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Article

Simultaneous Occurrence of Bioactive Compounds (Melatonin, Protocatechuic Acid and Hydroxytyrosol) Increases Their Neuroprotective Effects Against Alpha-Synuclein-Induced Proteotoxicity

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Abstract: The abnormal assembly of α-synuclein (α-Syn) is an initial step in the formation of Lewy bodies in the brain, which finally causes the neurona

l death, being considered as a pathological hallmark in Parkinson's disease (PD). Certain food bioactives or their metabolites at very low concentrations can trespass the blood brain barrier (BBB) that might, thereafter, act simultaneously. The aim of this work was to evaluate the inhibitory and destabilising capacities on α-Syn kinetics and the neuroprotective effects of three well-known bioactive compounds able to cross the BBB and present in foods; melatonin (MEL), protocatechuic acid (PCA) and hydroxytyrosol (HT), and their combinations. For this purpose, different in vitro techniques (Thioflavin T (ThT), Transmission Electronic Microscopy (TEM), electrophoresis and MTT assay) were used. All tested compounds and their combinations were able to abolish the toxicity induced by α-Syn. In addition, the combination of PCA (100 μM) +HT (100 μM) showed the highest inhibitory effect against α-Syn fibril formation and destabilises α-Syn fibrils (88 and 62%, respectively). This is the first time that MEL, PCA and HT prove a joint effect against α-Syn aggregation and toxicity when they are tested together.

Keywords: melatonin, protocatechuic acid, hydroxytyrosol, α-synuclein, toxicity, amyloid aggregation, neurodegeneration, Parkinson´s disease.

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1. Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease after Alzheimer's disease [1]. This disorder is characterised by several symptoms that considerably impair the quality of life of PD patients including bradykinesia, tremor, rigidity, impaired postural reflexes, depression or psychosis, and autonomic and gastrointestinal dysfunction. [2]. Physiopathologically, PD affects mainly the extrapyramidal motor system in the brain. Specifically, the major lesion compromises the dopaminergic neurons of the substantia nigra, as well as other brain stem nuclei including locus coeruleus and dorsal motor vagal nucleus with appearance of Lewy Bodies (LBs) [3-5]. LBs constitute the main histopathological feature of PD being they comprised by misfolded α-synuclein protein (α-Syn) [6,7]. α-Syn protein aggregates are known to be strongly neurotoxic (especially for dopaminergic neurons) but also, they give rise to other events such as mitochondrial dysfunction and microglia activation producing an inflammatory response [8]. For this reason, there is an interest in searching drugs/bioactives that can interfere or block the

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α-Syn aggregation or promote the destabilization of already formed aggregates. Both effects can, consequently, avoid the cascade of toxic facts that finally lead to neuronal death.

Certain bioactive compounds such as polyphenols (i.e. resveratrol, (+)-catechin, and curcumin [9]) have demonstrated to inhibit the aggregation of α-Syn protein and avoid its toxicity by both in vitro and in vivo studies [10,11]. One of the most important aspect to take into account is that the bioactive under study possesses the capacity to cross the blood brain barrier (BBB) [12,13] and, equally important, that the concentration reached in the target organ is enough to present activity. These issues are crucial when evaluating bioactives that are present in foods in dietetic doses and furthermore, they are extensively metabolized [14,15] being those molecules which finally exert an effect, if any. In Addition to those facts, this work focus on three bioactive compounds (MEL, HT and PCA) for the following reasons.

Melatonin (MEL) (N-acetyl-5-methoxytryptamine) is a neurohormone secreted by the pineal gland in mammals, involved in the regulation of circadian and seasonal rhythms, in oncostasis, and in osteoblast differentiation [16, 17]. Additionally, this compound is present at low concentrations in a number of vegetables, fruits, seeds, medicinal herbs, or fermented products [18-24]. Hence, nuts, tomatoes, beetroots, cucumber, banana, strawberry, cherry, apple, walnut, pistachio, bread, cocoa powder, green coffee, mustard seeds, feverfew, olive oil, wine and beer have been reported as foods containing MEL at concentrations varying between 5pg/g or mL to 230 µg/g or mL [25-35]. Considering melatonin bioavailability (up to 56%, with a mean value of 19%) [35–38] and plasma volume (5 L), the circulating melatonin would be between 0.15 and 21 ng/mL (for mean melatonin bioavailability = 19%) [39]. It is worth mentioning that melatonin concentration in human serum is 10–30 pg/mL [40,41]. These data reveal that melatonin intake could be between 15 and 700 fold higher than endogenous melatonin [39].

MEL has received a great deal of attention as a potential bioactive compound to prevent cardiovascular diseases as well as, some types of cancer [42-45]. Indeed, it has proved to significantly inhibit VEGF-induced VEGFR-2 activation in human umbilical vein endothelial cells which is known to trigger subsequent angiogenesis [46] that is related to CVD and cancer. Moreover, Melatonin as well as certain related indolic compounds, mainly serotonin, show an inhibitory and destabilizing effect on amyloid-β peptide fibril formation as recently reported [47] showing neuroprotective properties. Likewise, Ono et al reported recently that MEL inhibits α-Syn assembly [48]; on the other hand, there are some studies that reveal that MEL is able to cross the BBB [49,50]; these data suggest that MEL strongly inhibits the protofibril formation.

Protocatechuic acid (PCA) is present in certain fruits (0.28–18.73 mg/100 g), fruits juices (1.14–6.70 mg/100 mL), jams and berry jams (0.07–9.36 mg/100 g) and vegetables (0.62–10.62 mg/100 g) [51,52]. Furthermore, it is the major phenolic acid metabolite formed from anthocyanins increasing its potential as bioactive [53]. Anthocyanins are well known phenolic compounds present in a number of foods, especially berries (0.62–794.13 mg/100 g) and red wine (0.21 mg/100 mL) [52,54]. PCA plasma concentrations after gastrointestinal digestion and microbiota degradation ranged from 0.2 to 2 µM, following the administration of 500 mg of cyanidin 3-glucoside in humans [55,56]. Recently, several investigations have shown that PCA presents anti-inflammatory [57,58], antidiabetic [59-60] and neuroprotective properties [61-63] among others, this fact partly attributed to its catechol structure. Indeed, in vivo studies have demonstrated that following the ingestion of a standard diet supplemented with PCA (2–4 g) for 12 weeks, there was an increase of PCA levels in plasma and tissues, such as heart, liver, and kidneys and in the brain of mice [53] demonstrating it can trespass the BBB. In addition, our group has recently demonstrated that PCA (100 µM) can interact with α-Syn protein inhibiting his aggregation and protecting PC12 cells in in-vitro assays [63].

