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# Melatonin, protocatechuic acid and hydroxytyrosol effects on vitagenes system against alpha-synuclein toxicity

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**Abstract:** Preventing the abnormal assembly of α-synuclein (α-Syn) and the correct modulation of vitagenes system exercise strong neuroprotective effects. It has been reported that melatonin (MEL), protocatechuic acid (PCA) and hydroxytyrosol (HT) reduce α-Syn toxicity. Their effect on the vitagenes system of PC12 cells have not been explored yet. These bioactive can cross the blood brain barrier (BBB). Therefore, this work aims to evaluate the inhibitory and destabilising capacities of MEL, PCA, HT, and their combinations on α-Syn kinetics and effects on vitagenes system (sirtuin-1 (SIRT-1), sirtuin-2 (SIRT-2), heme oxygenase (HO-1) and heat shock protein 70 (Hsp70)). In vitro techniques (Thioflavin T (ThT), Transmission Electronic Microscopy (TEM), electrophoresis, MTT assay and qPCR) were used. Compounds, both individually and simultaneously were able to decrease the toxicity induced by α-Syn. Concurrently, occurrence of PCA (100 μM) +HT (100 μM) showed the highest inhibitory effect against α-Syn fibril formation and destabilisation of α-Syn fibrils (88 and 62%, respectively). Moreover, these compounds increased the expression of SIRT-2, HO-1 and Hsp70, contributing to a neuroprotective effect. In addition, the most important result is the

*Abbreviations:* blood brain barrier, BBB; alzheimer's disease, AD; heme oxygenasa, HO-1; heat shock protein 70, Hsp-70; hydroxytyrosol, HT; melatonin, MEL; Parkinson's disease, PD; protocatechuic acid, PCA; sirtuin-1, SIRT-1; sirtuin-2, SIRT-2; α-synuclein, α-Syn; Thioflavin T, ThT; Transmission Electronic Microscopy, TEM

increase on the expression of SIRT-2 caused by the combination of MEL + HT + PCA in the absence of α-Syn fibrils.

*Keywords:* melatonin; protocatechuic acid; hydroxytyrosol; Parkinson´s disease; vitagenes system; α-synuclein.

## 1. Introduction

The principal pathological hallmark of Parkinson's disease is the presence of extracellular spherical deposits formed mainly by α-synuclein protein (α-Syn) aggregates. These are known to be highly neurotoxic, especially for dopaminergic neurons. They also give rise to other events such as mitochondrial dysfunction and microglia activation producing an inflammatory response (Goedert, 2001). For this reason, there is great interest in searching drugs/bioactives that can interfere or block the α-Syn aggregation or promote the destabilisation of already formed fibrils (Masuda et al., 2006; Oliveri, 2019; Singh et al., 2017). Both effects can prevent the cascade of toxic events that finally lead to neuronal death. On the other hand, mitochondrial dysfunction produces harmful levels of reactive oxygen species which leads to cellular oxidative stress (free-radical theory of aging). Oxidative stress is highly damaging to cellular macromolecules and it is also a major cause of the loss and impairment of neurons in neurodegenerative disorders (Niedzielska et al., 2016; Liu et al., 2017). There is a growing body of evidence suggesting that modulation of vitagenes system impacts positively on reducingboth α-Syn misfolding and oxidative stress, which in turn results in a pronounced neuroprotective effect. The vitagenes system includes sirtuins, heat shock protein (Hsp) family and heme oxygenase-1 (Calabrese et al., 2010; Srivastava and C. Haigis, 2011).

Reduced SIRT1 levels were observed in the parietal cortex of the AD (Alzheimer's disease) patients and an inverse correlation has been observed between SIRT1 levels and accumulation of the tau protein in the advanced stages of AD in humans (Julien et al., 2010). Additionally, SIRT2 may play and important role in regulating neuronal motility

(migration, outgrowth, etc.) (Li et al., 2007) and, therefore SIRT2 may play important roles in neurodegeneration.

Heat shock proteins (Hsps) have a key role in cell repair and protective mechanisms (Sottile and Nadin, 2018). Moreover, Hsp70 play an important role in protein folding, quality control of misfolded proteins (Csermely et al., 2007), aggregation prevention, dissolving and refolding of aggregated proteins, as well as protein degradation (Broadley and Hartl, 2009; Goloubinoff and Rios, 2007).

Heme oxygenases (HO) are known to be dynamic sensors of cell oxidative stress and modulators of redox homeostasis throughout the phylogenetic spectrum. HO-1 induces the nuclear factor-erythroid 2 (Nrf2), one of the most important systems that enhance cellular protection against oxidative stress.

Several studies have demonstrated that bioactive food compounds such as resveratrol and curcumin increase HO-1 expression in PC12 cells and endothelial cells, among other cell lines. In addition, an overexpression of SIRT-1 was produced by resveratrol, reducing Aβstimulated NFκB signalling, proving its strong neuroprotective effects on AD (Hui et al., 2018; Zhou et al., 2019; Xu et al., 2019; Bucolo et al., 2019). Pivotal is the fact that the key bioactives have the capacity to cross the BBB. Of equal importance is the fact that in the target organ the active concentration can be reached through a balanced diet (Figueira et al., 2017). These issues are crucial when evaluating the potential of bioactives present in foods. As it is their metabolites which finally exert an effect, if there is such an effect, it is worth taking into consideration whether they are extensively metabolised (Angelo et al., 2001; Fernández-Pachón et al., 2009; Noguer et al., 2012; Wu et al., 2009), being their metabolites which finally exert an effect, if any. For this reason, only bioactives proven to cross the BBB such as MEL, PCA and HT (Carloni et al., 2018; Angelo et al., 2001; Zhang et al., 2011; Wu et al., 2009) have been considered in the present study.

