



ORIGINAL ARTICLE

Sesamol as a potential small molecule in inhibiting the induction of neurotoxic amyloids fibrils involved in the pathophysiology of alzheimer's disease



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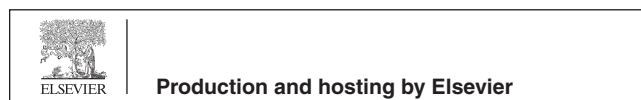
Abstract The presence of possibly toxic forms of intracellular α -synuclein, binding of aggregated α -synuclein and monomeric A β , and the potential co-localization of α -synuclein with intracellular neurofibrillary tangles as well as A β plaques in the brain could be the main causes in serious clinical symptoms mostly developed in Alzheimer's disease (AD) patients. Amyloid formation of α -synuclein can also induce neurotoxic effects through stimulation of endoplasmic reticulum stress (ERS). Therefore, the inhibition of protein aggregation can be of great importance for the control of pathophysiology of AD. Here, we assessed the use of sesamol as bioactive small molecule in inhibition of α -synuclein fibrillogenesis and neurotoxicity by different biophysical [ThT/Nile red/Congo red (CR)/circular dichroism (CD)] and cellular (MTT and qRT-PCR) analyses. From the spectroscopic analyses, we found that sesamol displays potential effect on the inhibiting the α -synuclein fibril formation. Also, sesamol showed no significant neurotoxicity and co-incubation of α -synuclein with sesamol apparently mitigated the ERS-mediated apoptosis induced by α -synuclein amyloids through regulation of IRE1, PERK, ATF6, and caspase-3 mRNA. Overall, sesamol-based compounds can be further developed and assessed for the regulation of pathophysiology of AD in which α -synuclein amyloids are predominantly involved.

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1. Introduction

A significant number of human disorders, including nervous system diseases, are associated with the formation of stable protein aggregates known as amyloid fibrils (Ross and Poirier, 2004). One of these disorders is a neurodegenerative disease called synucleinopathy, which is associated with the formation of amyloid accumulations of the α -synuclein in neurons, neurofibers, or glial cells known as Lewy bodies (Fujiwara et al., 2002; Jeon et al., 2020). The three main types of these diseases are Parkinson's disease (PD), dementia with Lewy body disease (LBD), and multiple system atrophy (MSA) (Twohig and Nielsen, 2019). It has been revealed that α -synuclein contributes in the pathophysiology of Alzheimer's disease (AD), whereas it was reported that Lewy-related pathology, mainly has α -synuclein, is existed in a most of autopsied brains suffering from AD (Twohig and Nielsen, 2019).

AD is known as one the most common types of dementia in people over 65 years of age (Jalbert et al., 2008). Pathological studies have suggested the formation of fibrous plaques and accumulated masses of α -synuclein in the central nervous system as one of the causes of pathophysiology of AD and inhibition of this process as an effective treatment for this type of disease (Twohig and Nielsen, 2019). It has been well reported that protein misfolding results in multiple assembly pathways through transiently formation of oligomers and protofibrils, which assemble into polymorphic fibrils after longer incubation (Goldsbury et al., 2005).

α -Synuclein as a protein with 140 amino acids belongs to the group of proteins with random coil structure (Lashuel et al., 2013). This protein has an amino terminal part, which contains a repetitive sequence with α -helix structure in the vicinity of the membrane, a middle part, which is completely hydrophobic and plays a major role at the beginning of the aggregation process, and carboxyl terminal part, with an acidic sequence plays a protective role against aggregations (Lashuel et al., 2013). In fact, α -synuclein contains one histidine and one tyrosine in the part which forms β -sheet-reach core of the fibril consisted mostly of the aliphatic amino acids (Lashuel et al., 2013).

Indeed, it has been shown that small molecules can prevent the amyloid formation of α -synuclein through regulation of aggregation kinetic parameters (Marchiani et al., 2013). Also, it has been reported that amyloid structures can interact with cell membranes and induce significant cytotoxicity through different signaling pathways and organelle stress which accelerate the induction of neurodegenerative disease (Marchiani et al., 2013; Pena-Diaz et al., 2020). For example, it has been displayed that α -synuclein amyloid fibrils triggered calcium release, generation of free radicals and mitochondrial deficit during neurodegeneration (Angelova and Abramov, 2017). Also, it has been reported the caspase activation through endoplasmic reticulum stress (ERS) is mediated by amyloid- β (Nakagawa et al., 2000).

