α-synuclein inhibitor from endophytic fungi

A

thesis submitted

in the partial fulfilment of the requirements for the award of degree of

DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

Submitted by

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December, 2023

Certificate

Certified that the thesis entitled "α-synuclein inhibitor from endophytic fungi" submitted by Ms. Sheetal Vats, Reg. no. 901700008 in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a record of candidate's own independent and original research work carried out by herself under my supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree.

Sanjai Saxena
21 Dec. 2023

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled "α-synuclein inhibitor from endophytic fungi" in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy at Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is an authentic record of my own work during the period from January 2018 to June 2023, under the supervision of Prof. Sanjai Saxena, Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology. This report has not been submitted for the award of any degree or certificate in this or any other university.

Place: Patiala, Punjab

Date: December, 2023

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Sheetal Vats

Date: December, 2023

Place: Patiala

DEDICATED

TO MY

FATHER

Publications

1. Sheetal Vats and Sanjai Saxena (2023): Endophytic *Fusarium* species, a unique bioresource for disaggregator of misfolded alpha-synuclein. Arch Microbiol 205(6), 224. https://doi.org/10.1007/s00203-023-03575-z (**SCI-IF: 2.8)**
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- International conference on Dec 7-8, 2018 "Food Security: Challenges & Opportunities" at department of Biotechnology, TIET, Patiala
- DST-SERB sponsored workshop on "Computational Drug design using Molecular docking and virtual screening" held on 27-29 April 2018, TIET, Patiala
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- Workshop "3rd Next generation sequencing (NGS)" on January 7-9, 2019 held at CDRI, Lucknow
- Online workshop "Microbial phylogeny – BACTERIA, FUNGI AND VIRUS" 16 Dec-18

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S. No.	Symbol	
1.	-	minus
2.	%	percentage
3.	~	approximately
4.	<	Lesser than
5.	>	Greater than
6.	±	plus minus
7.	×	multiplication
8.	≥	equals to or greater than
9.	°	degree
10.	°C	degree celsius
11.	µg	microgram
12.	µl	microliter
13.	µm	micrometer
14.	µM	micromolar
15.	Å	Angstrom
16.	bp	base pair
17.	cm	centimeter
18.	g	gram
19.	hr	hour
20.	k	kelvin
21.	Kb	kilo base pair
22.	kDa	kilo Dalton
23.	l	litre
24.	M	Molar
25.	mA	milliampere
26.	mg	milligram
27.	min	minute
28.	ml	milliliter
29.	mm	millimeter
30.	mM	millimolar
31.	mm^2	millimeter square
32.	mPa	mega pascel
33.	ng	nanograms
34.	nm	nanometer
35.	nM	nanomolar
36.	ns	nanosecond
37.	ps	picosecond
38.	ppm	parts per million
39.	psi	pounds per square inch
40.	rpm	revolutions per minute
41.	s	second
42.	U	unit
43.	v	volume
44.	v/v	volume by volume
45.	w/v	weight by volume
46.	X	times
47.	α	alpha
48.	β	beta
49.	γ	gamma

50.	Δ	Delta
51.	µg /ml	microgram per milligram
52.	µM	micromolar
53.	µs	microsecond
54.	π	pi