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Hydroxytyrosol (HT) is present in olive oil and wine being a natural antioxidant [64]. In addition, it is also a metabolite of dopamine, being endogenously synthesized in humans a dopamine by product [65,66]. HT has been identified in wine in concentrations ranging from 1.50-25 mg/L [67-70].

Oleuropein is known to be the precursor of HT in olives, subsequent hydrolysis either during olive oil production or during gastric conditions may yield HT [71]. The mean intake of HT from the consumption of just virgin extra virgin oil (50 mL) and wine (100-200 mL/day) ranges between 0.15-30 mg/day [71]. Considering HT bioavailability (40-95%) [72-74] and plasma volume (5L), the circulating HT would be between 0.15 and 37 μM [71].

HT possesses antioxidant, cardioprotective, antiinflammatory, antimicrobial, antidiabetic and antiatherogenic properties [72-75] and it trespass the BBB [12,13]. In addition, we have recently demonstrated that HT (100 μM) can interacts with α-Syn protein inhibiting his aggregation and protecting PC12 cells [71].

A common approach when trying to elucidate a molecular mechanism is to test a pure compound. However, an intrinsic difficulty to nutritional studies is food composition complexity. Indeed, it must be highlighted that the human diet is formed by a great variety of foods which are source of varied bioactives and attributing the effects to just one compound might lead to a very restricted view. Therefore, the focus of the present work is to consider the putative collaborative effect among those bioactive which have proved to trespass the BBB. On the other hand, the concentrations at which the bioactive compounds are found in the body after food intake, as we have seen for example with MEL (10-30 pg/ mL) [30], PCA (0.2 to 2 μM) [63] and HT (between 0.15 and 37 μM) [71], are relatively low but the possibility that they could present an additive effect might be a promising picture closer to a dietetic perspective. We have already verified that these compounds are active separately, so it is interesting to see what happens when they interact simultaneously as a consequence of a diet that contains eg olive oil, wine and plant foods. For this reason, it seems an interesting field to investigate the effect of the combinations of different compounds, mimicking as much as possible the real situation. In this work, we propose to study the inhibitory effects on α-Syn fibril formation and the capacity to destabilize the α-Syn preformed fibrils by MEL, PCA and HT and their combinations.as well as their neuroprotective potential against α-Syn-induced proteotoxicity.

2. Materials and Methods

2.1 Chemicals

Standards and reagents were purchased from the following suppliers: Sigma Aldrich, Steinheim, Germany (Melatonin (MEL), protocatechuic acid (PCA), hydroxytyrosol (HT), thioflavin T (ThT), dimethyl sulfoxide (DMSO), trypsin-EDTA, Dulbecco´s modified Eagle´s medium (DMEM)-Glutamax, thiazolyl blue tetrazolium bromide (MTT), phosphate-buffered saline (PBS), L-glutamine, fetal horse serum, fetal bovine serum and streptomycin); Alexotech, Umeå, Sweden (Alpha-Synuclein, Human, Recombinant); Panreac Castellar del Vallès, Barcelona, Spain (Na_2HPO_4/NaH_2PO_4 and NaCl); ATCC, Manassas, VA, USA (PC12 cells); Bio Rad Munich, Germany (2-mercaptoetanol, 10X Tris/glycine/SDS (10X premixed electrophoresis buffer contains 25 mM), 10X Tris/glycine (10X premixed electrophoresis buffer, pH 8.3), 4-20% polyacrylamide Stain-Free Gel Mini-PROTEAN TGX, Immun-Blot PVDF membrane, and Coomassie Blue); EMS, Hatfield, PA, USA (carbon-coated grids (300 mesh, copper).

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2.2 Measurement of α-Syn fibril formation and destabilization assay (ThT Assay).

The α-Syn aggregation and destabilization assays were performed according to the method of Ono et al. [76] with slight modifications. In this assay, we used the fluorescent molecule ThT due to its capacity to emit fluorescence when it is linked with misfolded proteins.

For the inhibition assay, a stock solution of α-Syn protein at 140 µM was prepared in buffer Na2HPO4/NaH2PO4 (25 mM)/NaCl (140 µM), adjusted to pH 7.4, and then diluted to 70 µM. On the other hand, for the disaggregation assay, α-Syn fibrils were formed. To this end, α-Syn solution at 140 µM (in the above described buffer) was incubated for 6 days, at 37 °C, under continuous agitation.

Stock solutions of MEL, PCA and HT were prepared at 150, 150 and 162 mM, respectively, in DMSO and subsequently diluted with buffer until reaching the desired final concentrations, which previously demonstrated to be active against α-Syn fibrils [46, 61, 69] (see Table 1). ThT at final concentration of 25 µM was added to each well in a black clear bottom, 96 well plates.

Table 1 shows the different concentrations employed for both assays: inhibition and destabilization of α-Syn fibrils. Thus, it was possible to evaluate if the observed effects were due to a synergic or additive effect. Equal volumes of each MEL, PCA and HT solutions were added in each well (compounds alone and their combinations at high concentrations: 250 µM, 100 µM and 100 µM and at low concentrations: 62.5 µM, 70 µM and 70 µM, respectively) (see Table 1) and α-Syn or α-Syn fibrils (70 µM final concentration). Thus, a total of fourteen experiments were carried out by triplicate.

Fluorescence emission data were recorded every 2 h during 144 h, using a multidetector microplate reader fluorescence spectrophotometer (Synergy HT, Biotek), set at 450 nm for excitation and 485 nm for emission wavelengths. For the assay of destabilization, measurements were recorded as explained above but without continuous agitation. At least three measurements were performed for every assay. Following, the next step was to kept samples at −80 °C until microscopy analysis (TEM).

