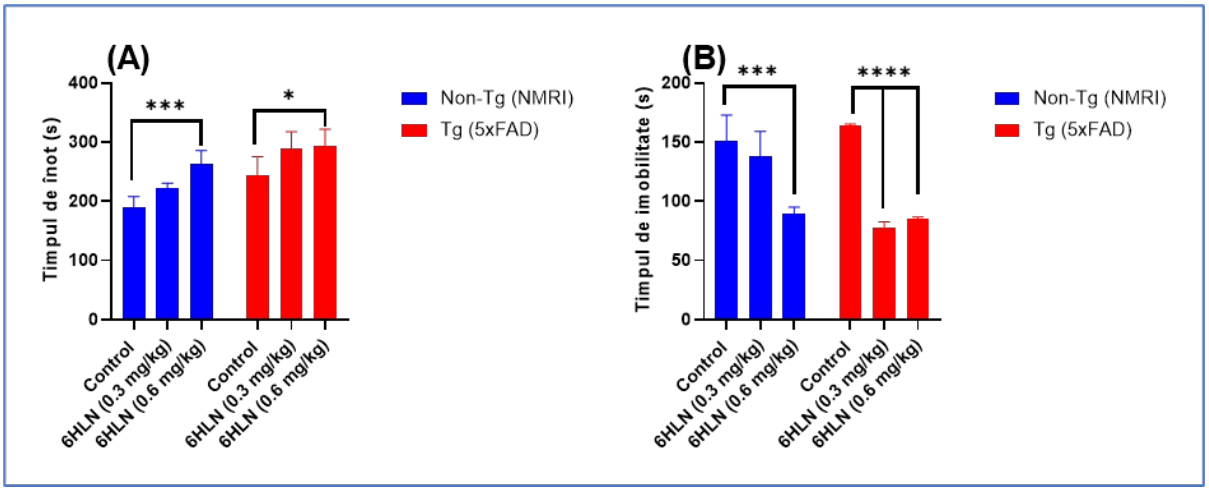

Fig. 10. Effects of 6-hydroxy-L-nicotine administration (6HLN, 0.3 and 0.6 mg/kg, bw, i.p.) on the depressive response in the forced swimming test. (A) Swimming time (s); (B) Immobility time. NMRI mice and transgenic Tg (5xFAD) mice were used. Results are expressed as means ± E.S.M. (n=5), using two-way ANOVA followed by Tukey's post hoc test (*p<0.01, ***p<0.0001 and ****p<0.00001).

A.3. *In vivo* evaluation of 6HLN against Aβ1-42-induced neuroinflammation.

Performing immunostaining for GFAP and IBA1 for astrocytes and microglia. Glial fibrillary acidic protein (GFAP) is a major protein found in astrocytes in the brain and is a marker of astrogliosis, a pathological feature of Alzheimer's disease. Inflammatory allograft factor 1 (IBA1) is a macrophage-expressed protein overexpressed in various brain pathologies, including Alzheimer's disease. To evaluate the neuroinflammatory response, immunostaining for GFAP and IBA1 was performed to stain astrocytes and microglia, and Aβ1-42 staining was performed with thioflavin S. Thus, the expression of these biomarkers was intensified in the hippocampus of 5xFAD mice, suggesting neuroinflammatory processes (astrogliosis and microgliosis), simultaneously with the reduction of the neuroinflammatory response as a result of 6HLN administration, especially at the dose of 0.6 mg/kg (**Fig. 11**).