List of abbreviations

S. No.	Abbreviation	Full form
1.	AA	Amino acid
2.	AD	Alzheimer's disease
3.	ADP	Adenosine diphosphate
4.	Ala	Alanine
5.	ANOVA	Analysis of variance
6.	APcI	Ammonium perchlorate
7.	ATP	Adenosine monoosphate
8.	Aβ	Amyloid beta
9.	BLAST	Basic Local Alignment Search Tool
10.	BRCA	Breast cancer gene
11.	C=C	Carbonyl
12.	C=O	Carboxyl
13.	Ca^{2+}	Calcium ions
14.	Chl	Chloroform
15.	Cl	Chloride
16.	CLA	Corn leaf agar
17.	CNS	Central nervous system
18.	CoQ	Coenzyme Q
19.	COX	Cyclooxygenase
20.	CRBA	Cooke rose Bengal agar
21.	C-terminal	Carboxy terminal
22.	DCF	Dichlorofluorescein
23.	Dcfh-Da	Dichloro-dihydro-fluorescein diacetate
24.	DCM	Dichloromethane
25.	DLB	Dementia with Lewy body
26.	DMSO	Dimethyl sulfoxide
27.	DNA	Deoxyribonucleic acid
28.	dNTP	Deoxynucleotide triphosphate
29.	DPPH	2,2-diphenyl-1-picrylhydrazyl
30.	DPX	Dibutylphthalate polystyrene xylene
31.	EA	Ethyl acetate
32.	EDTA	Ethyl diamine tetra acetic acid
33.	EF	Endophytic fungi
34.	TEF1α	Translational elongation factor 1 alpha
35.	EGCG	Epigallocatechin gallate
36.	ELISA	Enzyme linked immunosorbent assay
37.	ESI	Electrospray ionization
38.	EUR	European
39.	FC	Folin-Ciocalteu
40.	FDA	Food and drug administration
41.	Fig	Figure
42.	FTIR	Fourier transform infrared spectroscopy
43.	GA	Gallic acid
44.	gDNA	Genomic DNA
45.	GFP	Green fluorescent protein
46.	Glu	Glutamate
47.	GLUT	Glucose transporter
48.	Gly	Glycine

49.	GSH	Glutathione
50.	H_2O_2	Hydrogen peroxide
51.	HCl	Hydrogen chloride
52.	HIS3	Histidine 3
53.	HIV	Human Immunodeficiency virus
54.	HPLC	High performance liquid chromatography
55.	IC_{50}	Inhibitory concentration 50
56.	IDP	Intrinsically disordered protein
57.	Ile	Isoleucine
58.	IR	Infra-red
59.	ITS	Internal transcribed spacer
60.	iYPGRB	induced yeast peptone galactose raffinose broth
61.	Kan	Kanamycin
62.	LC	Liquid chromatography
63.	L-dopa	L-dopamine
64.	Lys	Lysine
65.	Mat a	Mating alpha
66.	MD	Molecular dynamics
67.	MGL	Molecular graphics laboratory
68.	ML	Machine learning
69.	MS	Mass spectroscopy
70.	MSA	Multiple sequence alignment
71.	Na	Sodium
72.	NaCl	Sodium chloride
73.	NAD(P)H	Nicotinamide adenine dinucleotide phosphate hydrogen
74.	NaOCl	Sodium hypochloride
75.	NBT	Nitroblue tetrazolium
76.	NDGA	Nordihydroguaiaretic acid
77.	NFCCI	National fungal culture collection of India
78.	NMR	Nuclear magnetic resonance
79.	NNI	Nearest neighbour interchange
80.	No.	Number
81.	NPT	Number pressure temperature
82.	NVT	Number volume temperature
83.	O_2	Oxygen
84.	OD	Optical density
85.	OH	Hydroxyl
86.	PBS	Phosphate buffer saline
87.	PD	Parkinson’s disease
88.	PDA	Potato dextrose agar
89.	PDB	Potato dextrose broth
90.	PDB-ID	Protein bata bank identification
91.	PDD	Parkinson’s disease dementia
92.	Phe	Phenylalanine
93.	PLA	Pine leaf agar
94.	PMCA	Plasma membrane calcium ATPase
95.	R	Reference
96.	RMSD	Root mean square deviation
97.	RMSF	Root mean square fluctuation
98.	rDNA	Recombinant DNA
99.	R_f	Retention factor