2.3 TEM (Transmission electron microscopy) images.

A total of 10 µL of ThT samples obtained in the above mentioned assays (sections 1.2) were placed on a 300 mesh carbon-coated Formvard grid and incubated for 20 min. Subsequently, excess fluid was removed with the aid of a filter paper and 5 µL of 2.5% glutaraldehyde (v/v) was placed on the grid, and incubated for an additional 5 min. Later, the grids were negatively stained for 1 min, with 5 µL of 0.5% uranyl acetate solution. Excess fluid was removed, and the samples were viewed using a Zeiss Libra 120 TEM, operating at 80 kV.

2.4 Electrophoresis.

To confirm the effect of MEL, PCA, HT and their combinations on the inhibition of α-Syn fibril formation and their disaggregation capacity, an electrophoresis analysis was performed. A total of 15 µL of ThT samples was diluted with 5 µL of loading buffer. Then, samples were boiled at 50 °C for 3 min and loaded on 4–20% Tris−glycine gel, for 1 h, at 100 V. Next, the gels were stained with Coomassie Blue (0.1% Coomassie R250, 10% acetic acid, and 40% methanol) and finally destained to visualize the bands.

2.5 PC12 cell culture.

PC12 cells (rat pheochromocytoma cells) were obtained from the American Type Culture Collection (ATCC). They were cultured in 75 cm2 culture flasks, containing 20 mL of DMEM−Glutamax,

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supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 15% (v/v) of fetal horse serum, and 2.5% (v/v) of fetal bovine serum, at 37 °C, with 5% CO_2. Cells were detached every 3 days using 1× trypsin−EDTA. A total of 25000 cells per well were seeded on 96 well plates for the cell viability experiments.

2.6 Cell cytotoxicity and neuroprotective assays (MTT Assay).

In order to test if MEL, PCA, HT were toxic for PC12 cells and to study if these compounds and their combinations can prevent the cell death triggered by α-Syn, MTT reduction assay [77] was carried out.

To prevent cell death by α-Syn itself, its concentration was reduced to 7 μM, as previously reported [71].

MEL (250 μM), PCA (100 μM), HT (100 μM) and their combinations were prepared and mixed in eppendorfs in equal volumes with α-Syn (140 μM) and then, incubated for 144 h on a thermoblock, with continuous agitation. Afterwards, they were diluted with serum free DMEM -Glutamax culture medium (1:10) MEL (25 μM), PCA (10 μM) and HT (10 μM) in all conditions tested, and then put in contact with PC12 cells during 24 h.

Then, cells were treated with 200 μL per well of MTT solution (final concentration, 0.5 mg/mL in DMEM−Glutamax medium) for 3 h, at 37 °C, with 5% CO_2. The dark blue crystals formed were solubilized with 100 μL per well of DMSO for 30 min. Finally, the absorbance was measured at 540 nm, with a microplate reader (Synergy HT, Biotek). Results were expressed as the percentage of MTT reduction in relation to the absorbance of control cells at 100%.

2.7 Statistical analysis.

Statistical analyses were carried out using Graphpad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Student´s t test was used to test significant differences between samples.

3. Results

3.1 Effects of MEL, PCA, HT and their combinations on α-Syn fibril formation.

The capacity of MEL, PCA, HT and their combinations to inhibit the α-Syn aggregation was measured by ThT fluorescence assay, since ThT emits fluorescence when binds to misfolded proteins (fibrils). When α-Syn was incubated alone, it can be observed that 142h is the time required to obtain the highest fluorescence signal corresponding to the maximum α-Syn fibrils formation (Figure 1A). However, when α-Syn was incubated with, PCA, HT and the combinations described in Table 1 for 142h, ThT fluorescence significantly decayed indicating a lower α-Syn fibrils formation (Figure 1A). The obtained percentages of inhibition are shown in Table 2. As can be seen, MEL did not cause a significant inhibition on ThT fluorescence as compared with α-Syn alone (Figure 1 A and Table 2). However, the incubation of PCA and HT alone produced a high and very similar inhibitory effect (76-81%, respectively). Every compound has been evaluated independently and similar results to those already reported were obtained [48, 63, 71].

On the other hand, the combination of MEL (250 μM) either with PCA (100 μM) or HT (100 μM) did not show significant differences compared with PCA and HT alone. Moreover, the combination of the three compounds (MEL + PCA + HT) presented no statistical differences compared with HT or PCA alone, if we consider the higher concentrations under study (Table 2).

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As the percentage of inhibition obtained with the concentration of the compounds alone are high, a possible synergic effect might be disregarded. Therefore, compounds concentrations were reduced and, consequently, inhibition percentages as well (Table 2).

Results show that despite the concentration was reduced three times, the effectiveness was only twice smaller, except for MEL + PCA + HT (Table 2). Furthermore, for the mix MEL + PCA + HT, there were not significant differences whatever the concentration employed. Moreover, MEL+ PCA+ HT presents a 73% inhibitory effect, which is higher than PCA+HT (57%). Therefore, MEL seems to reinforce the inhibitory effect of PCA+HT (57%) when MEL is combined with them leading to MEL+PCA+HT (73%).

TEM was used to observe the aggregation state of α-Syn alone or mixed with MEL, PCA and HT and their combinations after 142h of incubation to confirm the ThT results above mentioned. When α-Syn was incubated alone for 142h, numerous aggregates with fibrillar form were observed (Figure 1B). However, an outstanding decrease in the number of aggregates for the samples incubated with MEL, PCA and HT can be observed (Figure 1C−E). When these compounds are combined, we can see that the number of aggregates clearly diminished proving the enhanced inhibitory effects (Figure 1F- I).

Gel electrophoresis experiments confirm the previous results based on the resulting protein size after 142h of incubation of α-Syn with the compounds under study at the different concentrations. In all cases, either compounds alone or mixed, the bands corresponding to α-Syn monomers (14.5 KDa) are more intense as compared with the band of α-Syn alone (Figure 1J). In addition, we can also observe a decrease in the intensity of the 25 KDa bands (α-syn dimers) for all tested conditions in comparison with α-syn alone. These results agree well with the observed inhibitory effect by ThT assay and supported by TEM images.