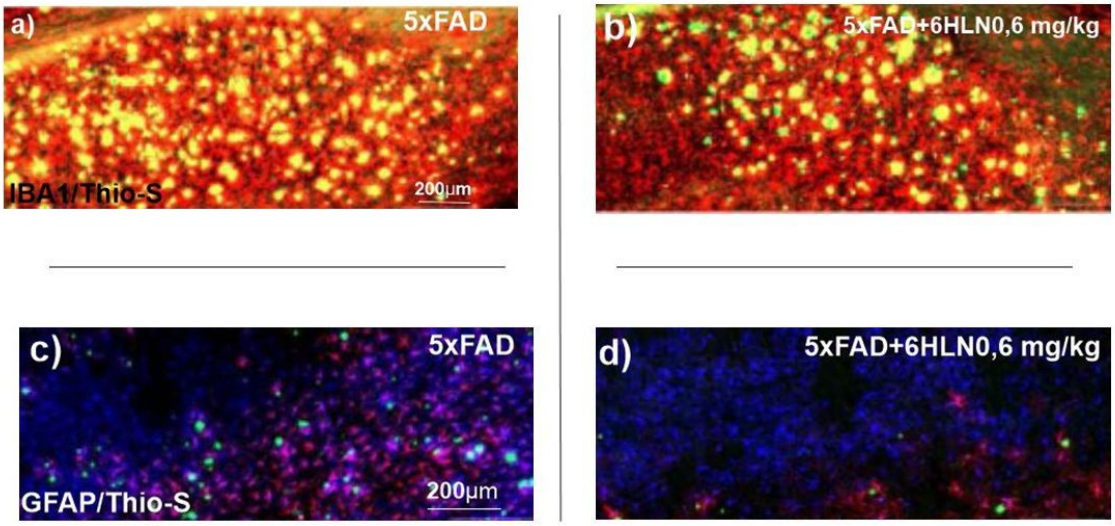

Fig. 11. Immunostaining of microglia and astrocytes. Mice brains were sectioned and immunostained for IBA1 and GFAP to highlight changes occurring in hippocampal microglia and astrocytes. (a,b) representative images from the hippocampus of 5xFAD and 5xFAd+0.6 mg/kg mice with

immunostaining for IBA1/Thio-S; (a,b) representative images from the hippocampus of 5xFAD and 5xFAd+0.6 mg/kg mice immunostained for GFAP/Thio-S.

Evaluation of the level of inflammatory markers: TLR4, NF-κβ, TNF-α, and IL6 in mice hippocampal homogenates after exposure to 6HLN. The involvement of TLR4 in Alzheimer's disease is well documented. TLR4 is upregulated in transgenic mice overexpressing amyloid precursor protein (APP). Increased immune reactivity for TLR4 was observed in glial cells surrounding plaques in the postmortem brains of Alzheimer patients. Aβ1-42 can also induce TLR4-dependent activation of microglia. In **Fig. 12A** the level of TLR4 in hippocampal homogenates of 5xFAD mice is shown. One-way ANOVA test demonstrated a significant effect of 6HLN treatment on TLR4 level. At the same time, a significant level of TLR4 was found in 5xFAD mice compared to the control group ($p<0.00001$), suggesting an intense neuroinflammatory response. In contrast, exposure of 5xFAD transgenic mice to 6HLN attenuated the neuroinflammatory response in a dose-dependent manner ($p<0.001$ for 0.3 mg/kg and $p<0.00001$ for 0.6 mg/kg).

Considering that Alzheimer's disease is characterized by cognitive decline accompanied by inflammation and neuronal loss, the effects of 6HLN treatment on the level of other inflammatory factors in hippocampal homogenates of 5xFAD mice, such as NF-κB, TNF-α, and IL6. The factor NF-κB plays an essential role in regulating the survival, activation, and differentiation of immunocytes, while dysregulation of this factor contributes to the pathogenic processes of various inflammatory diseases. Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine expressed by microglia, astrocytes, and neurons in the brain and by mononuclear cells in the peripheral blood circulation. Its production increases in several pathological conditions, including Alzheimer's disease. Activation of proinflammatory pathways in the brain, including the IL6 pathway, constitutes a potential point of convergence between memory dysfunction and metabolic changes in Alzheimer's disease.

