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In vitro Evidence that an Aqueous Extract of *Centella asiatica* Modulates α-Synuclein Aggregation Dynamics

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Abstract. α-Synuclein aggregation is one of the major etiological factors implicated in Parkinson's disease (PD). The prevention of aggregation of α-synuclein is a potential therapeutic intervention for preventing PD. The discovery of natural products as alternative drugs to treat PD and related disorders is a current trend. The aqueous extract of *Centella asiatica* (CA) is traditionally used as a brain tonic and CA is known to improve cognition and memory. There are limited data on the role of CA in modulating amyloid-β (Aβ) levels in the brain and in Aβ aggregation. Our study focuses on CA as a modulator of the α-synuclein aggregation pattern *in vitro*. Our investigation is focused on: (i) whether the CA leaf aqueous extract prevents the formation of aggregates from monomers (Phase I: α-synuclein + extract co-incubation); (ii) whether the CA aqueous extract prevents the formation of fibrils from oligomers (Phase II: extract added after oligomers formation); and (iii) whether the CA aqueous extract disintegrates the pre-formed fibrils (Phase III: extract added to mature fibrils and incubated for 9 days). The aggregation kinetics are studied using a thioflavin-T assay, circular dichroism, and transmission electron microscopy. The results showed that the CA aqueous extract completely inhibited the α-synuclein aggregation from monomers. Further, CA extract significantly inhibited the formation of oligomer aggregates and favored the disintegration of the preformed fibrils. The study provides an insight in finding new natural product for future PD therapeutics.

Keywords: α-Synuclein, anti-aggregation, *Centella asiatica*, circular dichroism, natural products, Parkinson's disease, transmission electron microscopy

INTRODUCTION

Parkinson's disease (PD) arises from the loss of dopaminergic neurons in the substantia nigra region of the brain [1]. The etiology of PD is complex and the mechanisms of the development of the disease are still not clear [2, 3]. Among the different mechanisms proposed, the aggregation pathway of α-synuclein is considered as the classical pathway leading to the disease [4–6]. In this pathway, the central event is that monomers of α-synuclein form soluble oligomers, which finally lead to the formation of mature insoluble fibrils in the brain through a multistep process [7, 8]. Inhibition of α-synuclein aggregation has become a major therapeutic target [9]. Various compounds, both synthetic and natural, are being used against the neurodegenerative diseases, targeting protein aggregation as a central pathway [10]. Currently, screening of potential compounds for preventing α-synuclein aggregation is a drug target. Natural compounds are a good source of potential compounds to modulate neurodegenerative diseases [11–16].

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Centella asiatica (CA), a herb grown in India and other Asian countries, is considered to be a potential candidate drug against neurodegenerative diseases. The plant is used widely in Indian traditional medicine as a brain tonic [17, 18]. There are limited studies showing that CA has the ability to increase memory and cognition [19, 20]. Gadahad et al. [21] have shown that CA has neuronal dendritic growth stimulating properties. Xu et al. [22] showed that a CA extract enhanced the phosphorylation of cyclic AMP response element binding protein and thus played a role as a memory enhancer. An aqueous extract of CA is found to prevent streptozotocin-induced cognitive deficits in rats [23]. The CA extract decreased the amyloid load in the brains of animal models with Alzheimer's disease [24]. All of the above studies and traditional knowledge indicate that compounds in the leaf extract of CA may have neuroprotective potential. However, there are no studies to indicate whether CA could be of potential use against PD.

PD is characterized by the presence of Lewy bodies (α-synuclein aggregates) in the substantia nigra. α-Synuclein aggregates into fibrils through multiple steps involving soluble oligomers and insoluble protofibrils and fibrils [7, 8]. We analyzed the potential role of a CA leaf aqueous extract in inhibiting the α-synuclein aggregation under *in vitro* conditions. We used an *in vitro* aggregation model as a quick battery test for the screening of compounds for their anti-aggregating potentials. We analyzed the anti-aggregating properties of CA leaf extract in the three steps of α-synuclein aggregation dynamics: (i) Phase I: monomer to aggregates, (ii) Phase II: oligomer to aggregates, and (iii) Phase III: disintegration of preformed fibrils of α-synuclein aggregation.