In mammals, Melatonin (MEL) (N-acetyl-5-methoxytryptamine) is a neurohormone secreted by the pineal gland. It is involved in the regulation of circadian and seasonal

rhythms, in oncostasis, and in osteoblast differentiation (Freyssin et al., 2018; Pévet et al., 2006). Low concentrations of this compound is also present in a number of vegetables, fruits, seeds, medicinal herbs, or fermented products (Chen et al., 2003; Dubbels et al., 1995; Hattori et al., 1995; Manchester et al., 2000; Murch et al., 1997; Reiter, 1991; Reiter et al., 2015). Hence, nuts, tomatoes, beetroots, cucumber, banana, strawberry, cherry, apple, olive oil, wine, beer among others have been reported as foods containing MEL at concentrations varying between 5 pg/g or mL to 230 μg/g or mL (de la Puerta et al., 2007; Di Bella and Gualano, 2006; Hornedo-Ortega et al., 2016a,b; Iriti and Varoni, 2016; Lei et al., 2013; Maldonado et al., 2009; Oladi et al., 2014; Reiter et al., 2005; Rodriguez-Naranjo et al., 2011; Stürtz et al., 2011; Zhao et al., 2013). In fact, circulating MEL in humans due to its intake from certain food has been estimated between 15 and 700 fold higher (0.15 and 21 ng/mL respectively) than endogenous MEL (Cerezo et al., 2017).

Recently, MEL and certain related indolic compounds, mainly serotonin, have been proven to inhibit and destabilise amyloid-β peptide fibril formation and α-Syn assembly, (Hornedo-Ortega et al., 2018b).

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) (Lin et al., 2011; Vitaglione et al., 2007). Furthermore, PCA is the major phenolic acid colonic metabolite formed from anthocyanins increasing its bioactive potential (Zhang et al., 2019). 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 (Czank et al., 2013; De Ferrars et al., 2014). 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 (De Ferrars et al., 2014) demonstrating it can cross the BBB. In addition, our group has recently proved that PCA (100 μM) can interact with α-Syn protein inhibiting his aggregation and

protecting PC12 cells in in vitro assays (Hornedo-Ortega et al., 2016a). Hydroxytyrosol (HT) is present in olive oil and wine being a natural antioxidant (Vissers et al., 2018). In addition, it is also a metabolite of dopamine, being endogenously synthesized in humans as its product (Meiser et al., 2013; Rodríguez-Morató et al., 2016). HT has been identified in wine in concentrations ranging from 1.50 to 25 mg/L (Boselli et al., 2006; Di Tommaso et al., 1998; Piñeiro et al., 2011; Proestos et al., 2005).

The mean intake of HT from the consumption of extra virgin oil (50 mL) and wine (100–200 mL/day) ranges between 0.15 and 30 mg/ day (Hornedo-Ortega et al., 2018a). Considering HT bioavailability (40–95%) (Tuck and Hayball, 2002; Visioli et al., 2000; Vissers et al., 2018) and plasma volume (5 L), the circulating HT would be between 0.15 and 37 μM (Hornedo-Ortega et al., 2018a).

In addition, we have recently demonstrated that HT (100 μM) can interact with α-Syn protein inhibiting its aggregation and protecting neuronal PC12 cells (Hornedo-Ortega et al., 2018a).

An intrinsic difficulty of 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 an array of bioactives. Therefore, attributing the effects to just one single compound might lead to a very restricted view, so the focus of the present work is to consider the putative collaborative effect among those bioactives that have proven to cross the BBB. For this reason, it seems an interesting field to investigate the simultaneous effect of the combinations of different compounds, mimicking as much as possible the real situation.

The hypothesis of this work is that the neuroprotective effect of certain bioactives and their simultaneous occurrence can be based on their action on vitagene system as well as in α-Syn aggregation and destabilisation. Therefore, we propose to study the inhibitory effects on α-Syn fibril formation and the capacity to destabilise pre-formed α-Syn fibrils by MEL, PCA and HT and their combinations as well as their neuroprotective potential against α-Syn-induced proteotoxicity. Additionally, the putative effect of these compounds on the vitagenes system on PC12 cells is evaluated.

## 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), SIRT-1, SIRT-2, Hsp70, HO-1, and β-actin primers (Table 1), 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 streptomycin9]; Alexotech, Umeå, Sweden [Alpha-Synuclein, Human, Recombinant]; Panreac, Castellar del Vallès, Barcelona, Spain [Na2HPO4/NaH2PO4 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 [carboncoated grids (300 mesh, copper]; Bioline, London, UK [TRIsure reagent and SensiFAST TM SYBR R® No-ROX Kit].

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

The α-Syn aggregation and destabilisation assays were performed according to the method of Ono et al., 2004 with slight modifications. In this assay, we used the fluorescent molecule ThT due to the increase in ThT fluorescence when it is in the presence of fibrils. For the inhibition assay, a stock solution of α-Syn protein at 140 μM was prepared in Na2HPO4/NaH2PO4 (25 mM)/NaCl buffer (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 inhibitors of α-Syn fibril formation (Hornedo-Ortega et al., 2018a, 2016a) (see Table 2). First, ThT at a final concentration of 25 μM was added to each well in a black clear bottom, 96 well plates. Subsequently, equal volumes of every MEL, PCA and HT solutions were added also in each well. MEL, PCA and HT were added as single compounds, as high concentrations combinations: 250 μM, 100 μM and 100 μM, respectively and at low concentrations: 62.5 μM, 70 μM and 70 μM, respectively (see Table 2). Finally α-Syn or α-Syn fibrils (70 μM final concentration) was added. α-Syn or α-Syn fibrils alone were used as control. A total of fourteen experiments were performed in 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 destabilisation assay, measurements were recorded as explained above but without continuous agitation. At least three measurements were performed for every assay. to the samples were keept 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. Excess fluid was then 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. Subsequently, 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.*

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

202 *2.5. PC12 cell culture.*

203 PC12 cells (rat pheochromocytoma cells) were obtained from the American Type Culture
204 Collection (ATCC). They were cultured in 75 $cm^3$ culture flasks, containing 20 mL of
205 DMEM−Glutamax, supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin,
206 15% (v/v) of fetal horse serum, and 2.5% (v/v) of fetal bovine serum, at 37 °C, with 5%
207 CO2. Cells were detached every 3 days using 1 × trypsin−EDTA. A total of 25,000 cells
208 per well were seeded on 96 well plates for the cell viability experiments.

209 *2.6. Cell cytotoxicity and neuroprotective assays (MTT Assay).*

210 In order to test if MEL, PCA, HT were toxic for PC12 cells, and to study if these
211 compounds and their combinations can prevent the cell death triggered by α-Syn an MTT
212 reduction assay (Mosmann, 1983) was carried out.