Aggregated species of proteins with their unique features, widely produced in central nervous system, have adverse effects on biological systems. Various mechanisms have been proposed to justify the harmful processes of amyloid species, but increasing the levels of reactive oxygen species (ROS) which interact directly with DNA, proteins, and cellular lipids, disrupting the vital function of cellular components such as the nucleus, mitochondria, lysosomes, and ER (Li et al., 2021). The ER is involved in regulating protein production, toxin neutralization, carbohydrate metabolism, lipid production, and calcium homeostasis. Amyloid species-induced ERS leads to mitochondrial dysfunction and cell apoptosis (Li et al., 2021).

Therefore, finding new and potential small molecules with promising anti-amyloid properties to control the protein aggregation-induced cytotoxicity through regulation of ERS can be of great importance in the treatment of neurodegenerative diseases including AD.

Sesamol as a natural organic compound the main active compound of Sesame (*Sesamum indicum* L.) seeds has demonstrated a wide range of pharmacological perspectives (Bosebabu et al., 2020). For example,

it has been reported that sesamol can show protective effects against memory and cognitive impairments (Ren et al., 2018), depression (Kumar et al., 2011), and AD in rat model (Liu et al., 2021).

Therefore, the main novelty of this work is to explore the inhibitory effect of sesamol against α -synuclein amyloid production and associated cytotoxicity against neuron-like cells (PC-12). Also, the mechanism of cytotoxicity mediated by α -synuclein aged in the amyloidogenic buffer with or without sesamol through ERS was aimed to be evaluated.

2. Materials and method

2.1. Materials

α -Synuclein, Thioflavin T (ThT), Congo red (CR), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) were obtained from Sigma-Aldrich Co. (USA). Fetal bovine serum (FBS) and antibiotics were purchased from Gibco Co. (USA).

2.2. Amyloid preparation

α -Synuclein concentration after centrifugation (100,000 g for 15 min) was calculated spectrophotometrically at 275 nm with an extinction coefficient of $5120 \text{ M}^{-1} \text{ cm}^{-1}$ (Meng et al., 2010). The formation of α -synuclein amyloid fibrils by dissolving α -synuclein monomers (100 μM) in the amyloidogenic buffer (20 mM sodium phosphate pH 7.4) was performed at 37 °C (Maqbool et al., 2020) supplemented with 5, 50, and 100 μM sesamol prepared in DMSO, where the final concentration of DMSO was not exceed than 1% (v/v). As a control, DMSO was added to the buffer. For all assays, α -synuclein samples aged for 60 h were withdrawn and used for further analyses.

2.3. ThT fluorescence assay

The fluorescence analysis was done with a fluorescence spectrophotometer (Cary Eclipse VARIAN, Australia) at 25 °C. To explore the inhibitory effect of sesamol with different concentrations of 5, 50, and 100 μM on the formation of α -synuclein amyloid fibril after 60 h, 15 μL of α -synuclein samples (100 μM) were added to 895 μL of 10 μM ThT solution, vortexed, and incubated for 7 min. The fluorescence emission data were then detected at excitation of 440 nm and the emission was read in the range of 460–550 nm. The excitation and emission slit widths were both set at 5 nm. All data were corrected against sesamol fluorescence intensity along with inner filter effect (Nielsen et al., 2001).

2.4. Nile red fluorescence assay

The set up for Nile red fluorescence analysis was similar as reported for ThT assay. The excitation wavelength was set at 550 nm and emission spectra were read between 580 and 760 nm. Both excitation and emission slit widths were set at 5 nm.

2.5. Congo red (CR) absorption assay

Aliquots of α -synuclein samples (10 μM), previously co-incubated with or without various concentrations of sesamol

for 60 h, were mixed with CR solution (960 μL , 20 μM). After 45 min of incubation at 25 $^{\circ}\text{C}$, CR absorbance was read between 440 and 600 nm using a UV-Vis spectrophotometer (Hitachi U 2000).

2.6. Circular dichroism (CD) assay

Far-UV CD (190–260 nm) spectra of different α -synuclein samples aged alone or with different concentrations of sesamol for 60 h were detected using a spectropolarimeter (Aviv Associates, USA), equipped with a 1 mm path cell at 25 $^{\circ}\text{C}$. The protein concentration was set at 10 μM .

2.7. MTT assay

PC-12 cells (5×10^3 cells/well) were cultured into 96-well plates in DMEM/F12 cell culture medium supplemented with FBS (10%) and antibiotics (1%). After 24 h, the cells were replaced and added by the same medium containing α -synuclein samples or mixture of protein (10 μM) and sesamol with final concentrations of 0.5, 5 and 10 μM (aged for 60 h) for 24 h. The medium was then replaced with 5 mg/mL MTT solution and incubated for another 3.5 h at 37 $^{\circ}\text{C}$. The MTT solution was then gently removed and then 150 μL of DMSO was poured

into each well for 5 min and the optical density was read at 570 nm employing a microplate reader (SpectraMax M5, USA). Cells without any protein or sesamol incubation used as the control sample and the data were reported as a percentage of control.