Using the one-way ANOVA test, the significant effects of 6HLN treatment on the level of NF-κB [$F(3,8)=98.32$, $p < 0.0001$) (**Fig. 12B**), TNF-α [$F(3,8)=61.15$, $p < 0.0001$) (**Fig. 12C**) and IL6 [$F(3,8)=18.64$, $p < 0.0001$) (**Fig. 12D**). Administration of 6HLN decreased the neuroinflammatory response in the hippocampus of 5xFAD mice evidenced by the decrease in the level of NF-κB ($p < 0.00001$ for 0.3 mg/kg and 0.6 mg/kg), TNF-α ($p < 0.0001$ for 0.3 mg/kg and $p < 0.00001$ for 0.6 mg/kg) and IL6 ($p < 0.00001$ for 0.3 mg/kg and 0.6 mg/kg).

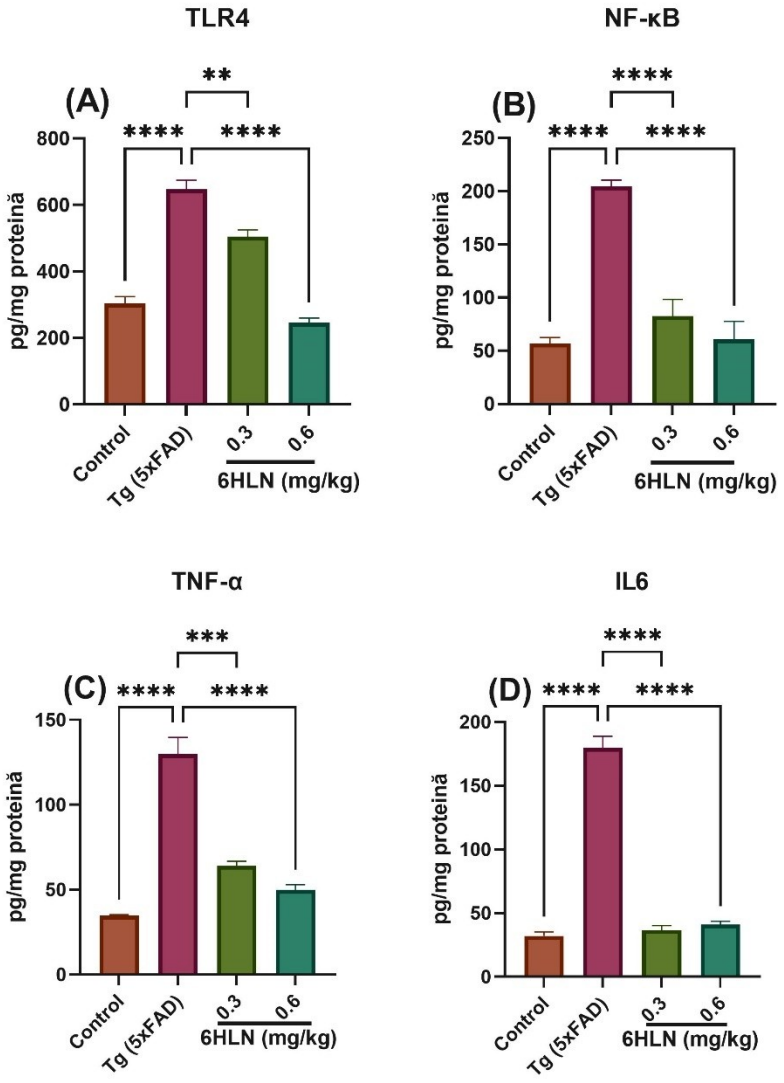

Fig. 12. Effects of 6HLN administration (0.3 and 0.6 mg/kg) on the level of (A) TLR4, (B) NF-κB, (C) TNF-α, and (D) IL6 in hippocampal homogenates of 5xFAD mice. Data are presented as means ± E.S.M (n=3), ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$ (Tukey post hoc test).