3.2 Effects of MEL, PCA, HT and their combinations on destabilization of preformed α-Syn fibrils.

In the same way, ThT assay was developed using preformed α-Syn fibrils with the purpose of testing the destabilization effect of MEL, PCA, HT, and their combinations. To this end, α-Syn fibrils were incubated at all described conditions (Table 1). Figure 2A and B show the increase on ThT fluorescence when α-Syn was incubated with MEL, PCA, HT and their combinations. The percentages of destabilization obtained are shown in Table 3. Each of the three compounds was evaluated independently. When MEL was incubated alone, we obtained a low percentage of destabilization (20%). Conversely, the incubation of PCA and HT alone produced a high destabilizing effect (53 and 71 %, respectively). If we compare the destabilizing effect of MEL + PCA and PCA individually, no significant differences are observed (*$p<0.05$). However, the joint effect of MEL and HT, on the destabilization of α-Syn fibrils is noticeable (Table 3). Additionally, if we compare the combination of MEL + PCA + HT with the individual compounds, the percentage of destabilization significantly improves (85%, Table 3). Similarly, the most effective combination was the mix of HT + PCA (89%) improving the results obtained by the isolated compounds (Table 3).

Likewise, and to confirm the destabilizing effect on preformed α-Syn fibrils by the compounds under study, TEM experiments were performed. Accordingly, Figure 2C shows that the incubation of α-Syn alone (6 days, 37ºC, continuous agitation) results on the formation of fibrils. Concerning the incubation of MEL, PCA and HT separately with α-Syn fibrils, we have confirmed that MEL lacks a significant destabilizing effect (Figure 2D). However, PCA and especially HT, clearly reduce the number of fibrillar aggregates being these thinner and disperse (Figure 2E-F). The effect is clearly noticeable after the coincubation of MEL + PCA, MEL + HT, PCA + HT and MEL+PCA+HT (Figure 2 G-J). Moreover, electrophoresis revealed that, when PCA and HT are incubated separately, and in the case of the following combinations: MEL + HT, HT + PCA and MEL + PCA + HT, all the bands (14.5, 25 and 45 KDa) corresponding to α-Syn monomers, dimers and trimers

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show very low intensity and even almost disappeared (Figure 2K) proving the destabilizing activity of the studied compounds.

In the same way as in the inhibition of α-Syn fibrils formation, the concentrations were reduced and tested by ThT experiments (Table 1). The calculated destabilization percentages are displayed in Table 3. We can see that by reducing the concentrations three times, in the case of MEL, PCA or HT alone, this effect is twice smaller (8, 30 and 49% respectively). Similarly, the combination of the compounds resulted in the destabilization percentage twice lower (26%, 47%, 62% and 60%, Table 3). Likewise, based on the data at low concentrations the combination of compounds results in an enhancement of the destabilizing effect.

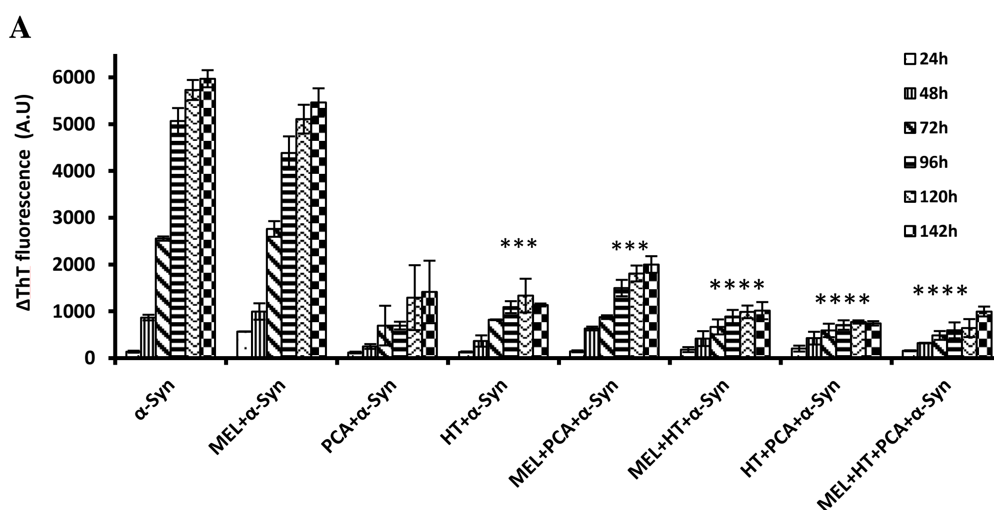
3.3 Effects of MEL, PCA, HT and their combinations against α-Syn toxicity on PC12 cells.

The cytotoxic effect of MEL, PCA, HT, and their combinations has been studied using the MTT assay. None of the conditions tested turned out to be toxic by itself to PC12 cells (Figure 3A). [78].

Figure 3B displays cell viability expressed as a relative percentage to the untreated control cells. After exposure to α-Syn alone, viability decreased by about 46%, compared to the control proving its strong neurotoxicity. After treating PC12 cells with MEL, PCA, HT and their combinations + α-Syn during 24h, a significant increase on the cell viability was observed in comparison with α-Syn alone in all conditions (Figure 3B). The percentages of increase on the PC12 cell viability are shown in Table 4. The most effective condition was MEL + HT with an increase on the cell viability of 37%. It can be observed that HT alone or HT combined with MEL and PCA produce similar increases on cell viability (34. 4 and 30%, respectively). In the case of PCA and MEL, cell viability is enhanced when they were combined with each other or with HT (35.4 and 30%, respectively).

All these results from the abovementioned experiments support the notion of the existence of important interaction between MEL, PCA and HT (or their combinations) with α-Syn protein, preventing the α-Syn fibril formation and consequently diminishing its neurotoxicity.