MATERIALS AND METHODS

α-Synuclein was purchased from rpeptide, USA. Tris buffer, glycine, sodium hydroxide, hydrochloric acid, and uranyl acetate were procured from BDH Laboratory chemicals Division. Thioflavin-T was purchased from ICN Biomedicals Pvt. Ltd, USA. Copper grids (200 mesh size) were purchased from Sigma Chemicals, USA.

Preparation of aqueous extract from the leaves of Centella asiatica

CA was grown under controlled conditions. The plant was cleaned with triple-distilled water and the leaves were separated and freeze-dried. The leaf aqueous extract was prepared by boiling the leaves in triple-distilled water and lyophilized and stored at −80°C.

Experimental design

We tested for anti-aggregating properties of the CA leaf aqueous extract in three phases of the aggregation pathway. First, we sought to understand the prevention of α-synuclein aggregation from monomers to aggregates (Phase I). α-Synuclein (50 μM) was dissolved in 20 mM Tris-HCl pH (8.4), and 100 μg of freshly dissolved CA leaf aqueous extract was added and continuously stirred using magnetic beads. We studied the aggregation kinetics to follow monomer-oligomer-aggregate phases as a function of time (0–96 h). Aliquots were taken at regular intervals of 8 h starting for 0 h to 96 h for the thioflavin-T and transmission electron microscopy (TEM) analyses. A control experiment of α-synuclein aggregation alone, in the absence of leaf extract, was carried out as above. α-Synuclein was dissolved in triple-distilled water and centrifuged at 10,000 rpm at 4°C before incubating for the aggregation. The supernatant was taken for the monomers of α-synuclein and any aggregates remained in the precipitate. Second, we sought to understand the prevention of α-synuclein aggregation from oligomer to aggregates (Phase II). α-Synuclein oligomers were prepared according to Danzer et al. [25] with little modification. α-Synuclein (50 μM) was dissolved in Tris buffer and incubated at 37°C with constant stirring for 24 h (at which point oligomers appeared). The incubation mixture was then centrifuged at 10,000 rpm to remove any protofibrils formed, and the supernatant, which contained the oligomers, was removed. We added 100 μg of CA leaf aqueous extract to the oligomers and incubated the mixture for aggregation at 37°C with constant stirring. Aliquots were taken at time intervals of 0 h, 24 h, 48 h, and 96 h for thioflavin-T and TEM analyses. Finally, we conducted a third experiment to understand the efficiency of the CA extract to disintegrate the pre-formed fibrils (Phase III). Here, freshly prepared α-synuclein was allowed to form mature fibrils for 96 h and then 100 μg extract CA was added to the mature fibrils. We then followed the disintegration of fibrils for 10 days.

Thioflavin-T assay

The thioflavin-T assay was conducted to understand the protein aggregation kinetics. Thioflavin-T specifically binds to aggregates. 25 μl of (1 mM)

thioflavin-T was added to 1000 µl of total reaction volume. Thioflavin-T fluorescence was measured at excitation and emission wavelengths of 446 nm and 482 nm, respectively, using a F4500 Hitachi fluorescence spectrometer. The background thioflavin-T fluorescence intensity was subtracted from the experimental values.

Transmission electron microscopy study

TEM was used to detect the presence or absence of aggregates. 10 µL of incubated sample was placed on a carbon-coated copper grid (200 mesh size) and allowed to rest for 1 min. Excess sample was wicked off with lens paper and then negatively stained by transferring the grid face down to a droplet of (2% (w/v) uranyl acetate for 1 min before wicking off the solution. Then the grids were air dried for 1 h. Four individual experiments were carried out for each sample. The grids were completely dried to avoid moisture and then scanned under JOEL 1010 TEM.

Circular dichroism (CD) studies of the aggregation of α-synuclein

A CD study was conducted to understand the binding and to observe any secondary conformation changes of α-synuclein in the three phases of the aggregation process. 20 µL of incubated sample was taken and spectra were recorded on a JASCO J700 Spectropolarimeter at 25°C, with 2 mm cell length in the wavelength range between 200–320 nm in Tris-Cl buffer (5 mM, pH 7.4). Each spectrum was calculated as the average of four scans.