2.8. Quantitative real time PCR (qRT-PCR) assay

After seeding and treating the cells with the different solutions of α -synuclein fibrillation products (10 μM) for 24 h, total RNA was extracted with the TRIzol reagent (Invitrogen Life Technologies, USA) and cDNA was synthesized employing the relevant RT kit (Invitrogen Life Technologies, USA) based on the manufacturers' instructions. SYBR-Green PCR master mix (Invitrogen Life Technologies, USA) was used for qRT-PCR analysis. The program and data analysis were set up based on the previous reports (Livak and Schmittgen, 2001; Tu et al., 2013).

2.9. Statistical analysis

The data were reported as mean \pm standard error of five independent assays. Student's *t*-test and one-way ANOVA were

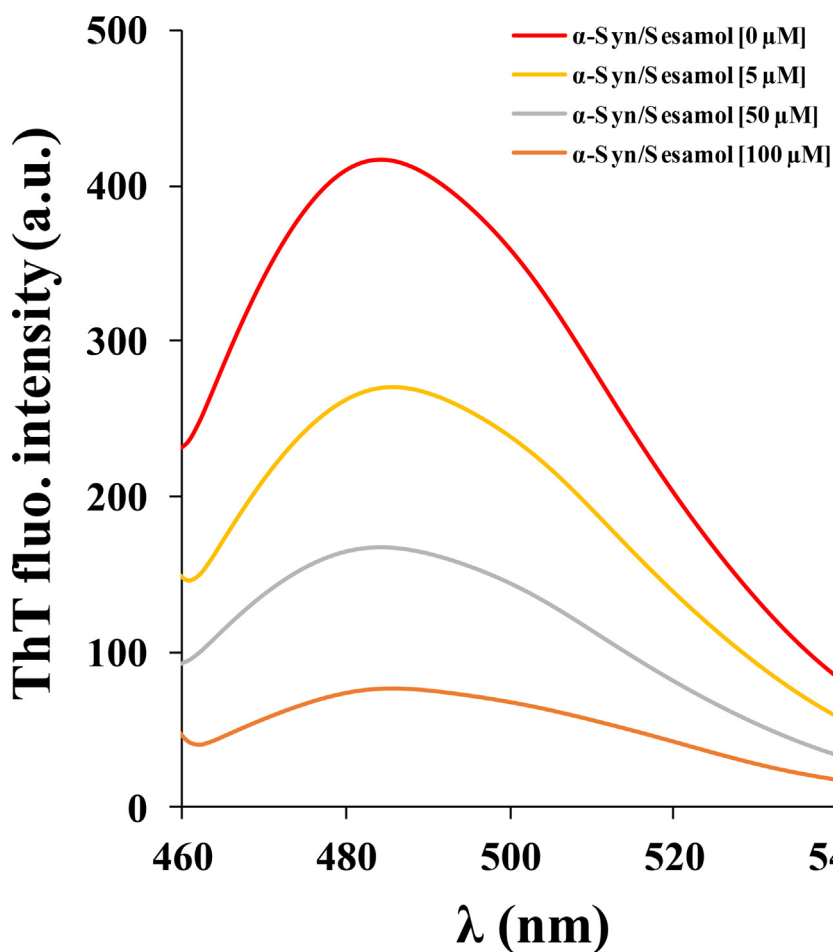


Fig. 1 ThT fluorescence analysis of α -synuclein aged for 60 h in the aggregation buffer with or without different concentrations of sesamol (5, 50, 100 μM).

performed to assess the statistical difference (P -value < 0.05) using SPSS software.

3. Results

3.1. Determining α -synuclein fibrillization with or without sesamol

The progress of α -synuclein fibrillization (100 μ M) was revealed by using ThT at 25 $^{\circ}$ C by reading fluorescence emission in the range of 460–550 nm. To examine the inhibitory effect of sesamol, α -synuclein fibrillization was performed in the presence of different concentrations of sesamol. As displayed in Fig. 1, sesamol resulted in an apparent decrease in the ThT fluorescence in a concentration-dependent manner. It can be suggested that ThT fluorescence is probably related to the level of amyloid fibrillization and the decrease in the ThT fluorescence can be determined to assess the inhibitory potential of sesamol (Naiki et al., 1989; Jameson et al., 2012). An apparent decline in ThT fluorescence would suggest that sesamol can prevent the α -synuclein fibrillization.