213 MEL, PCA, HT and their combinations were mixed in eppendorfs in equal volumes with
214 α-Syn reaching the concentration of 250 µM, 100 µM, 100 µM and 70 µM, respectively.
215 Then, they were incubated for 144 h on a thermoblock, with continuous agitation.
216 Afterwards, they were diluted with serum free DMEM-Glutamax culture medium (1:10)
217 to reach the final concentrations as follows: MEL (25 µM), PCA (10 µM), HT (10 µM) and
218 α-Syn (7 µM) in all conditions tested. They were then placed into contact with PC12 cells
219 for 24 h. In this case to prevent cell death, the incubated solution of α-Syn concentration
220 was reduced to 7 µM as previously reported (Hornedo-Ortega et al., 2018a). To test the
221 compounds cytotoxicity the same conditions were used but without αSyn.

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% CO2. The dark blue crystals formed were solubilised with 100 µL per well of DMSO for 30 min. Finally, 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. qPCR Analysis.*

Total RNA of PC12 cells after different treatments (α-Syn alone (5 µM)/α-Syn (5 µM) + MEL (25 µM), PCA (10 µM) and HT (10 µM) and their combinations/MEL (25 µM), PCA (10 µM) HT (10 µM) and their combinations) was extracted using the TRIsure reagent (Bioline) and following the manufacturer's instructions. Using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), 1 µg of total RNA was transformed into cDNA. qPCR was performed using the SensiFAST TM SYBR ® No-ROX Kit (Bioline) and SIRT-1, SIRT-2, Hsp70, and HO-1 primers (β-actin was used as a housekeeping gene) (Table 1). Results were calculated using the percentages of the powers of each condition.

*2.8. Statistical analysis.*

Results are expressed as mean ± SD based on data from three independent experiments. Statistical analyses were performed using Graphpad Prism 6.01 software (GraphPad Software, Inc., San Diego, CA, USA). Student's t-test was used to test significant differences between samples.

## 3. Results

*3.1. The combination of MEL, PCA, HT is most effective against 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. As mentioned above, ThT was used due to the fact that its fluorescence increases in the presence of fibrils. It can be observed that

142 h is the time required to obtain the highest fluorescence signal corresponding to the maximum α-Syn fibrils formation (Fig. 1A) when α-Syn was incubated alone. However, when α-Syn was incubated with, PCA, HT and the combinations described in Table 2 for 142 h, ThT fluorescence significantly decayed indicating a lower α-Syn fibrils formation (Fig. 1A). The obtained percentages of inhibition are shown in Table 3. As can be seen, MEL did not cause a significant inhibition on ThT fluorescence as compared with α-Syn alone (Fig. 1A and Table 3). However, incubating PCA and HT alone produced a high and very similar inhibitory effect (76–81%, respectively). Every compound was evaluated independently and results similar to those already reported were obtained (Ono et al., 2012; Hornedo-Ortega et al., 2018a, 2016a).

Compared with PCA and HT alone, the combination of MEL (250 μM) with either PCA (100 μM) or HT (100 μM) did not show significant differences. Moreover, if we consider the higher concentrations under study (Table 3) the combination of the three compounds (MEL + PCA + HT) presented no statistical differences compared with HT or PCA alone. 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 (Table 3). Results show that despite the concentration was reduced three fold, the effectiveness was only reduced by a factor of two, except for MEL + PCA + HT that showed not significant differences whatever the concentration employed (Table 3). Moreover, MEL + PCA + HT presents a 73% inhibitory effect, which is higher than PCA + HT combination (57%). Therefore, MEL seems to reinforce the inhibitory effect of PCA + HT.

TEM was used to observe the aggregation state of α-Syn alone or mixed with MEL, PCA and HT and their combinations after 142 h of incubation in order to confirm the ThT results above mentioned. When α-Syn was incubated alone for 142 h, numerous aggregates with fibrillar form were observed (Fig. 1B). However, an outstanding decrease in the number of aggregates for the samples incubated with MEL, PCA and HT

was observed (Fig. 1C−E). When these compounds are combined, we can see that the number of aggregates clearly diminished proving that this combination enhanced their inhibitory effects (Fig. 1FI).

Gel electrophoresis experiments confirm the previous results based on the resulting protein size after 142 h of incubating of α-Syn with different concentrations of the compounds under study. In all cases, with either single or mixed compounds, the bands corresponding to αSyn monomers (14.5 KDa) are more intense when compared with the band of α-Syn alone (Fig. 1J). In addition, we can also observe a decrease in the intensity of the 25 KDa bands (α-Syn dimers) for all conditions tested 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. Combinations of PCA + HT and MEL + PCA + HT are most effective on destabilization of preformed α-Syn fibrils.*

The ThT assay was developed using pre-formed α-Syn fibrils with the purpose of testing the destabilisation effect of MEL, PCA, HT, and their combinations. To this end, α-Syn fibrils were incubated in all described conditions (Table 2). Fig. 2A and B show the decrease on ThT fluorescence when α-Syn was incubated with MEL, PCA, HT and their combinations compared with α-Syn alone. Each of the three compounds was evaluated independently. When MEL was incubated alone, we obtained a low destabilisation percentage (20%). Conversely, the incubating of PCA and HT alone produced a high destabilising effect (53 and 71%, respectively). If we compare the destabilising 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 destabilisation of α-Syn fibrils is noticeable (Table 4). Additionally, if we compare the combination of MEL + PCA + HT with the individual compounds, the destabilisation percentage significantly improves (85%). Similarly, the most effective combination was the mix of HT + PCA (89%) improving the results obtained by the isolated compounds.

Similarly, and to confirm the destabilising effect of the compounds under study on pre-formed α-Syn fibrils, TEM experiments were performed. Accordingly, Fig. 2C shows that the results of incubating α-Syn alone (6 days, 37 °C, continuous agitation) on the formation of fibrils. With respect to incubating of MEL, PCA and HT separately with α-Syn fibrils, we have confirmed that MEL lacks a significant destabilising effect (Fig. 2D). However, PCA and especially HT, clearly reduce the number of fibrillar aggregates these latter being thinner and dispersed (Fig. 2E–F). The effect is clearly noticeable after co-incubating MEL + PCA, MEL + HT, PCA + HT and MEL + PCA + HT (Fig. 2G–J). Moreover, electrophoresis revealed that, as well as the following combinations: MEL + HT, MEL + PCA, HT + PCA and MEL + PCA + HT, when PCA and HT are incubated separately, all the bands (14.5 KDa) corresponding to α-Syn monomers, show a high intensity (Fig. 2K) proving the destabilising activity of the studied compounds.