SDS-polyacrylamide gel electrophoresis

To further understand whether molecules in the CA extract bind to α-synuclein, we conducted SDS-PAGE for the samples [26]. 15 µl of pre-incubated samples were mixed with 15 µl of SDS-PAGE sample buffer and loaded onto a 12% SDS- polyacrylamide gel without boiling. Low molecular weight markers were used as molecular weight standards. The samples were electrophoresed at 100 V at room temperature for 4 h. The gels were stained for protein with 0.1% Coomassie Brilliant Blue.

Estimation of polyphenols in the CA water extract

The total phenol content of the CA water extract was estimated using Folin-Ciocalteu (F-C) reagent [27]. The polyphenols in the aqueous extract of CA were separated on a reverse-phase C18 column (4.6 × 2.50 mm) using HPLC system (agilent-Model 1200 series) and a diode array detector (operating at 280 nm and 320 nm). The solvent system consisted of a mixture of water: methanol: acetic acid (83 : 15 : 2), which was used as mobile phase (isocratic) at a flow rate of 1 ml/min [28]. The known quantities reference phenolic acid standards such as caffeic acid, p-coumaric acid, cinnamic acid, ferulic acid, gallic acid, gentisic acid, protocatechuic acid, syringic acid, and vanillic acid were studied for identification and quantification of phenolic acids present in the extract. We also estimated the levels of asiatic acid by HPLC and selenium by ICP-AES.

Statistical analysis

All data were analyzed using SAS/STAT® Software, USA.

RESULTS

Phase I

Thioflavin-T assay

Thioflavin-T fluorescence of aggregation kinetics followed a sigmoidal curve. Figure 1A shows three phases of aggregation kinetics of α-synuclein as a function of time (0–96 h). The thioflavin-T data indicated a lag period for the first 24 h, where thioflavin-T fluorescence intensity was static, indicating the presence of monomers only. After 24 h, there was an intermediate phase during which oligomers and other intermediate forms might have formed until 90 h. After 90 h we observed a saturated phase, where fully matured fibrils were formed, and here thioflavin-T fluorescence reached a plateau. In the presence of the CA aqueous extract, the sigmoidal pattern of α-synuclein aggregation kinetics was prevented. Here there was only a lag phase with possibly the presence of monomers only. Figure 1B shows the thioflavin-T fluorescence at 0, 24, 48, and 96 h time intervals. For α-synuclein alone, thioflavin-T fluorescence increases as a function of time, indicating the formation of aggregates from oligomers. But, in the presence of the CA aqueous extract, thioflavin-T fluorescence did not increase, indicating that the extract inhibited the aggregation of oligomers. We also conducted electrophoresis studies to understand the binding of molecules in the extract to monomers and aggregates. We did not find any shift in the mobility of bands or any other changes between