3.4 Figures, Tables and Schemes



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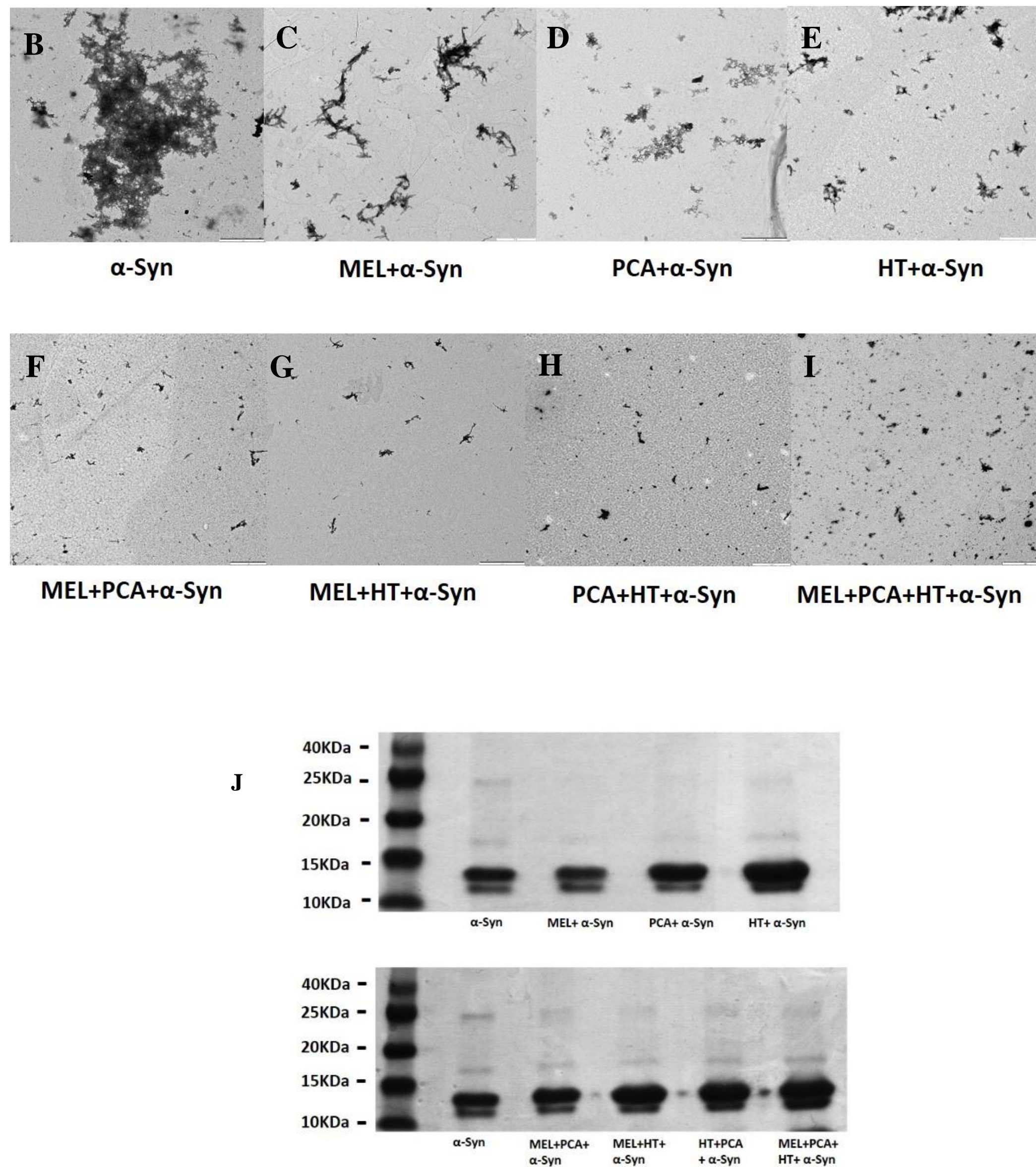
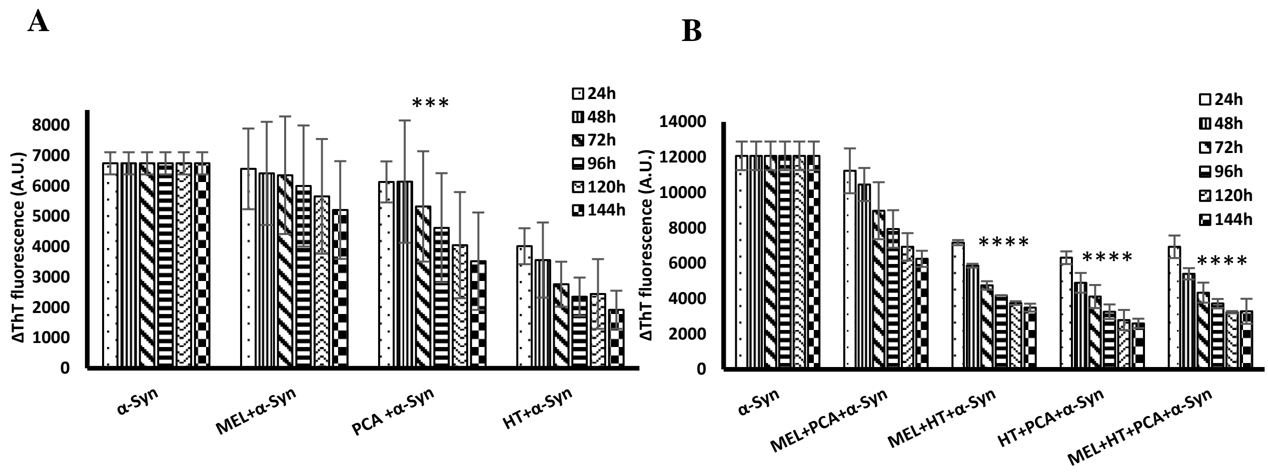
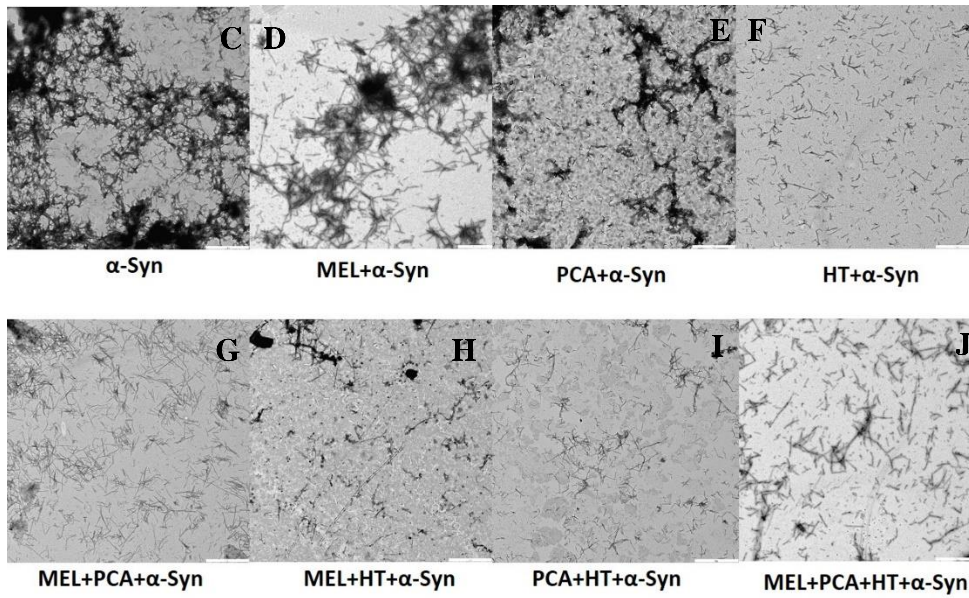