100.	RNA	Ribonucleic acid
101.	ROS	Reactive oxygen species
102.	RPM	Revolutions per minute
103.	RT	Room temperature
104.	SD	Standard deviation
105.	SDA	Sabouraud dextrose agar
106.	SDS- PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
107.	SNARE	Soluble N-ethylmaleimide sensitive factor activating protein receptor
108.	SNCA	Synuclein alpha
109.	SOD	Superoxide dismutase
110.	Sp.	Species
111.	SSE	Secondary structure element
112.	T	Theaflavin
113.	T33DG	Theaflavin-3-3-digallate
114.	T3G	Theaflavin-3'-gallate
115.	TBE	Tris borate EDTA
116.	TFC	Total phenolic content
117.	Thr	Threonine
118.	ThT	Thioflavin T
119.	TIP3P	Transferable intermolecular potential with 3 points
120.	TLC	Thin layer chromatography
121.	TOF-MS	Time of flight- Mass spectroscopy
122.	TPC	Total phenolic content
123.	TRP1	Tryptophan 1
124.	URA3	Uracil 3
125.	USA	United states of America
126.	USFDA	United states food and drug administration
127.	UV	Ultraviolet
128.	Val	Valine
129.	VMAT	Vesicular monoamine transporter
130.	WA	Water agar
131.	WHO	World health organisation
132.	YPDB	Yeast peptone dextrose broth
133.	YPGRB	Yeast peptone galactose raffinose broth

Executive summary

The present study was oriented towards the exploration of α-synuclein disaggregation potential of fungal endophytes isolated from *Camellia sinensis* and *Malus domestica* from Assam and Himachal Pradesh inhabiting biodiversity hotspots of India. Out of 79 endophytic fungal isolates, culture filtrates of 4 isolates were found to exhibit disaggregation potential due to anti-oxidant effect analysed via preliminary screening NBT assay. The cell free culture filtrate of "59CSLEAS" was further screened by using various *in vitro* assays viz. ThT, Sandwich ELISA and DCFH-DA and found as the most potent. The crude chloroform residue of endophytic fungal isolate 59CSLEAS was found to disaggregate oligomeric protein up to 56.5 % via Sandwich ELISA technique. The potential endophytic fungus was identified using morphological and molecular tools as *Fusarium oxysporum*. Another approach i.e., *in silico* analysis suggested role of antioxidants compounds present in *Camellia sinensis* in inhibition of protein oligomerization. Further, the crude chloroform residue of *Fusarium oxysporum* was fractionated into 11 major fractions using TLC and column chromatography.

The fraction 10 was found to exhibit maximum α-synuclein disaggregation potential with DC_{50} of 1.101 ± 0.04 µM. Fraction 10 appeared white, light sensitive, and analysed as potent antioxidant having high free radical scavenging properties IC_{50} 0.12 ± 0.04 mg/ ml ascertained via DPPH assay. Mass spectra of bioactive fraction showed parent ion peak at 459.1208 m/z. Further, IR spectra confirmed the presence of hydroxyl and aromatic carbonyl groups. ^{1}H-NMR and ^{13}C-NMR spectra of bioactive fraction 10 tentatively identified the compound to be gallate ester. The ESI-MS, FTIR, and NMR spectra of data of bioactive fraction 10 was found to be identical to Epigallocatechin gallate. This is the first report of a plant catechin being produced by an endophytic fungus which is also a potent alpha-synuclein disaggregator.

The present study establishes that endophytic fungi are prolific sources of novel inhibitors/ disaggregators against neurodegenerative disorders and oxidative stress. The isolation of Epigallocatechin gallate, as alpha-synuclein disaggregator from endophytic fungi further warrants its further evaluation by *in silico* simulation studies and optimizing QSAR (Quantitative Structure Activity Relationship) for its development into a pharmacophore in management of oxidative stress related disorders since Epigallocatechin gallate is already being used as a nutraceutical.

CHAPTER-1
INTRODUCTION

1.1 Background

Proteins are fundamental biomacromolecules which are assembled from amino acids based on the genetic information encoded in the genes. Proteins play roles which are diverse as well as essential. They serve as messengers of information as exemplified by adenylate cyclase (signal transduction), facilitate hormonal regulation as seen in Insulin and participate in immune response as immunoglobulins (Furthner et al., 2021; Ostrom et al., 2022; Schroeder and Cavacini, 2010). Furthermore, proteins also aid in transportation process of vital molecules such as Glucose transporters (GLUTs) and also participate in formation of structural scaffolds such as the actin and myosin (Morris et al., 2022; Navale and Paranjape, 2016).