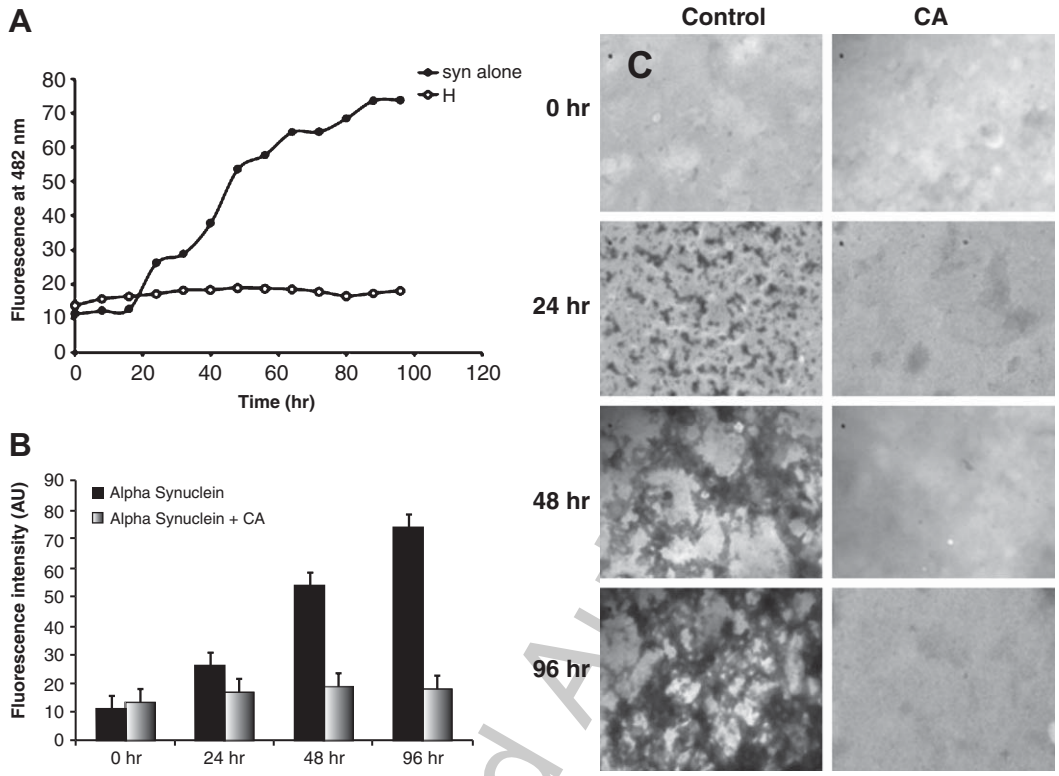

Fig. 1. Studies on the role of CA in modulating synuclein aggregation. A) Phase I (monomers to aggregates). B) Phase I: Bar diagram showing the Thioflavin-T fluorescence of α-synuclein in presence or absence of CA leaf extract. C) Phase I: transmission electron microscopic study.

the treatment and the control (data not shown). Hence, we conducted CD studies to understand the binding of the molecules in the extract to synuclein and to test for changes in the secondary conformations of synuclein if the molecules are binding.

TEM study

TEM pictures were analyzed for the aggregation kinetics at time intervals of 0, 24, 48, and 96 h, respectively (Fig. 1C). The TEM data clearly showed that there were no aggregates at 0 and 24 h in the control experiment. The aggregates started growing from 48 and 96 h onwards (Fig. 1C). But, in the presence of the CA leaf extract, there was no presence of aggregates at time intervals of 24, 48, and 96 h, indicating that the CA leaf extract completely prevented the formation of aggregates (Fig. 1C). Both Thioflavin-T and TEM data clearly supported that the CA extract totally prevented the formation of aggregates and the data are statistically significant ($p < 0.001$).

CD studies

The binding and secondary conformation of α-synuclein at time intervals of 0, 24, 48, and 96 h in the presence and absence of CA water extract are presented in Fig. 2A and B. Figure 2A indicates that α-synuclein exists in random coil conformation. The aggregated α-synuclein is in β-sheet conformation as evidenced by the decrease in the 200 nm negative peak and the increase in the 230 nm negative peak (Fig. 2B). In the presence of the CA water extract, α-synuclein stayed in the random coil conformation, indicating stability of monomers (Fig. 2A, B).

Phase II

Thioflavin-T assay

Thioflavin T fluorescence during the second phase of the aggregation process from oligomers is represented in Fig. 3A. In the control experiment, Thioflavin-T fluorescence increased as a function of time, indicating the formation of aggregates from oligomers. When the CA leaf extract was introduced at the time of oligomer formation, the intensity of Thioflavin-T decreased. This indicated that the CA leaf extract could able to prevent the formation of aggregates and the all the above data are statistically significant ($p < 0.001$).