Consistent results, therefore, have been obtained with the three techniques used in this study.

Similarly, the concentrations were reduced and tested by ThT experiments (Table 2). We can see that by reducing the concentrations three fold, in the case of MEL, PCA or HT alone, this effect was smaller by a factor of two (8, 30 and 49% respectively). The combination of the compounds resulted in the destabilisation percentage being lower by a factor of two (26%, 47%, 62% and 60%, Table 4). Based on these data, at low concentrations combinating compounds results in an enhancement of the destabilising effect.

*3.3. HT, MEL + HT and MEL+ PCA, reduce α-Syn toxicity on PC12 cells.*

The cytotoxic effect of MEL, PCA, HT, and their combinations have been studied using the MTT assay. None of the compounds and their combinations tested turned out to be toxic by itself to PC12 cells (Fig. 3A) (ISO 10993-5:2009(E)).

Fig. 3B shows cell viability expressed as a relative percentage compared with 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 for 24 h, a significant increase in cell viability was observed in comparison with α-Syn alone in all conditions (Fig. 3B). The most effective condition was MEL + HT with a 37% increase in cell viability (Table 5). It can be observed that HT alone or HT combined with MEL and PCA produce similar increases in cell viability (34.4 and 30%, respectively). In the case of PCA and MEL, cell viability was enhanced when they were combined with each other or with HT (35.4 and 30%, respectively).

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

*3.4. SIRT-1, SIRT-2, Hsp 70 and HO-1 expression by MEL (25 μM), PCA (10 μM) HT (10 μM) and their combinations.*

In order to prove the effect of MEL, PCA, HT and their combinations on vitagenes expression, SIRT-1, SIRT-2, HO-1, and Hsp70 gene expression were analysed in both the absence and the presence of α-Syn (Fig. 4A–D). For every gene under study, two controls were performed (untreated cells and α-Syn incubated alone). For both control conditions the same effect is observed in the expression of each gene.

As can be observed, SIRT-1 gene expression does not increase after the treatment with any of the compounds or their combinations.

PCA; MEL + HT; HT + PCA and MEL + HT + PCA in the absence of α-Syn fibrils increased SIRT-2 gene expression significantly (Fig. 4B). In addition, HT and HT + PCA incubated with α-Syn significantly increased SIRT-2 gene expression. It is remarkable

that the condition that produces the greatest increase in the SIRT-2 gene expression were those of the three compounds combinations in the absence of α-Syn fibrils.

Similarly, HT; MEL + HT; HT + PCA and MEL + HT + PCA in the absence of α-Syn fibrils increased HO-1 gene expression significantly (Fig. 4C). Additionally, a combination of HT and HT + PCA incubated with α-Syn also increases the expression significantly. The largest increase occurs in the case of MEL + HT without α-Syn fibrils.

Finally, HT + PCA (Fig. 4D) significantly increased Hsp70 gene expression in the absence of α-Syn fibrils. Moreover, HT and HT + PCA, MEL + HT incubated with α-Syn significantly increased Hsp70 gene expression. In summary, the most important result to highlight is the increase in SIRT-2 expression caused by the combination of MEL + HT + PCA in the absence of α-Syn fibrils. This effect is observed to a lesser extent in the case of HO-1 gene expression. The condition HT and HT + PCA incubated with α-Syn increase the expression of SIRT-2, HO-1 and Hsp-70 gene. Moreover, MEL + HT without α-Syn fibrils is the one that produces the greatest increase in HO-1 gene expression. In the case of Hsp-70 gene expression, HT with α-Syn fibrils is the one that produces the greatest increase.

## 4. Discussion.

A substantial body of evidence suggests that MEL may inhibit the fibril formation of some amyloidogenic proteins (β-amyloid peptide and tau, α-Syn) (Hornedo-Ortega et al., 2018a; Ono et al., 2012). MEL was able to attenuate arsenite-induced apoptosis via a reduction of aggregated α-Syn levels in rat brain (Nobre-Júnior et al., 2009) as confirmed by Western blot analysis. In contrast, our results have demonstrated that MEL (250 μM) presents a discrete inhibitory (8%) and destabilisation effect (20%) of the already formed α-Syn fibrils. The aggregation and destabilisation experiments were performed according to the method of Ono and collaborators with slight modifications: instead of α-Syn from Recombinant Peptide Technologies, LLC (Borgat, GA, USA), we used α-Syn from

Alexotech, Umeå, Sweden. Furthemore, they used ThS as a fluorescent molecule, whereas we used ThT.

PCA (100 μM) and HT (100 μM) seem to be promising neuroprotective molecules, since they showed a potent inhibitory and destabilising effect on α-Syn fibrils (around 80% and 60%, respectively for PCA; and 80% and 71%, respectively for HT) (Tables 3 and 4). These results are in accordance with a recent article published by of our research group (Hornedo-Ortega et al., 2018a 2016a) in wich we reported that, individually PCA and HT also presents a high inhibitory effect on α-Syn aggregation (from 70 to 80% and 85%, respectively) and a high destabilising effect of α-Syn fibrils (around 80% and 65%, respectively) (Hornedo-Ortega et al., 2018a, 2016a). It is worth mentioning that both PCA and HT also can reach the brain (Szwajgier et al., 2017).

The effects of other polyphenolic compounds against the aggregation and disaggregation of α-Syn fibrils such as oleuropein aglycone (Palazzi et al., 2018), curcumin (Ono and Yamada, 2006) or Epigallocatechin-3-gallate (EGCG) (Bieschke et al., 2010) hace also been studied. Nevertheless, although these compounds are effective against aggregation and destabilisation of α-Syn fibrils, they are unable to cross the BBB (Bieschke et al., 2010; Ono and Yamada, 2006; Palazzi et al., 2018).

Furthermore, this is the first time that the combination of MEL, PCA and HT have proved to be more effective against α-Syn fibrils formation and destabilisation when they were combined than when they were acting individually First of all, we found that the most effective combination, using the compounds at high concentrations, was PCA + HT as compared with PCA or HT alone, with values of around 90% inhibition on α-Syn fibril formation (Table 2). Similarly, the destabilisation assay of α-Syn fibrils has revealed that the mix of PCA + HT was also the most effective combination (89%), the values being much higher than those obtained from HT or PCA alone (53 and 71%, respectively) (Table 3).