3.2. Nile red fluorescence assay

Based on probable conformational changes of proteins that may occur during fibrillation, it has been well-established that

different stimuli and aggregation buffers can trigger the formation of denatured structures in protein, characterized by appearance of relatively mobile hydrophobic patches on the protein surface (Gilan et al., 2019; Pang et al., 2021). Nile red fluorescence analysis as a hydrophobic reporter dye, showed formation of apparent hydrophobic groups on the surface of the α -synuclein amyloid (Fig. 2). As exhibited in Fig. 2, the presence of sesamol declined the Nile red fluorescence intensity along with a significant red shift, indicating the prevention of the formation of hydrophobic moieties in α -synuclein amyloid structure by sesamol in a probable concentration-dependent manner. Taking these data together, it seems quite likely that sesamol inhibits α -synuclein amyloid fibrillization by preventing the structural changes of protein.

3.3. CR analysis

To further investigate whether sesamol influences the process of α -synuclein fibrillization, CR absorption assay was done. It was seen that although α -synuclein fibrillization resulted in a significant increase along with a blue shift in CR absorbance, sesamol was effective in inhibiting amyloid fibril formation of α -synuclein in a concentration dependent manner, as evidenced by the apparent reduction in both CR absorbance and blue shift (Fig. 3).

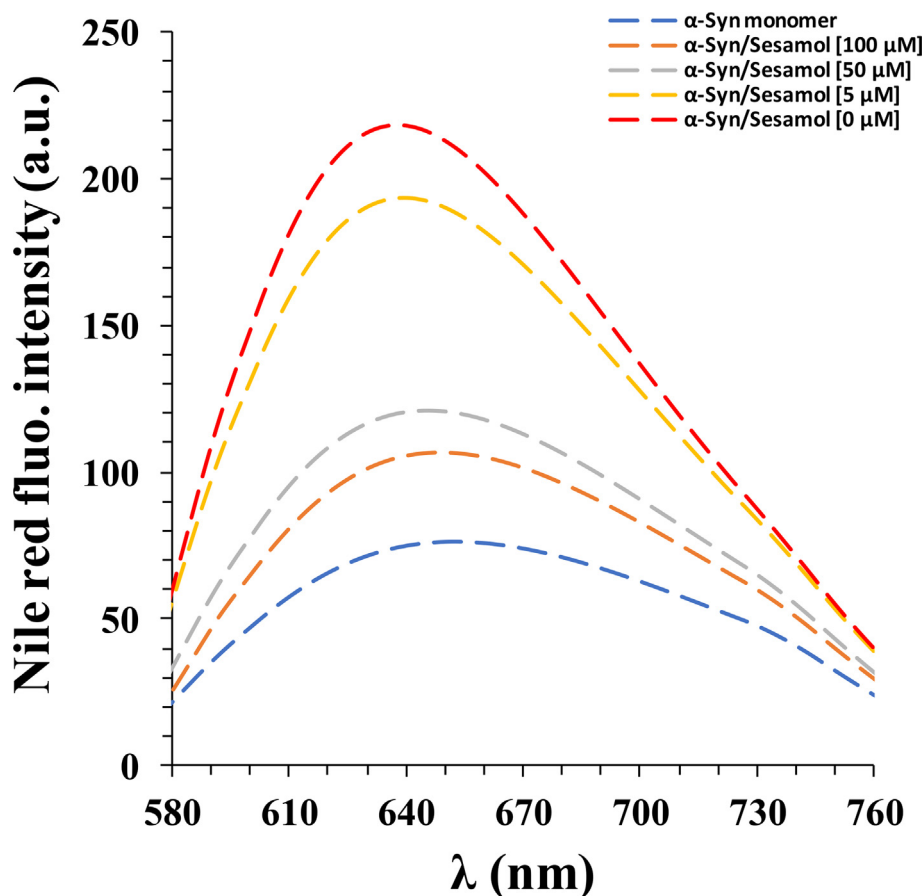


Fig. 2 Nile red fluorescence analysis of α -synuclein aged for 60 h in the aggregation buffer with or without different concentrations of sesamol (5, 50, 100 μ M).

3.4. Far-UV CD analysis

To investigate whether secondary conformational alterations of α -synuclein induced by the aggregation buffer could be inhibited by sesamol, far-UV CD analysis of the α -synuclein was performed for different α -synuclein samples at the end of fibrillation process. As manifested in Fig. 4, the far-UV CD band of α -synuclein, after incubation in an aggregation buffer, alters from showing a minimum around 199 nm, features of the random coil conformation of the α -synuclein monomer, to a deep band around 218 nm, as reported for intermolecular β -sheet structures (Zand et al., 2019; Khodabandeh et al., 2020). However, sesamol with the studied concentrations afforded apparent inhibition on the formation of intermolecular β -sheet structures. Also, based on the characteristic alteration of the bands, namely the reduction of the intensity of minimum around 218 nm, this reduction in ellipticity intensity was further induced in the presence of higher concentrations of sesamol than that of lower concentrations.