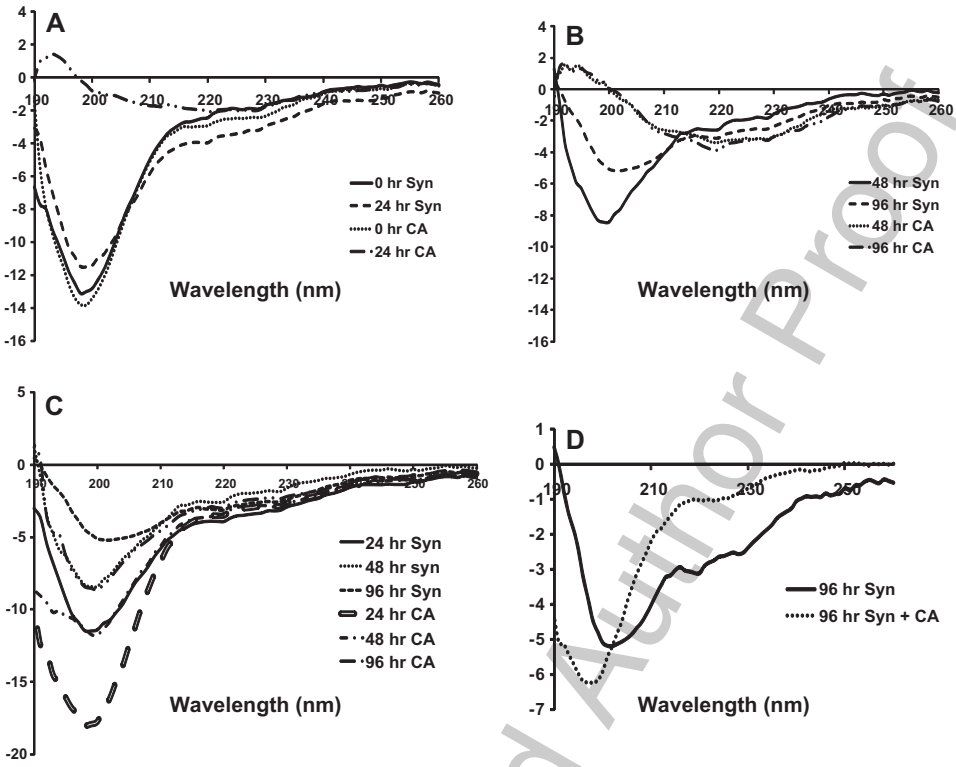

Fig. 2. Studies on the role of CA in modulating synuclein conformation. Circular dichroism (CD) spectra of α-synuclein aggregation. A) CD spectra of α-synuclein aggregation (Phase I, monomers to aggregates) at time intervals 0 h (solid line) and 24 h (dashed line), and in the presence of the CA water extract at 0 h (dotted line) and 24 h (dashed and double dotted line). B) CD spectra of α-synuclein aggregation (Phase I: monomers to aggregates) at time intervals 48 h (solid line) and 96 h (dashed line), and in the presence of CA water extract at 0 h (dotted line) and 24 h (dashed and double dotted line). C) CD spectra of α-synuclein aggregation (Phase II: oligomers to aggregates) at time intervals 24 h (solid line), 48 h (dotted line), and 96 h (dashed line), and in the presence of the CA water extract at time intervals 24 h (hollow dashed line), 48 h (dashed and double dotted line), and 96 h (dashed and dotted line). D) CD spectra of α-synuclein aggregation (Phase III: disintegration of fibrils): 96 h aggregates (solid line) and 96 h aggregates treated with CA plant extract (dotted line).

TEM study

Figure 3B shows the results of TEM of Phase II. The fibrils formation increased with time from 24 h to 96 h in the treatment with α-synuclein alone (oligomer to fibrils). The CA extract appeared to effectively prevent the formation of fibrils as very few fibrils were observed and the data is significant at $p < 0.001$.

CD studies

α-Synuclein went into the β-sheet conformation in the aggregation kinetics from 24 to 96 h. In the presence of the CA water extract, α-synuclein was in a mixed conformation (random coil and β-sheet) (Fig. 2C).

Phase III

Thioflavin-T assay

Figure 4A shows the results of Thioflavin-T fluorescence assay of Phase III. The fluorescence intensity after 96 h of the aggregated sample treated with the CA extract for 10 days showed an approximately 50% decrease relative to the 96 h aggregated control sample. This indicates that CA may disintegrate preformed fibrils up to 50% and the data is significant at $p < 0.05$.

TEM study

Figure 4B shows the results of TEM of Phase III. The α-synuclein (96 h incubated) aggregates showed a high density of mature fibrils. The incubation of these fibrils with the CA extract for 10 days resulted in a reduced density of fibrils, indicating that the CA extract may be able to disintegrate the fibrils by up to 70%, and the data is significant at $p < 0.001$.