Evaluation of specific activities of acetylcholinesterase (AChE) and antioxidant enzymes (SOD, GPX, CAT) and total reduced GSH content in mouse hippocampal homogenates after exposure to 6HLN. Numerous studies have repeatedly demonstrated that oxidative stress contributes to the etiology of several neurodegenerative disorders, including Alzheimer's disease.

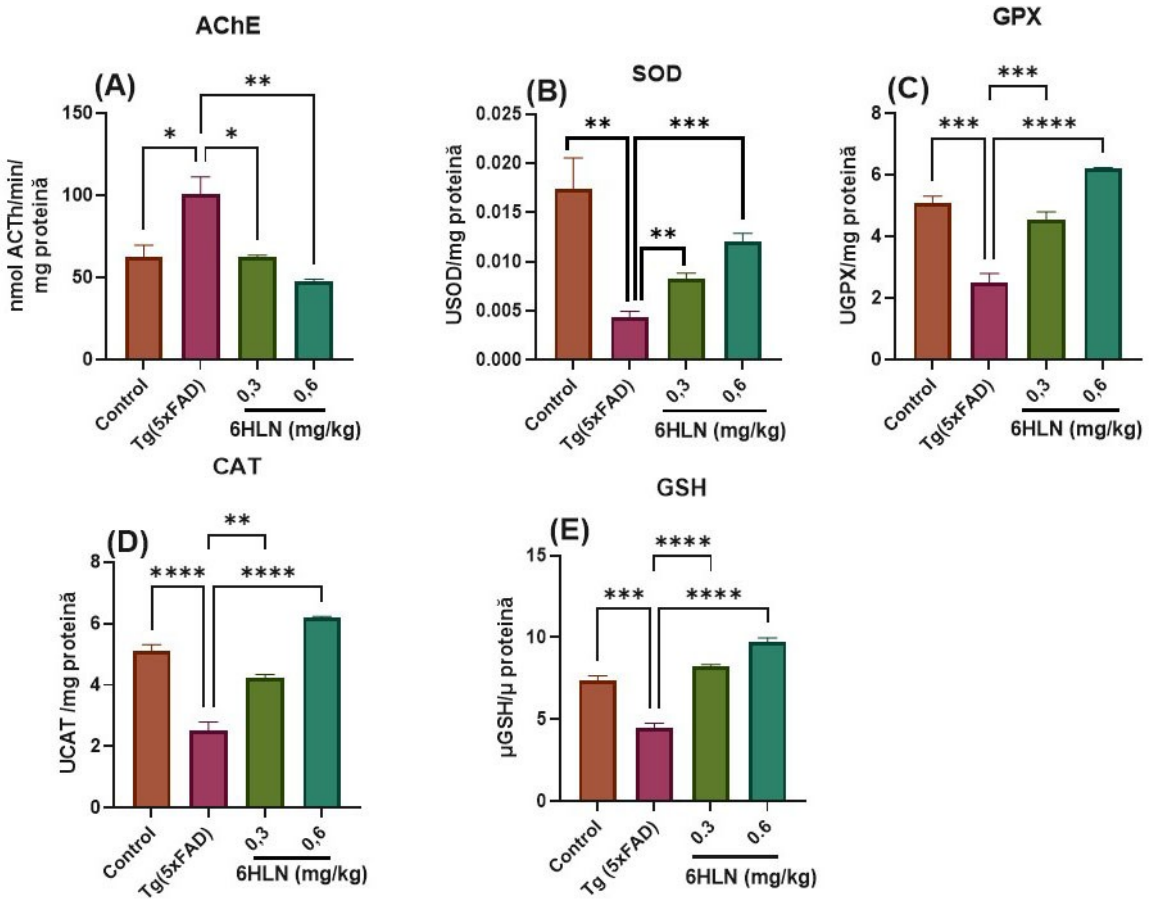

Fig. 13. Effects of 6HLN administration (0.3 and 0.6 mg/kg) on the specific activities of (A) AChE, (B) SOD, (C) GPX, and (D) CAT and the level of GSH reduce (E) in hippocampal homogenates of 5xFAD mice. Data are presented as means ± E.S.M (n=3), * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$ (Tukey post hoc test).