However, the mechanism of action remains unclear and it appears to be based on the modulation of multiple pathways. A proposed mechanism for similar bioactive compounds is through the modulation of toxic oligomer formation by binding and stabilising 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 (Dhouafli et al., 2018; Ji et al., 2016).

The most important biological consequence of misfolded α-Syn association is the production of neurotoxic structures that finally cause cell death (Ardah et al., 2014; Steiner et al., 2011). Our results show that PCA, HT and their different combinations are effective against αSyn-induced toxicity (Table 5), preventing PC12 cell death due to their ability to inhibit notably the formation of α-Syn fibrils. Thus, all tested conditions resulted in an increase of between 20 and 30% in living cells in comparison with α-Syn alone (Table 5). Previous studies have shown the protective individual effects of MEL, PCA and HT against α-Syn neurotoxicity. Treatment of α-Syn with MEL increased cell viability to approximately 86% (Ono et al., 2012). However, this result does not match with our results wich show a more modest effect for MEL (20%). With respect to the neuroprotective effects of PCA and HT, our results are in accordance with previous articles (Hornedo-Ortega et al., 2018a, 2016a). In fact, PCA (1–50 μM) increased cell viability between 7 and 13%, while HT its ability to reverse completely the toxic effect of α-Syn reaching values of control viability at 10–25 μM (Hornedo-Ortega et al., 2018a, 2016a). Our results show a significant increase in cell viability. This was approximately between 30 and 40% for all compounds tested, as well as their combinations in comparison with α-Syn alone (Table 5 and Fig. 3B). The most potent neuroprotective compound was HT (34%) followed by MEL and PCA (20 and 18%, respectively). However, when MEL and PCA were combined with HT we observed an increment of at least 10%.

We have, moreover, investigated the effect of MEL, PCA, HT and their combinations on SIRT-1, SIRT-2, Hsp70, and HO-1 gene expression in order to evaluate their putative effect against α-Syn-induced toxicity.

SIRT-2, acting via NFκB, is considered a target in neurodegenerative processes. It is known to reduce cellular oxidative stress by promoting Forkhead box O transactivation activity by increasing the expression of the antioxidant mitochondrial superoxide dismutase and reducing cellular ROS levels (Wang et al., 2007). Our results show that PCA, MEL + HT, HT + PCA and MEL + HT + PCA in the absence of α-Syn fibrils increased SIRT-2 gene expression significantly, the last combination obtaining the highest SIRT-2 expression (Fig. 4B). These results highlight the preventive potential of the compounds studied with regard to neurodegenerative diseases. Additionally, we have demonstrated that in the presence of α-Syn fibrils not only are HT and HT + PCA able to destabilise them increasing PC12 cells viability, but also they are able to increase SIRT-2 gene expression simultaneously. Therefore, these bioactive compounds do not present a unique mechanism but act as neuroprotective molecules on multiple levels.

HO-1, known as Nrf2, is one of the most important systems that enhance to induce cellular protection against oxidative stress. In fact, Nrf2 drives the transcription of many of the genes involved in free radical scavenging, especially those concerning the critical tripeptide glutathione and it is even involved in the production of the free radical scavenger bilirubin (Abraham et al., 2005). Exposure of PC12 cells to curcumin and resveratrol have been shown to increase HO-1 significantly in brain cells (Scapagnini et al., 2006; Rojo et al., 2008). Recent studies show that MEL can promote the nuclear transcription of Nrf2 and the expressions of target genes such as HO-1 (Yu et al., 2019). Consistent with the previous studies, PCA significantly upregulated HO1 expression and increased the activity of antioxidant enzymes (Han et al., 2018). Equally, PCA increased HO-1 expression in HUVEC (Funakohi-Tago et al., 2018) and neuroblastoma cells (Han et al., 2018). In the recent years, significant progress has been made in determining the

458 diverse roles of HO-1 in brain senescence and, agingrelated human neurodegenerative
459 disorders (Kitamuro et al., 2003). We have observed that HT, MEL + HT, HT + PCA and
460 MEL + HT + PCA in the absence of α-Syn fibrils increase the HO-1 gene which could be
461 considered as a protective action (Fig. 4C). On the other hand, HT and HT + PCA
462 incubated with α-Syn also increased its expression, reinforcing their potential.

463 It has been reported that Hsp70 may have a role both in refolding and in degrading
464 misfolded α-Syn molecules, thus providing protection against α-Syn toxicity. Hence,
465 molecular chaperones may be involved in regulating the biochemical characteristics and
466 toxicity of α-Syn (Ebrahimi-Fakhari et al., 2012). Accordingly, we have demonstrated that
467 HT, MEL + HT, HT + PCA in the presence of α-Syn increase Hsp70 gene expressing
468 (Fig. 4D). These data support the abovementioned hypothesis (Fig. 2, Table 3).
469 Moreover, our results show that the co-incubation of HT + PCA with PC12 cells in
470 absence of α-Syn also increase Hsp70 gene expression.

471 This is the first time that the role of the combination of MEL, PCA and HT against α-Syn
472 kinetics and toxicity has been studied, approximating reality in terms of the simultaneous
473 presence of several compounds in our diet and counteracting the low in vivo
474 concentration of these compounds.

475 Moreover, the present study shows that a number of mechanisms may be involved in
476 the neuroprotection properties observed against αSyn-induced toxicity of MEL, PCA, HT
477 and their combinations. The most important result is the increase on the expression of
478 SIRT-2 by the combination of MEL + HT + PCA in the absence of α-Syn fibrils. This effect
479 is observed to a less extension in the case of HO-1 gene expression.It is also worth
480 mentioning that the increase in gene expression in the absence of α-Syn since diet could
481 be a useful preventive mean of counteracting α-Syn toxicity, delaying neurodegenerative
482 processes.