CD studies

α-Synuclein went into a β-sheet conformation at 96 h. In the presence of the CA water extract,

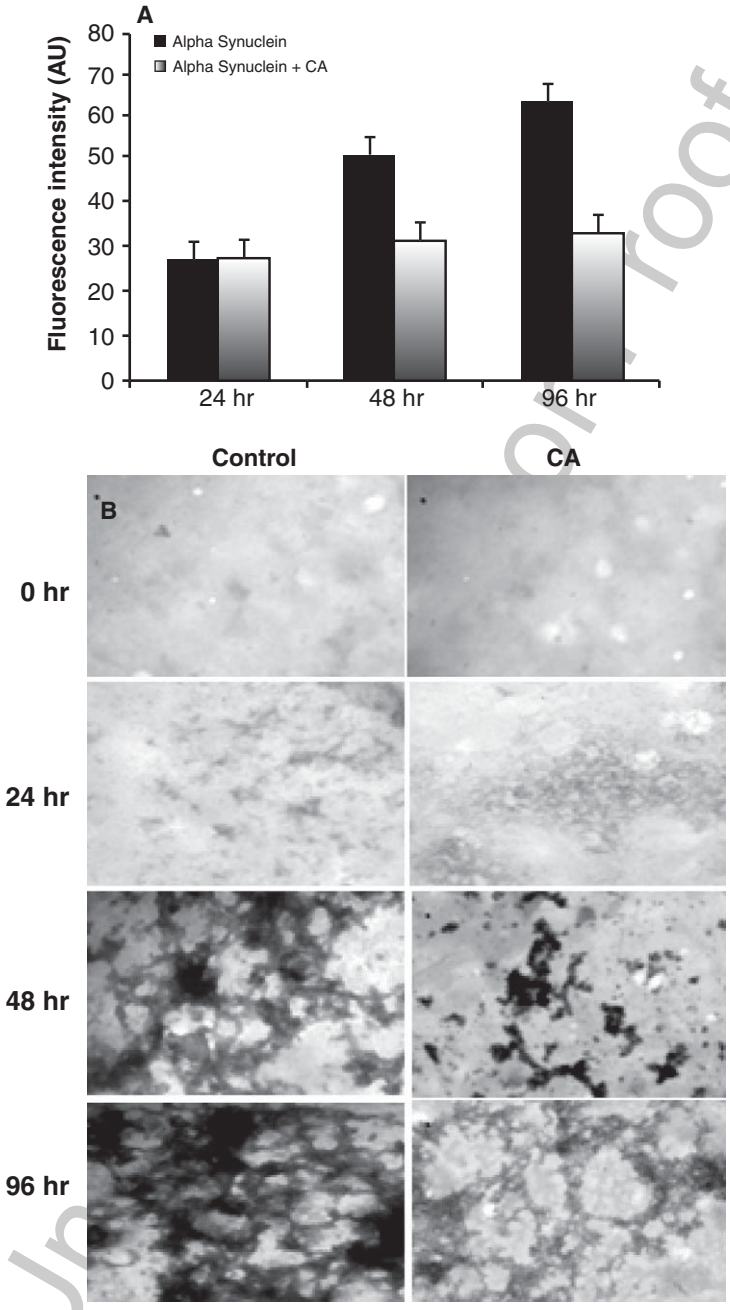

Fig. 3. Studies on the role of CA in modulating synuclein aggregation. A) Phase II (oligomers to aggregates): Bar diagram showing the Thioflavin-T fluorescence of α-synuclein as a function of time. B) Phase II (transmission electron microscopy study): Aliquots were analyzed for the presence or absence of aggregates using electron microscopy.

α-synuclein was predominantly in a random coil (random coil and partly β-sheet form) (Fig. 2D).

Polyphenol content in CA extract

The total phenolic content of the CA water extract was 1.83 mg/ml. The extract was found to have molecules of caffeic acid followed by p-coumaric acid, gentisic acid, protocatechuic acid, chlorogenic acid, gallic acid, asiatic acid, ferulic acid, and selenium (Table 1). We still believe that there may be additional unidentified small molecules in the extract, which is under exploration currently.