In **Fig. 13A** the specific activity of AChE is shown. One-way ANOVA test demonstrated that 6HLN treatment had a significant impact on the specific activity of AChE [$F(3,8)=13.50$, $p < 0.001$). Compared to the control group, AChE activity significantly increased in the 5xFAD group ($p < 0.01$). At the same time, 6HLN treatment significantly decreased AChE activity, especially in the group treated with the 0.6 mg/kg dose ($p < 0.01$ for 0.3 mg/kg and $p < 0.001$ for 0.6 mg/ kg). Experimental results suggest the positive impact of 6HLN in restoring cholinergic system activity in the hippocampus of 5xFAD mice.

Considering that oxidative stress significantly influences the cognitive status of patients with Alzheimer's disease, the effects of 6HLN treatment on the specific activity of antioxidant enzymes (SOD, GPX and CAT) were investigated. One-way ANOVA test demonstrated the significant effects of 6HLN treatment on the specific activity of SOD [$F(3,8)=11.42$, $p < 0.001$) (**Fig. 13B**), GPX [$F(3, 8)=49.62$, $p < 0.0001$) (**Fig. 13C**), and CAT [$F(3,8)=66.60$, $p < 0.0001$) (**Fig. 13D**). Administration of 6HLN restored the antioxidant status in the hippocampus of 5xFAD mice as evidenced by the enhancement of the specific activities of SOD ($p < 0.001$ for 0.3 mg/kg and $p < 0.0001$ for 0.6 mg/kg), GPX ($p < 0.0001$ for 0.3 mg/kg and $p < 0.00001$ for 0.6 mg/kg) and CAT ($p < 0.001$ for 0.3 mg/kg and $p < 0.00001$ for 0.6 mg/kg). There was also a decrease in reduced GSH levels in the hippocampus of 5xFAD mice compared to control animals ($p < 0.0001$), being stimulated by 6HLN administration ($p < 0.00001$ for 0.3 mg/kg and 0.6 mg/kg) (**Fig. 13E**).

Evaluation of MDA (lipid peroxidation) and carbonylated proteins (protein oxidation) levels in mouse hippocampus homogenates after exposure to 6HLN. In **Fig. 14A** and **Fig. 14B,** the levels of MDA and carbonylated proteins are shown. One-way ANOVA test demonstrated overall effects of 6HLN treatment on the level of MDA [F(3,8)=15.31, p < 0.001) (**Fig. 14A**) and carbonylated proteins [F(3,8)=11 .95, p < 0.001) (**Fig. 14B**). The antioxidant activity of 6HLN was demonstrated by significantly decreasing MDA (p < 0.001 for 0.3 mg/kg and p < 0.00001 for 0.6 mg/kg) and carbonylated proteins (p < 0.01 for 0.6 mg/kg) compared to the Tg(5xFAD) group.