Figure 1. MEL, PCA, HT and their combinations inhibit α-Syn fibril formation (A) MEL (250 μM), PCA (100 μM), HT (100 μM), MEL+PCA, MEL+HT, HT+PCA and MEL+HT+PCA (the same compounds concentration were kept for the differents combinations) measured by ThT fluorescence assay at λEx 450 and λEm 485, for 142 hours, at 37 °C and 1000 rpm. ****$p < 0.0001$, ***$p < 0.001$ at 142h (B-I) TEM images of αsyn after incubation for 142 h with MEL, PCA and HT and their combinations. (J) Effects of MEL, PCA and HT and their combinations on αsyn fibril formation tested by electrophoresis.


A
B
ΔThT fluorescence (A.U.)
24h
48h
72h
96h
120h
144h
***
****
α-Syn
MEL+α-Syn
PCA +α-Syn
HT+α-Syn
MEL+PCA+α-Syn
MEL+HT+α-Syn
HT+PCA+α-Syn
MEL+HT+PCA+α-Syn


C
D
E
F
α-Syn
MEL+α-Syn
PCA+α-Syn
HT+α-Syn
G
H
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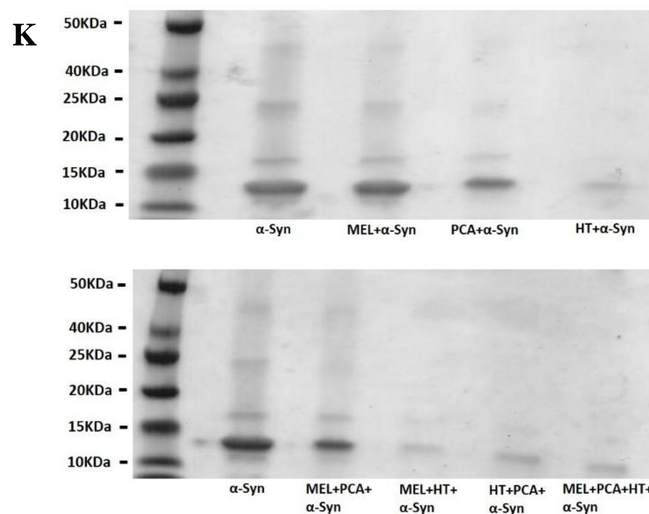
MEL+PCA+α-Syn
MEL+HT+α-Syn
PCA+HT+α-Syn
MEL+PCA+HT+α-Syn


K
50KDa
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α-Syn
MEL+α-Syn
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HT+α-Syn
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MEL+PCA+HT+α-Syn

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Figure 2. MEL, PCA, HT and their combinations destabilizes preformed fibrils of α-Syn (70 μM) (A) Effects of MEL (250 μM), PCA (100 μM) and HT (100 μM) on the kinetics of destabilization of α-Syn. (B) Effects of MEL+PCA, MEL+HT, HT+PCA and MEL+HT+PCA (the same compounds concentration were kept for the differents combinations) on the kinetics of destabilization of α-Syn (70 μM) measured by ThT fluorescence at λEx 450 and λEm 485, for 144 hours, at 37 °C and 1000 rpm. ****$p < 0.0001$, ***$p < 0.001$ at 144h (C-J) TEM images of αsyn fibrils after incubation for 144 h with MEL, PCA and HT and their combinations. (K) Effects of MEL, PCA and HT and their combinations on destabilization of α-Syn, tested by electrophoresis