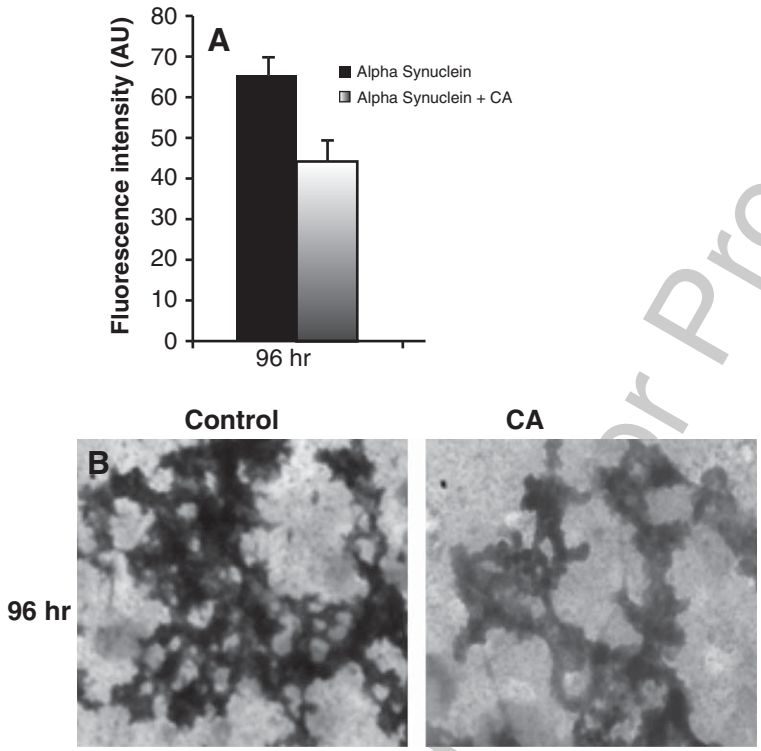

Fig. 4. Studies on the role of CA in modulating synuclein aggregation. A) Phase III (disintegration of preformed fibrils): Bar diagram showing the Thioflavin-T fluorescence of α-synuclein. B) Phase III (transmission electron microscopy study): The aliquots were analyzed for the presence or absence of aggregates using electron microscopy.

Table 1
Phenolic content in aqueous extract of *Centella asiatica* (CA). The values are expressed as mean and standard deviation

Sample	CA (μg/g dry extract)
Gallic acid	65.56 ± 2.33
Protocatechuic acid	135.65 ± 7.21
Gentisic acid	172.78 ± 8.51
Chlorogenic acid	109.56 ± 6.87
Caffeic acid	512.57 ± 9.34
P-Coumaric acid	410.21 ± 9.13
Ferulic acid	21.76 ± 1.93
Asiatic acid	1.4 ± 0.21
Selenium	2.8 ± 0.32

DISCUSSION

PD has complex pathology with multiple etiologies [1]. The aggregation of α-synuclein leading to the degeneration of dopaminergic neurons in substantia nigra is the central pathway involved in PD [1]. Currently, the drugs used for the treatment of PD are supplementing DOPA (a neurotransmitter secreted by the dopaminergic neurons) to stimulate the neurons of substantia nigra, and there are very few drugs targeting the aggregation pathway [29, 30]. Progressive neurodegeneration is seen even in patients taking drugs. The drugs used for PD relieve the symptoms but are not able to prevent neurodegeneration. After 5–10 years of treatment, patients become resistant to drugs because the number of neurons stimulated by the decreases over time. Discovering new drugs that can target the protein aggregation pathway will be a good alternative therapeutic approach.