Different paradigms have been proposed in relation to structure and function of proteins (Uversky, 2019). However, intrinsically disordered proteins (IDPs) defy the structure-function paradigm as they lack well defined structure but do exhibit some dynamical and structural ordering. Intrinsically disordered proteins or intrinsically unstructured proteins are characterized by their biased amino acid compositions and low complexity, as well as by their low proportion of bulky hydrophobic amino acids and high proportions of charged and hydrophilic amino acids. Despite being functional, IDPs are unable to fold spontaneously into stable, well defined, globular-3-D structures. They are dynamically disordered and fluctuate rapidly through a range of conformations (Dyson and Wright, 2005). Earlier thought to be mysterious entities, the role and functionality of IDPs in signalling and crucial cellular processes, including the regulation of transcription, translation and the cell cycle, have made IDP research as one of mostly dynamic and popular field in modern protein science (Galea et al., 2008; Iakoucheva et al., 2002; Mir et al., 2018; Wright and Dyson, 1999).

The functionality of IDP exists in different conformations such as unfolded, non-globular and extended. IDPs delve into multifarious physiological facets that span from intricate molecular recognition processes to the orchestration of molecular assemblies. Intrinsically disordered proteins wield significant influence in the regulation of central dogma of molecular biology and also contributing to vital task of providing structural stability to the cells – the building blocking of life (Chakrabarti and Chakravarty, 2022; Trivedi and Nagarajaram, 2022).

Much similar to their structured protein counterparts, the existence and maintenance of IDPs are governed by a finely coordinated and regulated phenomena. However, in event of imbalance this coordination may result in various pathological conditions (diseases). In fact a broad range of human diseases which are also known as protein misfolding or protein conformation disease arise from the failure of a specific peptide or protein to adopt a conformational state i.e., a

misfolded state. The obvious consequence of protein misfolding is aggregation (or fibril formation) leading to the loss of normal function and gaining a toxic function.

Several studies have suggested that IDPs such as α-feto protein (AFP) and p53 regulate the cellular processes and aberrations in these IDPs is manifested as Cancer. Similarly, breast cancer type 1 susceptibility protein (BRCA1) responds differentially to types of DNA damages thereby exemplifying their role in signal flow and development of breast cancer (Deng, 2006; Mark et al., 2005). Prion disease generally result in pathological accumulation of aggregated forms of Prion proteins (PrP). Creutzfelds Jacobs Disease (CJD), scrapie, bovine spongiform encephalopathy generally spread by the transmission of PrP aggregates from one individual or species to another (Watzlawik et al., 2006). Diabetes and Cardiovascular diseases also result due to IDP dysfunction/dysregulation. Hirudin is a thrombin specific inhibitor occurring in the salivary glands of medicinal leech (*Hirudo medicinalis*) while Thrombin is a blood protein involved in blood clotting and coagulation. Crystallographic studies have elucidated that the intrinsic disorder plays an important role during protein-protein interaction i.e., hirudin-thrombin complexation (Uversky et al., 2008).

Islet Amyloid Polypeptide (IAPP) / Amylin is an IDP additionally produced by β-cells of pancreatic islets apart from Insulin carries out normal function associated with energy metabolism. However, dysfunction of this IDP results in amyloid fibril formation which is linked to non-insulin dependent diabetes mellitus (NIDDM) or type II diabetes. Pathologically deposition of amyloid fibrils in the Islets is a common feature which slowly effects the insulin production (Cooper et al., 1987; Jaikaran et al., 2001).