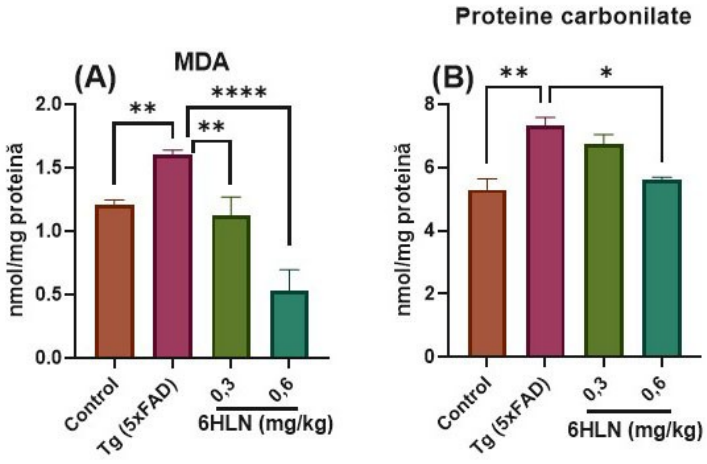

Fig. 14. Effects of 6HLN administration (0.3 and 0.6 mg/kg) on the level of MDA (A) and carbonylated proteins (B) in hippocampal homogenates of 5xFAD mice. Data are presented as means ± E.S.M (n=3), * p < 0.01, ** p < 0.001 and **** p < 0.00001 (Tukey post hoc test).

B. Achieved result indicators

The deliverables associated with each activity have been achieved and are presented in Table 1. The main proposed result indicators were:

-3 ISI articles in Web of Science indexed journals, red area (Q1) with Acknowledgment as follows:

1. NS El Sayed, S Abidar, M Nhiri, **L Hritcu**, WW Ibrahim, 2023, Aqueous extract of Ceratonia siliqua L. leaves elicits antioxidant, anti-inflammatory, and AChE inhibiting effects in amyloidβ42-induced cognitive deficit mice: Role of α7-nAChR in modulating Jak2/PI3K/Akt/GSK3β/β-catenin cascade, *Phytotherapy research*, 37(6):2437-2453, https://doi.org/10.1002/ptr.7766 (**Q1, IF 7,2, AIS 0,841**)
2. **Boiangiu RS, Brinza I, Honceriu I, Mihasan M, Hritcu L.**, 2024, Insights into Pharmacological Activities of Nicotine and 6-Hydroxy-L-nicotine, a Bacterial Nicotine Derivative: A Systematic Review. *Biomolecules*, 14(1):23. doi: 10.3390/biom14010023 **(Q1, IF 4,8, AIS 1,047)**
3. **Postu PA, Boiangiu RS, Mihasan M,** Stache AB, **Tiron A, Hritcu L.**, 2024, 6-hydroxy-L-nicotine induces distinct biological effects in representative cancer cell lines, *Molecules*, **(Q2, IF 4,2, AIS 0,677)**

3. The estimated impact of the results obtained, with an emphasis on the most significant result obtained.

Academic Impact: The most significant result provided by this project to the academic community was the demonstration for the first time of the antiaggregating potential of a nicotine metabolite - 6HLN from *P. nicotinovorans* pAO1 against the amyloid peptide. The project was based on the use of biotechnology to obtain 6HLN. The results of the project also confirmed the potential role of 6HLN in preventing or reducing the aggregation of amyloid peptides (particularly Aβ1-42), which is a hallmark of Alzheimer's disease. Furthermore, any evidence showing that 6HLN can attenuate memory

impairment, reduce oxidative stress, or decrease cytotoxicity *in vivo* models (5xFAD transgenic mice) of Alzheimer's disease would significantly contribute to validating its therapeutic potential.

Economic and social impact: Despite ongoing efforts, the development of an effective treatment for Alzheimer's dementia remains elusive. The social and economic impact of the proposal is clear: age-related brain disorders are a major global health problem. Alzheimer's disease is estimated to affect more than 74.7 million people worldwide by 2030, causing devastating suffering and enormous costs to families and society. Therefore, based on the results obtained in this project, the development of new strategies for improving health and well-being throughout the life course will be considered. Second, it would provide insight into some of the early pathological processes in the Alzheimer's brain and add further impetus to current efforts by pharmaceutical companies to develop similar drugs for the treatment of Alzheimer's disease.

The news regarding the publications and the participation of the team members in the conferences, along with the results obtained were permanently published and disseminated both on the project page http://cercetare.bio.uaic.ro/grupuri/bioactive/content/grants/PCE2022_hl.html.

Project manager,

Prof. univ. dr. habil. Lucian HRIȚCU