α-Synuclein is hypothesized to have multiple functions in the cell. α-Synuclein aggregation is responsible for neuronal dysfunction and PD [4–6]. However, α-synuclein aggregation dynamics are complex and multifactorial. Soluble α-synuclein monomers exist predominantly in the random coil secondary conformation. During the onset of aggregation, natively random coil folded monomers turn into misfolded oligomers. The drug molecules so far reported are targeted toward the prevention of aggregates without much attention to aggregation conformation dynamics. There are few mechanistic studies focused on understanding the neuroprotective role of CA and these studies focused primarily on cognition, decrease in amyloid load, etc. [19, 20, 22–24, 31, 32]. There are also reports indicating that the CA aqueous

extract cannot inhibit the Aβ aggregation from both monomer and oligomers, nor could it disintegrate the preformed fibrils under *in vitro* conditions. However, the reason for this behavior is not explored in the paper [33]. We hypothesize that Aβ has a single methionine at residue 35, while synuclein has four methionine residues in α-synuclein. As we elaborate below, natural compounds preferentially bind to methionine and can help modulate the assembly of synuclein. We postulate that the presence of a number of methionine residues may be responsible for the differential action of CA on Aβ and synuclein.

The present study was focused on understanding the modulation of aggregation and conformation patterns of the synuclein protein *in vitro*. We analyzed the ability of CA extract to prevent α-synuclein aggregation in three different phases. Our most significant result is that the CA aqueous extract prevented the aggregation of monomers to oligomers by retaining the random coil structure of synuclein. This indicates that the CA aqueous extract stabilized the monomer of α-synuclein, thereby preventing the aggregation. The CA aqueous extract also effectively inhibited the oligomer going into the aggregation step, and caused the disintegration of preformed fibrils to greater extent (70%). These results are promising in furthering our attempts to discover new drugs to treat PD.

There are few studies on the neuroprotective role of polyphenols in PD models. The conjugate of dopamine, 5-S-cysteinyl-dopamine, causes neurotoxicity, possibly leading to PD pathology. Vauzour et al. [34] reported that the hydroxycinnamates caffeic acid and p-coumaric acid, and the hydroxyphenethyl alcohol, tyrosol, are able to protect neurons against neurotoxicity induced by 5-S-cysteinyl-dopamine *in vitro*. Jimenez-Del-Rio et al. [35] reported that pure polyphenols such as gallic acid, ferulic acid, caffeic acid, coumaric acid, propyl gallate, epicatechin, epigallocatechin, and epigallocatechin gallate could protect the impaired movement activity induced by paraquat in *Drosophila*. In addition, the caffeic acid phenethyl ester has been found to prevent 6-hydroxydopamine-induced neurotoxicity in intrastriatal rodent models [36]. Ono and Yamada [37] reported that compounds like nordihydroguaiaretic acid, curcumin, rosmarinic acid, ferulic acid, tannic acid, myricetin, kaempferol (+)-catechin and (−)-epicatechin, rifampicin, and tetracycline not only prevent the formation of α-synuclein fibrils but also destabilize preformed fibrils. Recently, Caruana et al. [38] reported 14 natural polyphenols that are able to inhibit α-synuclein oligomer formation and also destabilize pre-formed α-synuclein oligomers. Our leaf water extract has compounds like caffeic acid, p-coumaric acid, gentisic acid, protocatechuic acid, chlorogenic acid, gallic acid, and ferulic acid, asiatic acid, and selenium, and we believe that these compounds act synergistically to prevent synuclein assembly *in vitro*, but further investigations are in progress.

However, the mechanisms through which natural compounds modulate synuclein assemblies remain unclear. Peptide mapping and mass spectrometric studies indicate that synuclein is oxidized at all four methionine residues when synuclein is incubated with polyphenol inhibitors namely exifone, gossypetin, and dopamine. These studies have shown that the inhibitors bind to N-terminal region, which plays a key role in synuclein dimerization and aggregation, while the C-terminal region plays a key role in retaining the random-coil character [39]. Other studies have shown that polyphenols have aromatic elements and vicinal hydroxyl groups on a single phenyl ring. These groups play a key role for polyphenol binding to monomer/oligomer and also for destabilizing self-assembly of synuclein [37]. It has also been proposed that polyphenols bind to synuclein by strong non-specific hydrophobic associations and also non-covalent pi-pi stacking interactions through aromatic residues present in synuclein sequence [40]. Still more investigations are needed to understand the mechanisms through which polyphenols prevent and destabilize synuclein assemblies.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1961).

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