483

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**Declaration of competing interest**: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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**Table**

**Table 1.** Primers for RT-PCR.

| mRNA | Primers |
|---|---|
| *β*-actin | Forward: 5’-TGTGATGGTGGGAATGGGTCA-3’ |
| | Reverse: 5’-TTTGATGTCACGCACGATTTCC-3’ |
| SIRT-1 | Forward: 5’TCATTCCTGTGAAAGTGATGACGA-3’ |
| | Reverse: 5’-GCCAATCATGAGGTGTTGCTG-3’ |
| SIRT-2 | Forward: 5’-TACCCAGAGGCCATCTTTGA-3’ |
| | Reverse: 5’-TGATGTGTGAAGGTGCCGT-3’ |
| Hsp70 | Forward: 5’- GGGCTCTGAGGAACCGAGC-3’ |
| | Reverse: 5’-CAGCCATTGGCGTCTCTC-3’ |
| HO-1 | Forward: 5’-ACTTTCAGAAGGGTCAGGTGTCC-3’ |
| | Reverse: 5’-TTGAGCAGGAAGGCGGTCTTAG-3’ |

**Table 2.** 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) |

**Table 3.** Percentages of inhibition of MEL, PCA, HT and their combinations against α-Syn fibril formation of three replicates (*n*=3) at high concentrations and low concentrations. (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 ± 0.2 | 73 ± 9 |

**Table 4.** Percentages of destabilization of MEL, PCA, HT and their combinations against α-Syn fibril formation of three replicates (n=3) at high concentrations and low concentrations. (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 5.** Percentages of increase on the cell viability of MEL, PCA, HT and their combinations against α-Syn- induced toxicity. Compounds concentrations: Mel (250 µM), PCA (100 µM) and HT (100 µM).

| Compounds | % Increase on the cell viability |
|---|---|
| MEL+α-Syn | 20 ± 3.2 |
| PCA+α-Syn | 18 ± 2.8 |
| HT+α-Syn | 34 ± 3 |
| MEL+PCA+α-Syn | 35 ± 1.3 |
| MEL+HT+α-Syn | 37 ± 2.5 |
| PCA+HT+α-Sy | 30 ± 2 |
| MEL+PCA+HT+α-Syn | 30 ± 1.2 |

Figure

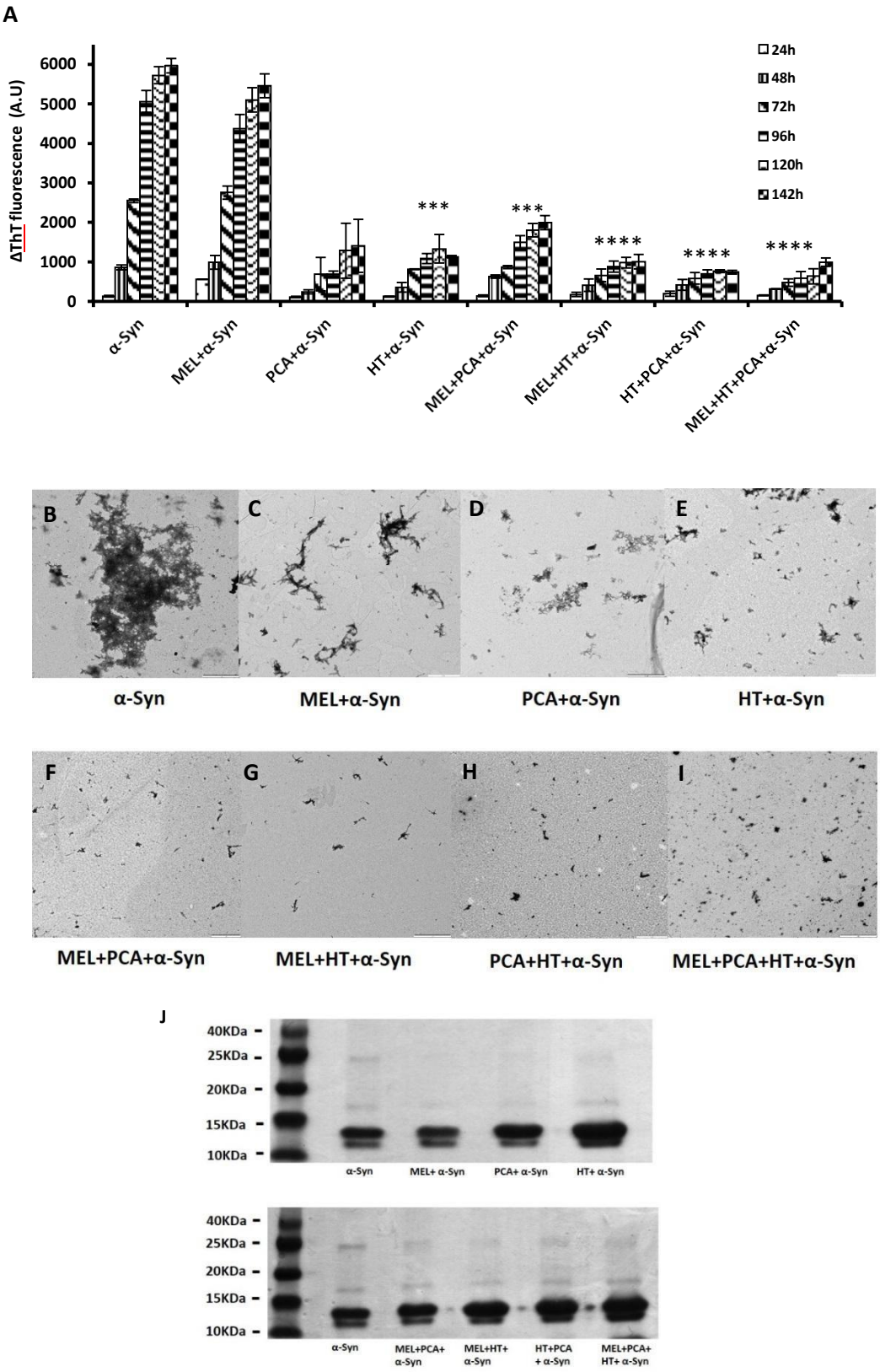


**Figure 1.** MEL, PCA, HT and their combinations inhibit α-Syn fibril formation (A) MEL (250 µM), PCA (100 µM), HT (100 µM), MEL+PCA, MEL+HT, HT+PCA and MEL+HT+PCA (the same compounds concentration were kept for the differents combinations) measured by ThT fluorescence assay at λEx 450 and λEm 485, 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.