As revealed by the analysis indicated above, sesamol protects α -synuclein against secondary structural alterations.

It has been well-reported that amyloid species as intermediates products in the early stage of fibrillation process, may be dominantly responsible for amyloid-induced cytotoxicity, rather than the mature amyloid fibrils (Yang et al., 2020).

Based on this fact, it is of great importance to assess whether the kinds of aggregates formed in the presence of sesamol are less cytotoxic products or not.

3.5. MTT assay

The cytotoxicity of the α -synuclein (100 μ M) species formed in the aggregation buffer with or without different concentrations of sesamol (5, 50, and 100 μ M) was assessed using the MTT assay (Fig. 5). For MTT assay, the protein samples and corresponding sesamol concentrations were diluted 10-fold. Therefore, PC-12 were cultured with different samples for 24 h and MTT assay was analyzed. The cytotoxicity of sesamol and α -synuclein monomers with the concentration of 10 μ M was found to be negligible as compared to the control group. Notably, the cytotoxicity of α -synuclein amyloid without sesamol was significantly higher than that of α -synuclein amyloid with the similar procedure in the presence of varying concentrations of sesamol. Also, it was shown the inhibitory effect of sesamol on the mitigation of α -synuclein amyloid formation and associated cytotoxicity was based on a concentration-dependent manner. This data suggests that the presence of a therapeutic sesamol can inhibit the α -synuclein aggregation and underlying neurotoxicity without a significant injury to neurons.

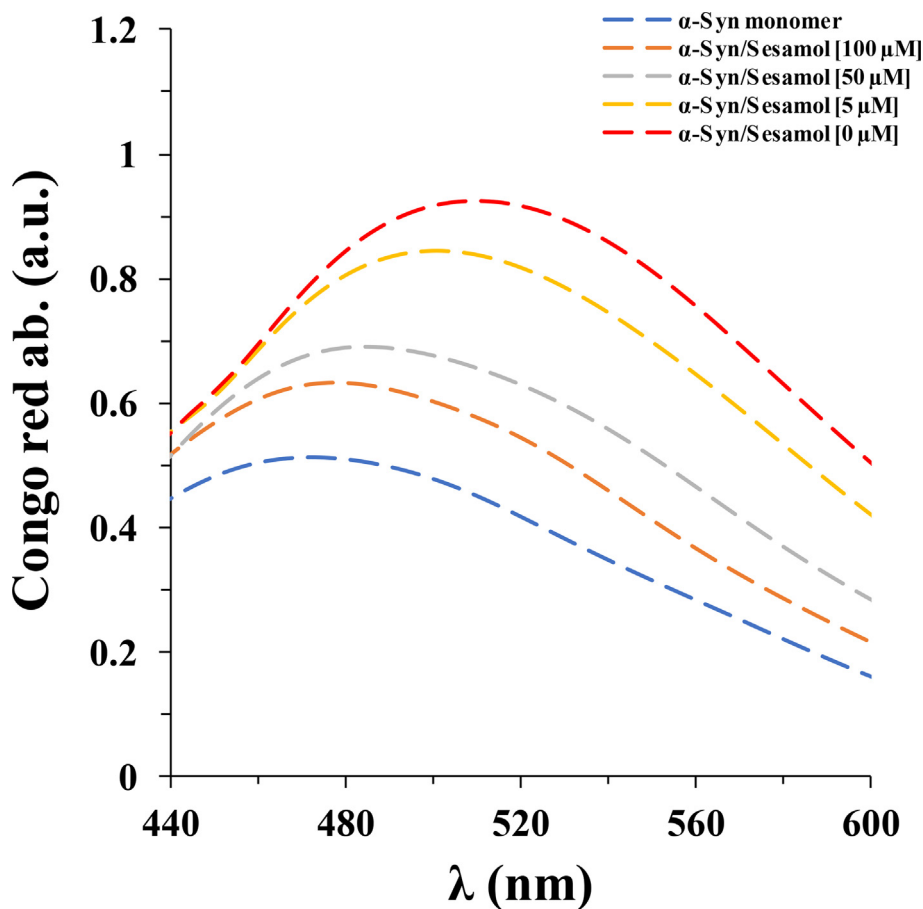


Fig. 3 CR absorption analysis of α -synuclein aged for 60 h in the aggregation buffer with or without different concentrations of sesamol (5, 50, 100 μ M).