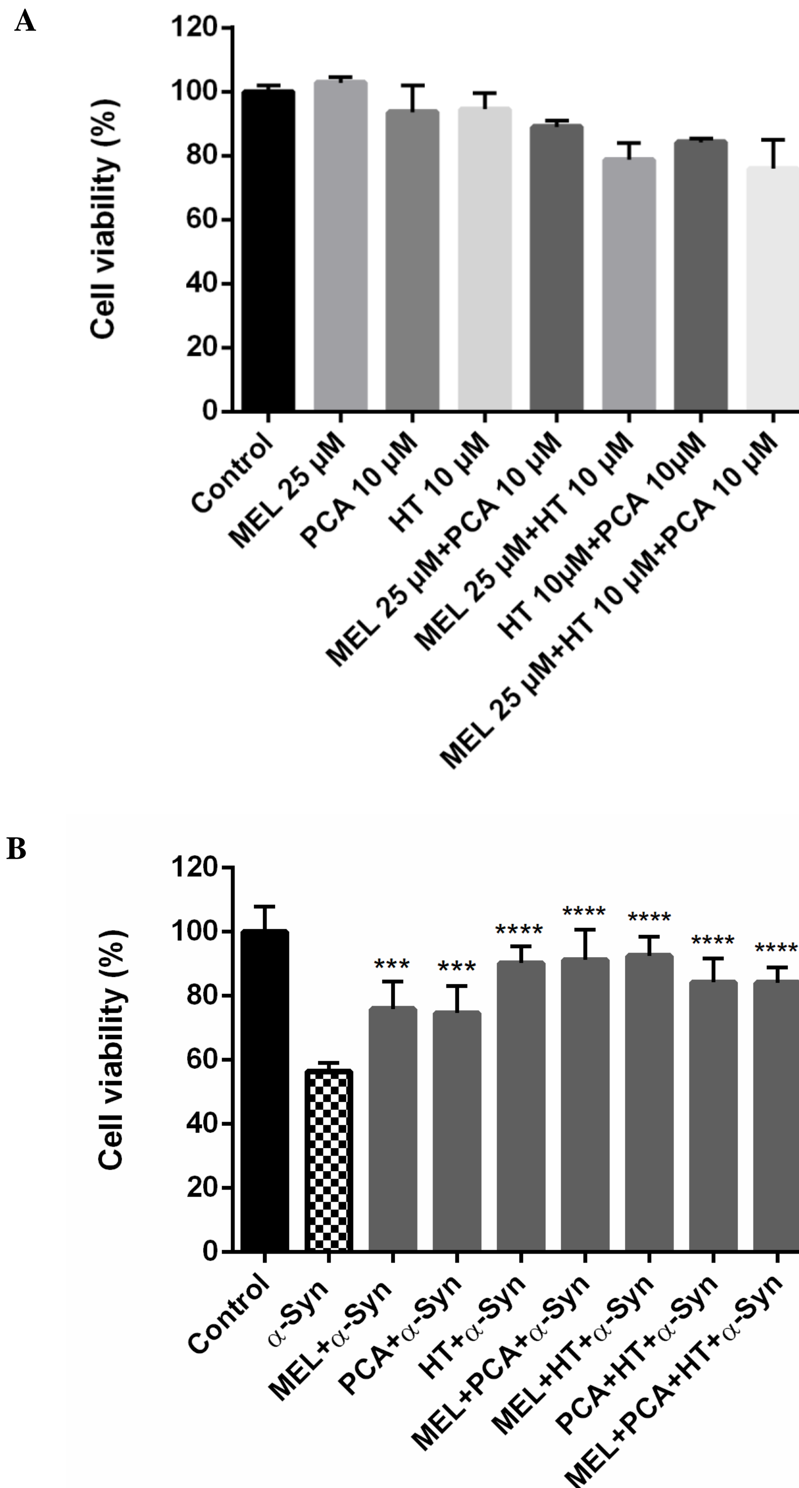

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Figure 3. PCA prevents cell death caused by α-Syn toxicity: (A) Cell viability (%) (MTT test) of MEL (25 μM), PCA (10 μM), HT (10 μM), and their combinations (the same compounds concentration were kept for the differents combinations); (B) Cell viability (%) (MTT test) of MEL (25 μM), PCA (10 μM), HT (10 μM), and their combinations with 24 h of pretreatment against α-Syn toxicity (7 μM). Data are expressed as mean ± standard deviation (SD) (n=3). ****$p < 0.0001$; ***$p < 0.001$.

Table 1. Tested concentrations of MEL, PCA and HT against α-Syn (70 μM) (ThT assay).

Compounds and their combinations high concentration	Compounds and their combinations low concentration
MEL (250 μM)	MEL (62.5 μM)
PCA (100 μM)	PCA (25 μM)
HT (100 μM)	HT (25 μM)
MEL (250 μM) + PCA (100 μM)	MEL (62.5 μM) + PCA (25 μM)
MEL (250 μM) + HT (100 μM)	MEL (62.5 μM) + HT (25 μM)
PCA (100 μM) + HT (100 μM)	PCA (62.5 μM) + HT (25 μM)
MEL (250 μM) + PCA (100 μM) + HT (100 μM)	MEL (62.5 μM) + PCA (25 μM) + HT (25 μM)

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Table 2. Percentages of inhibition of MEL, PCA, HT and their combinations against α-Syn fibril formation of three replicates (n=3). Compounds concentrations was as follows: High concentrations: Mel (250 μM), PCA 100 μM) and HT (100 μM). Low concentrations: Mel (62.5 μM), PCA 25 μM) and HT (25 μM). The same compounds concentrations were kept for the differents combinations. (a) *** $p<0.001$ MEL vs MEL+PCA, MEL+HT and MEL+HT+PCA. (b) *$p<0.05$ HT vs PCA+HT and MEL+PCA+HT.

Compounds	**%Inhibition ±SD**	
	High concentrations	**Low concentrations**
MEL+α-Syn (a)	8 ± 9	3 ± 7
PCA+α-Syn	76 ± 9	36 ± 7
HT+α-Syn (b)	81 ± 8	50 ± 8
MEL+PCA+α-Syn	66 ± 1.6	29 ± 9
MEL+HT+α-Syn	83 ± 2.3	52 ± 1
PCA+HT+α-Syn	88 ± 1.0	57 ± 1
MEL+PCA+HT+α-Syn	83 ± 0.23	73 ± 9

Table 3. Percentages of destabilization of MEL, PCA, HT and their combinations against α-Syn fibril formation of three replicates (n=3). Compounds concentrations was as follows: High concentrations: Mel (250 μM), PCA 100 μM) and HT (100 μM). Low concentrations: Mel (62.5 μM), PCA 25 μM) and HT (25 μM). The same compounds concentrations were kept for the differents combinations. (c) *$p<0.05$ MEL vs MEL+PCA. (d) **$p<0.01$ MEL vs MEL+HT. (e) MEL vs MEL+PCA+HT. (f)***$p<0.001$ PCA vs PCA+HT. (g) ****$p<0.0001$ PCA vs MEL+PCA+HT. (h)**$p<0.001$ HT vs PCA+HT. (i)*$p<0.05$ HT vs MEL+PCA+HT.