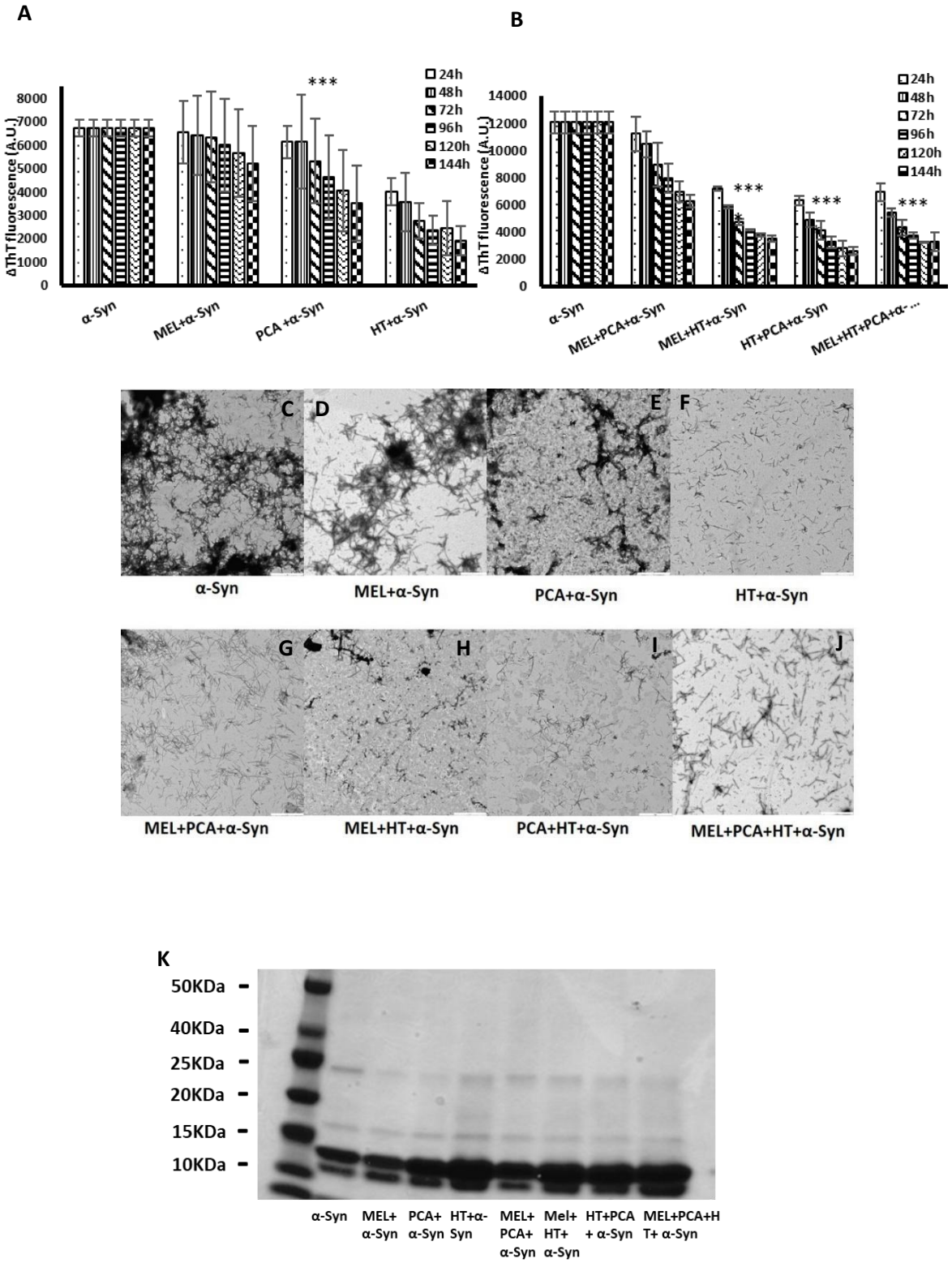


**Figure 2.** MEL, PCA, HT and their combinations destabilizes preformed fibrils of α-Syn (70 μM) (A) Effects of MEL (250 μM), PCA (100 μM) and HT (100 μM) on the kinetics of destabilization of α-Syn. (B) Effects of MEL+PCA, MEL+HT, HT+PCA and MEL+HT+PCA (the same compounds concentration were kept for the differents combinations) on the kinetics of destabilization of α-Syn (70μM) 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.