3.6. qRT-PCR assay

Different external stimuli including protein amyloids, can stimulated ERS which led to abnormal protein folding, *i.e.*, unfolded protein response (UPR) comprised the important signaling pathways through inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Kaneko et al., 2017; Amen et al., 2019). We then assessed the expression of these well-known ERS markers, to explore the impacts of α -synuclein amyloid aged for 60 h in the aggregation buffer with or without sesamol on ERS in PC-12 cells at 24 h. It was shown that α -synuclein amyloid alone apparently triggered overexpression of IRE1 (Fig. 6a), PERK (Fig. 6b), ATF6 (Fig. 6c), which results in induction of apoptosis through caspase-3-dependant pathway (Fig. 6d). However, α -synuclein amyloid aged in the presence of sesamol induced downregulation of all studied genes relative to α -synuclein amyloid alone. These data supported that α -synuclein amyloids lead to ERS and associated apoptosis induction in PC-12 cells and this kind of stress can be mitigated after incubation of cells with α -synuclein amyloid aged with sesamol in a concentration-dependent manner.

4. Discussion

In this study, we explored the protective effects of sesamol on the formation of α -synuclein fibrillization and associated neu-

rotoxicity *in vitro*. To the best of our knowledge, this is the first study to show that the neurotoxicity stimulated by α -synuclein amyloids can be probably alleviated by co-incubation of α -synuclein samples with sesamol in an aggregation buffer though inhibiting the ERS-mediated apoptosis.

AD is a recognized global health priority because it imposes high costs on health care and economic systems (Jalbert et al., 2008). AD is a neurodegenerative disease known as the one of the common forms of dementia that cognitive and behavioral disorders are its symptoms and one of the important symptoms of its pathology is the destruction and degeneration of neurons (Warren et al., 2023).

Pathological markers of AD include the presence of amyloid plaques in extracellular and intracellular of neurons. Small molecules can activate cellular pathways such as the ubiquitin protease system, autophagy, apoptosis, and chaperones to protect cells against protein amyloid-induced neurotoxicity (Pourhanifeh et al., 2019).

Interactions of amyloid species of proteins with cells and deregulation of autophagy, apoptosis, inflammation and oxidative stress are important factors in the onset of neurodegenerative diseases (Pourhanifeh et al., 2019). Organized studies on the biological effects of amyloid species of proteins and their mechanism result in developing potential platforms for treatment of neurodegenerative diseases. So far, little information has been obtained about the toxicity mechanism of amyloid species of proteins. In this paper, in addition to

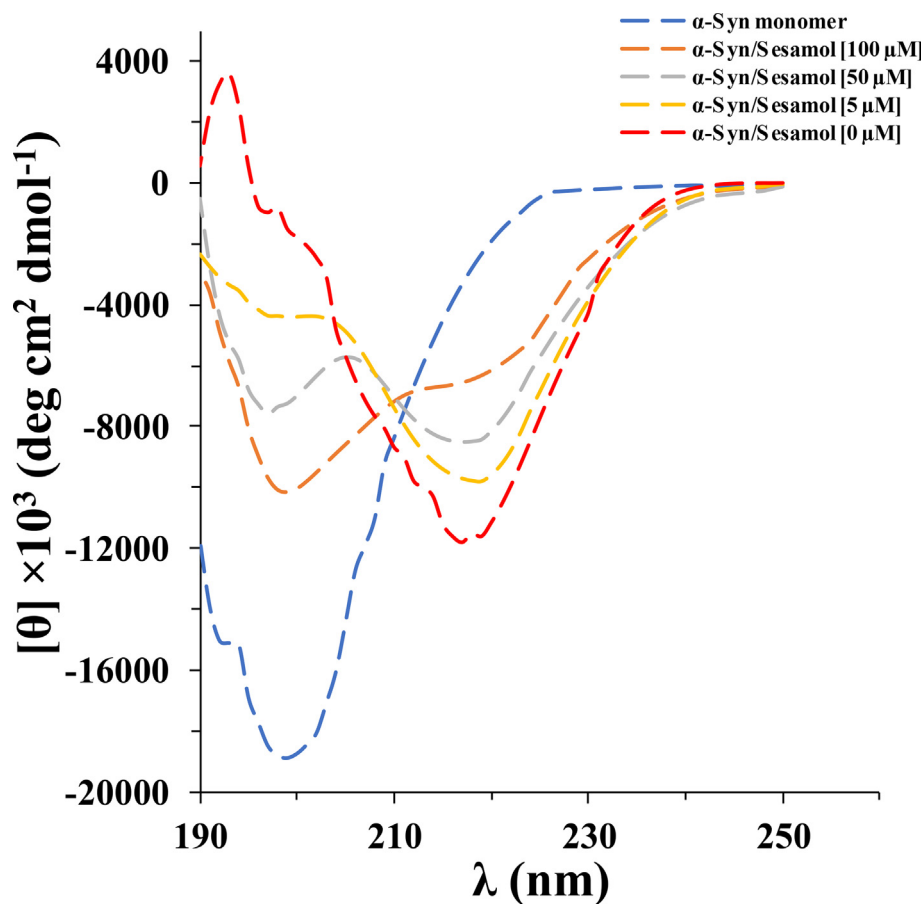


Fig. 4 Far-UV CD analysis of α -synuclein aged for 60 h in the aggregation buffer with or without different concentrations of sesamol (5, 50, 100 μ M).