Compounds	**% Destabilization ± SD**	
	High concentrations	**Low concentrations**
MEL+α-Syn (c) (d) (e)	20 ± 1.6	8 ± 7
PCA+α-Syn (f) (g)	53 ± 9	30 ± 5.2
HT+α-Syn (h) (i)	71 ± 4.3	49 ± 1.0
MEL+PCA+α-Syn	56 ± 2.0	26 ± 4.1
MEL+HT+α-Syn	83 ± 3.3	47 ± 3.2
PCA+HT+α-Syn	89 ± 3.2	62 ± 2
MEL+PCA+HT+α-Syn	85 ± 1.8	60 ± 1.0

Table 4. Percentages of increase on the cell viability of MEL, PCA, HT and their combinations against α-Syn- induced toxicity. Compounds concentrations was as follows: Mel (250 μM), PCA 100 μM) and HT (100 μM). The same compounds concentrations were kept for the differents combinations.

Compounds	% INCREASE ON THE CELL VIABILITY
MEL+α-Syn	20
PCA+α-Syn	18
HT+α-Syn	34
MEL+PCA+α-Syn	35
MEL+HT+α-Syn	37
PCA+HT+α-Syn	30
MEL+PCA+HT+α-Syn	30

4. Discussion

A substantial body of evidence suggests that MEL may inhibits the fibril formation of some amyloidogenic proteins (β-amyloid peptide and tau, α-Syn) [47,48]. MEL was able to attenuate arsenite-induced apoptosis via a reduction of aggregated α-Syn levels in rat brain [79] by Western blot analysis. Similarly, Ono and collaborators reported the potent inhibitory effects of MEL on α-Syn assembly [48]. In contrast, our results have demonstrated that MEL (250 μM) presents a discrete inhibitory (8%) and destabilization effect (20%) of the already formed α-Syn fibrils. The aggregation and destabilization experiments were performed according to the method of Ono and collaborators with slight modifications: instead of α-Syn from Recombinant Peptide Tecnologies, LLC (Borgat, GA, USA), we used α-Syn from Alexotech, Umeå, Sweden. Besides, they used as a fluorescent molecule ThS and we used ThT.

On the other hand, PCA can reach the brain [80]. We have obtained that PCA (100 μM) shows a potent inhibitor and destabilizer effect of α-Syn fibrils (around 80% and 60% of activity, respectively) measured by ThT assay, TEM images and gel electrophoresis. These results are in accordance with a recently published article of our research group [63]. In this work we reported that PCA also presents a high inhibitory effect on α-Syn aggregation (from 70 to 80%) and a high destabilizer effect of α-Syn fibrils (around 80%) [63].

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HT possesses the optimal characteristic to be studied as a possible neuroactive compound. Our results revealed that HT (100 μM) has a significant inhibitory effect of α-Syn fibrils formation (80%) and, also, a potent destabilizing activity (71%). These findings are in accordance with previous studies in which we obtained percentages of inhibition and destabilization around 85% and 65% [71].

The mechanism of action remains unclear and seems to be through the modulation of multiple pathways. A proposed mechanism is through the modulation of toxic oligomer formation by binding and stabilizing unfolded species of α-Syn reducing fibrillation and redirecting the aggregation pathway to form off-pathway, amorphous non-toxic aggregates, blocking seeding and further conformational changes that may result in aggregation and cytotoxicity [81,82].

In the same way, other polyphenolic compounds have been studied against the aggregation and disaggregation of α-Syn fibrils such as oleuropein aglycone, curcumin or Epigallocatechin-3-gallate (EGCG). Oleuropein aglycone stabilizes α-synuclein monomers thus hampering the growth of on-pathway oligomers and favouring the growth of stable and harmless aggregates [83]. Curcumin has been tested as an inhibitor of α-Syn aggregation in vitro and it efficiently destabilizes preformed α-Syn and avoids fibril formation in a dose-dependent manner [84]. EGCG has been reported to have promising effects on the reduction of aggregation and toxicity of α-Syn fibrils [85]. Nevertheless, although these compounds are effective against aggregation and destabilization of α-Syn fibrils, they are not able to cross the BBB [83-85].

However, this is the first time that the combination of MEL, PCA and HT have proved to be more effective than these compounds indepently against α-Syn fibrils formation and desestabilization.

First of all, we carried out ThT assay using the compounds at high concentrations as shown in Table 1. We found that the most effective combination was PCA + HT as compared with PCA or HT alone, with values around 90% inhibition on α-Syn fibril formation. Similarly, the destabilization assay of α-Syn fibrils has revealed that the most effective combination was the mix of HT + PCA (89%), whose values are much higher than those obtained from HT or PCA alone (53 and 71%, respectively).

The most important biological consequence of α-Syn misfolded association is the production of neurotoxic structures that finally cause cell death [86,87]. Our results show that PCA, HT and the different combinations showed in Table 1 are effective against α-Syn-induced toxicity, preventing PC12 cell death due to their ability to notably inhibit the formation of α-Syn fibrils. Thus, all tested conditions resulted in an increase between of 20 and 30% in living cells in comparison with α-Syn alone (Table 4). Previous studies had shown protective effects against α-Syn neurotoxicity of MEL, PCA and HT. Treatment of α-Syn with MEL increased cell viability to approximately 86% [48]. However, this result does not match with our results showing a more modest effect for MEL (20%). Concerning the neuroprotective effects of PCA and HT, our results are in accordance with previous articles [63, 71]. In fact, PCA (1-50 μM) increased cell viability between 7-13% [84], and HT demonstrated be able to totally reverse the toxic effect of α-Syn reaching to values of control viability at (10-25 μM) [53].

The evaluation of the neuroprotective activity against α-Syn by the combination of MEL, PCA and HT has not been reported previously. Our results show a significant increase on the cell viability approximately between 30-40% for all tested compounds and their combinations in comparison with α-Syn alone. The most potent neuroprotective compound was HT (34%) followed by MEL and PCA (20 and 18% respectively) on cell viability. ever, when MEL and PCA were combined with HT we observed an increment of at least 10 % more.

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This is the first time that the role of the combination of MEL, PCA and HT against α-Syn kinetics and toxicity has been studied, getting closer to the reality about the simultaneous presence of several compounds in our diet and counteracting the low concentration in vivo of these compounds. Additional experiments with the combinations of MEL, PCA and HT are needed to confirm and understand these structure-activity relationships. These combinations are a promising way to enhance the effect of these compounds separately and thus improve their neuroprotective properties taking into account its bioavailability and the capacity to cross the BBB

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