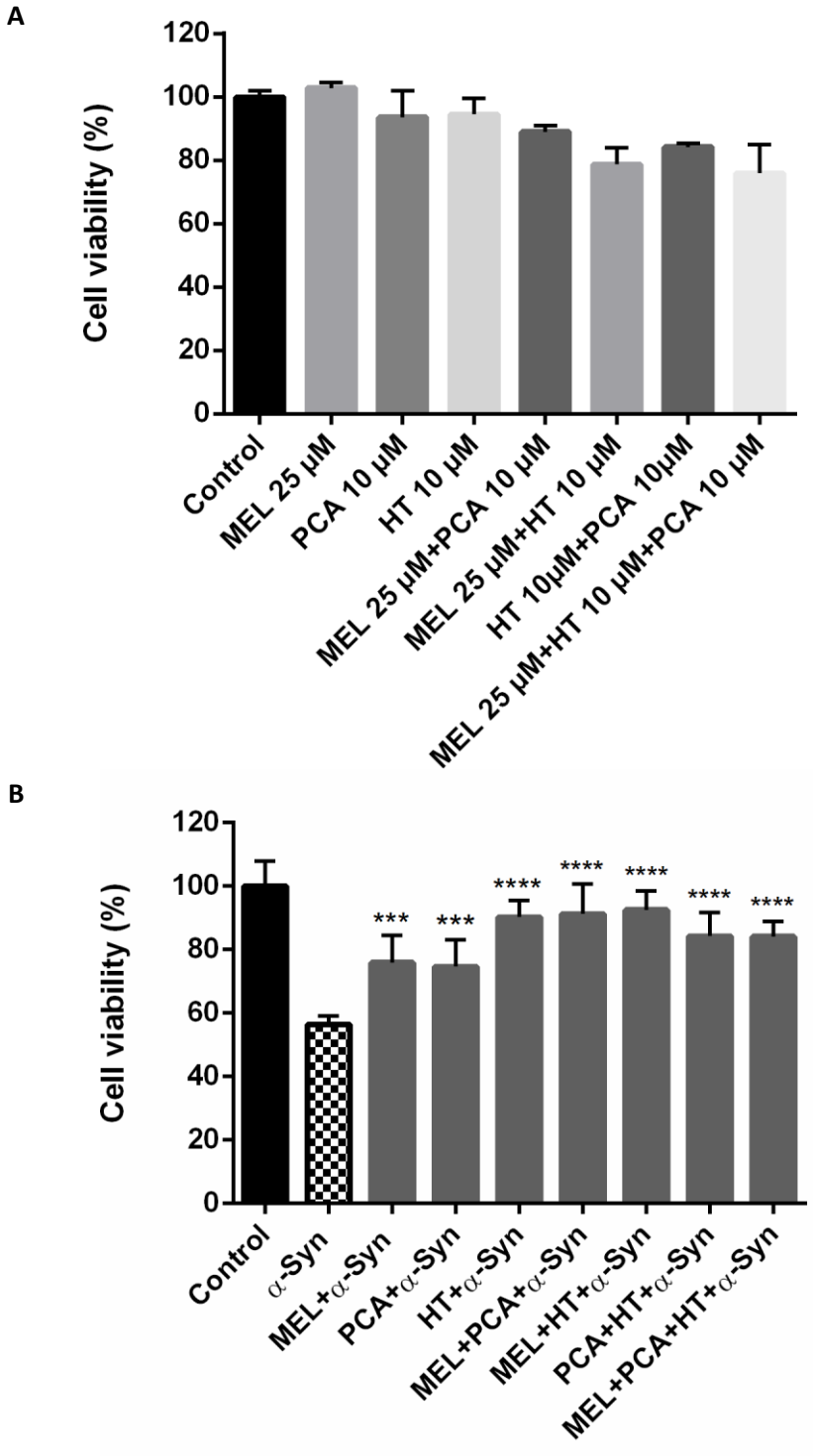


**Figure 3.** PCA prevents cell death caused by α-Syn toxicity: (A) Cell viability (%) (MTT test) of MEL (25 µM), PCA (10 µM), HT (10 µM), and their combinations (the same compounds concentration were kept for the differents combinations); (B) Cell viability (%) (MTT test) of MEL (25 µM), PCA (10 µM), 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). ****$p < 0.0001$; ***$p < 0.001$.

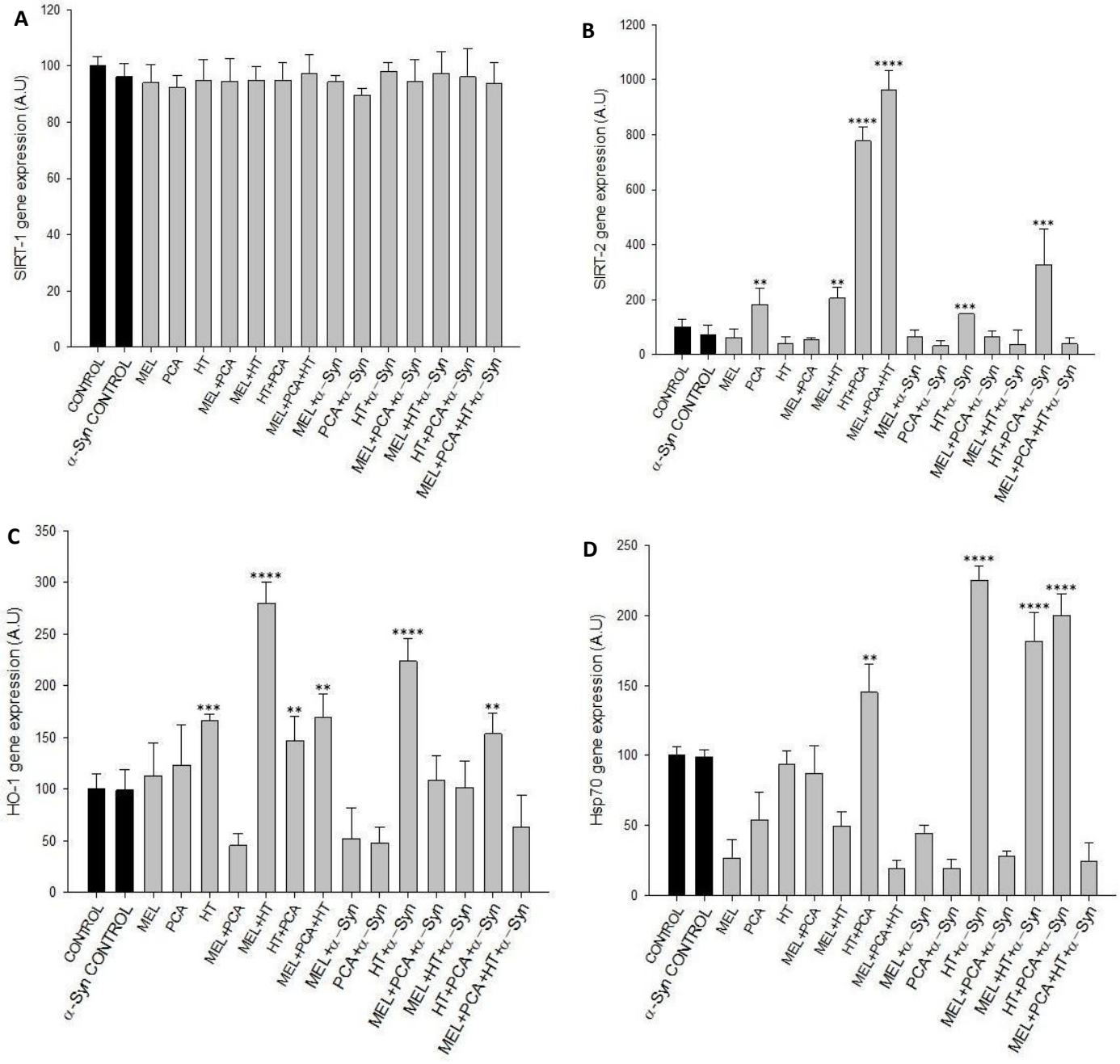


**Figure 4.** A) SIRT-1 gene expression (A.U); B) SIRT-2 gene expression (A.U); C) HO-1 gene expression (A.U); D) Hsp 70 gene expression (A.U) of α-Syn incubated alone (5 μM) or with MEL (25 μM), HT (10 μM), PCA (10 μM) and their combination and MEL (25 μM), HT (10 μM), PCA (10 μM) and their combination alone. Data are expressed as mean ± standard deviation (SD) (n=3). ****p < 0.0001; ***p < 0.001; **p <0.01) vs two controls (untreated cells and α-Syn fibrils alone).