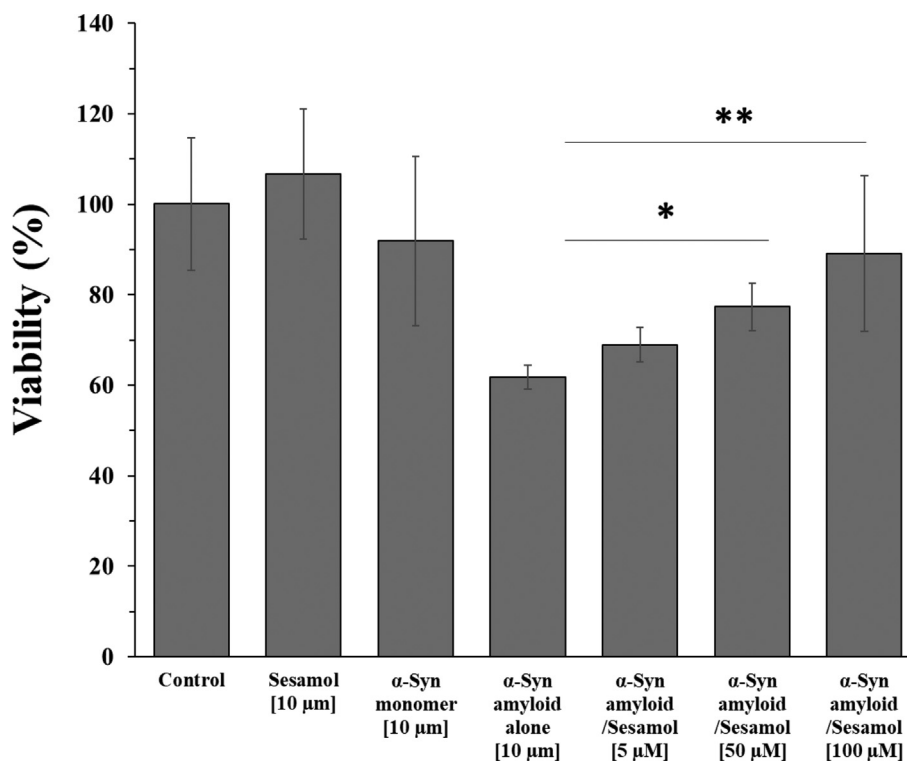


Fig. 5 MTT assay of PC-12 cells after incubation with α -synuclein amyloid aged for 60 h in the aggregation buffer without or with different concentration of sesamol after 24 h. * $P < 0.05$ and ** $P < 0.01$ relative to control samples.

characterization of amyloid fibrils formed in the presence of sesamol, we also examined the neurotoxicity of the aggregated species through ERS-mediated signaling pathway.

It has been indicated that ERS is considered as a crucial factor in the development of AD (Santos and Ferreira, 2018). Homeostasis of folded proteins in the ER is critical to cell function, and the presence of extracellular and intracellular abnormalities including protein amyloid fibrils cause accumulated proteins and incorrectly folded proteins in the ER (Huang et al., 2007). ERS has been observed in AD (Santos and Ferreira, 2018) which results in the activation of transcriptional protein response pathways, including the transcription factor 6 activator, inositol bound to protein 1, and the R-like protein kinase of the endoplasmic reticulum (Mahdi et al., 2016).

The role of ERS-induced apoptosis in neurodegenerative diseases has been well-reported (Xiang et al., 2017). Apoptosis can develop the onset and progression of many disorders including neurodegenerative diseases (Mattson, 2000). ERS-induced apoptosis is a process responsible for the destruction of damaged neurons (Huang et al., 2007).

It has been well-documented that formation of protein amyloids leads to ERS-induced apoptosis *in vitro* (Askanas and Engel, 2011) and some small molecules including Epigallocatechin gallate (Du et al., 2018) as well as bioactive compounds of kimchi (Woo et al., 2018) and syringic acid (SA) (Li et al., 2021) mitigate protein aggregated species-triggered neurotoxicity through inhibiting ERS-associated apoptosis.

Actually, it has been recently reported that SA can inhibit the fibrillation of tau protein as another important protein in the onset of neurodegenerative diseases through controlling

amyloid kinetic parameters and inhibition of structural changes of protein verified by a number of biophysical and imaging techniques (Li et al., 2021). Furthermore, the protein species that appeared in the presence of SA showed lower adverse effects on the induction of ERS than amyloid species alone by regulation of ATF-6, caspase-8 and caspase-3 genes (Li et al., 2021), which was in good agreement with the data found in this paper. In conclusion, it can be proposed that small molecules can be used as potential compounds in the development of effective platforms against protein aggregation. In fact, small molecules like sesamol can interact with monomeric states of proteins via different forces and inhibit the protein-protein interaction as a critical step in the protein aggregation pathway through delaying the rate constant of protein fibrillation. Also, it should be noted that some limitations of this study including the lack of exploring the direct interaction of sesamol with α -synuclein as well as the disaggregation of preformed amyloid species in the presence of sesamol, should be explored in the future studies *in vitro* and *in vivo*. However, based on this study we can claim that interaction of sesamol with monomeric species of α -synuclein are likely to inhibit the formation of amyloid fibrils and relevant neurotoxicity (ERS and apoptosis) evidenced by ThT/Nile red fluorescence, CR absorbance, CD, and cellular analyses.

Indeed, small molecules due to their unique structures can prevent the formation of amyloid fibrils due to potential interactions with proteins and resultant protein stability (Porat et al., 2006; Gazit, 2002). In fact, protein-protein interactions can play a key role in the formation of amyloid fibrils and any molecules that can target these forces, can be used as a potential anti-amyloid agent (Stains et al., 2007). Also, it should be

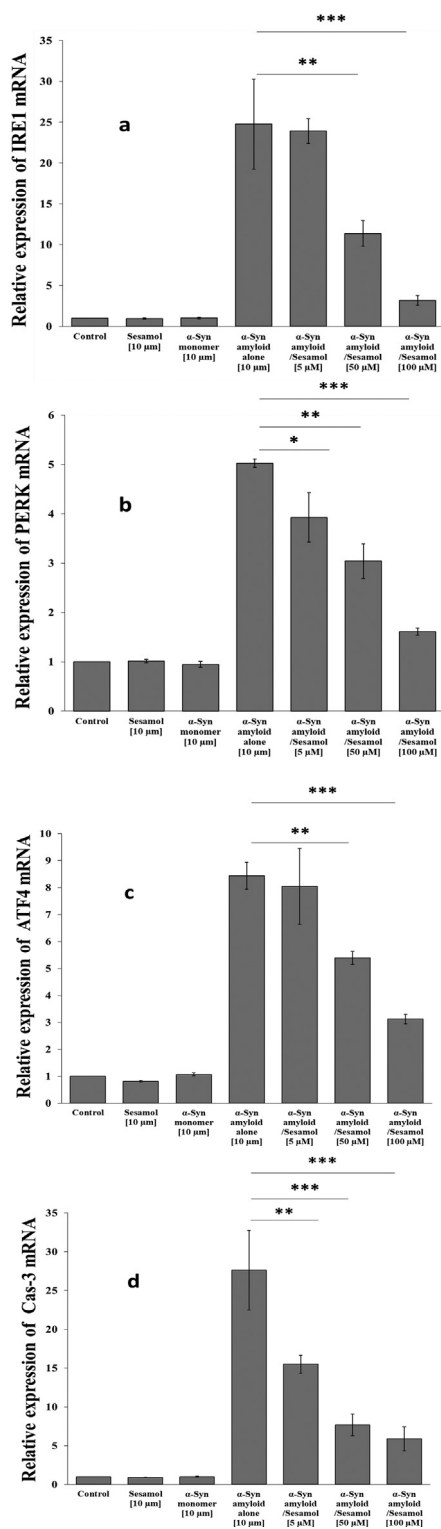


Fig. 6 (a) IRF1 mRNA expression, (b) PERK mRNA expression, (c) ATF6 mRNA expression, (d) caspase-3 mRNA expression in PC-12 cells after incubation with α -synuclein amyloid aged for 60 h in the aggregation buffer without or with different concentration of sesamol after 24 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: relative to control samples.

noted that the iron in varying concentrations (Ganguly et al., 2020) can play a key role in implications in the pathogenesis of neurodegenerative diseases through induction of mitochondrial dysfunction mediated via different signaling pathways (Park et al., 2020).

5. Conclusion

In this study we found that sesamol can prevent the formation of α -synuclein fibrillization and underlying ERS-mediated apoptosis in a concentration dependent manner. Our findings suggest that sesamol can be used as a potential candidate for prevention of α -synuclein amyloids involved in the pathophysiology of AD and relevant regulation of ER stress-associated apoptosis in neurons.

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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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