The term α-synucleinopathies is predominantly linked to an IDP, α-synuclein which possess no or little order under physiological conditions (Das and Eliezer, 2019). It is characterized by fibrillar aggregates of α-synuclein protein (misfolded state) in the cytoplasm of selective population of glia and neurons. Universal features of α-synuclein are intracytoplasmic inclusion of oligomeric proteins known as Lewy bodies and glial cytoplasmic inclusions (Schmitz et al., 2023). α-synucleinopathies comprise of DLB (dementia with Lewy bodies); Multiple Sclerosis Atrophy (MSA), neurodegeneration with brain iron accumulation type I and Parkinson's disease with dementia (PDD). DLB is a progressive disorder known to be an umbrella term for diagnosing PDD and DLB. Rapid eye movement, fluctuating cognition in alertness and attention, sleep behaviour disorder, and visual hallucinations are the main pathophysiological features of DLB (Armstrong, 2019). Parkinson's disease is the most common neurodegenerative disease due to the loss of dopaminergic neurons in the substantia nigra pars compacta (Choong and Mochizuki, 2022). The

disease's etiology is unknown; however, the formation of intracytoplasmic inclusions of α-synuclein protein known as Lewy bodies are reported in sporadic and familial forms of disease progression (Srinivasan et al., 2021). Bradykinesia, rest tremor, and rigidity are the main pathological features accounting for PD. α-synuclein containing inclusions are distinct morphologically into Lewy bodies, Lewy neurites (dystrophic neurites), glial and cytoplasmic inclusions and axonal spheroids (Jellinger, 2010; Uversky et al., 2008). Clinically α-synucleinopathies lead to chronic decline in motor, cognitive, behavioural and autonomic function based on the extent and distribution of lesions.

1.2 α-synuclein: an IDP

The protein α-synuclein is composed of 140 amino acid residues, however lacking cysteine and tryptophan. It is encoded by a single gene SNCA, consisting of seven exons located in specifically 4q21.3-q22 region in human's chromosome No. 4 (Chen et al., 1995). As the protein was identified to be neuron specific localized in the pre-synaptic nerve terminals and the nucleus it was named as synuclein (Maroteaux et al., 1988). The synuclein family in vertebrates comprises of three members, namely α-syn, β-syn, and γ-syn. All the three members are involved in neuronal function, and are involved in the long-term operation of the nervous system (Greten-Harrison et al., 2010). However, α-synuclein attracted much greater interest as mutation in the SNCA gene expressing the protein led to familial cases of early onset of familial Parkinsons disease (Polymeropoulos et al., 1997).

Based on the amino acid sequence, α-synuclein is divided into three distinct regions viz. (i) residues 1-60, containing four 11 AA imperfect repeats (which code for the amphipathic α-helix) having a conserved motif KTKEGV, (ii) residues 61-95 which contain hydrophilic and highly amyloidogenic NAC regions consisting of three additional KTKEGV repeats, (iii) while the third region is highly enriched in acidic residues and proline C terminal (Fig.1.1). The NAC domain is responsible for the aggregation of the protein. C-terminal forms the acidic tail of the α-synuclein protein with S129 as the main phosphorylating site. The C-region is largely regulating the aggregation of N-terminal being amphipathic in nature is responsible for binding to the membrane and generally is oriented as α-helix (Fan et al., 2021).

1.3 Oxidative stress as a primer for α-synuclein aggregation

Reactive oxygen species (ROS) are produced in all aerobic cells and comprise of chemically reactive molecules of oxygen. The ROS generally comprise of hydrogen peroxide (H_2O_2), superoxide anion radicals (O_2●-); hydroxy radicals (●OH), singlet oxygen ($^1O_2$), nitrogen dioxide (NO_2), hypochlorous acid, (HOCl) and peroxynitrate (ONOO−) which are generated via different

enzymatic and non-enzymatic pathways. When the ROS are produced in excessive amount, they trigger the redox signalling pathway under physiological conditions. However, when the ROS generation surpasses it becomes detrimental and can damage myriad cellular components such as proteins, DNA, lipids (Korovesis et al., 2023).

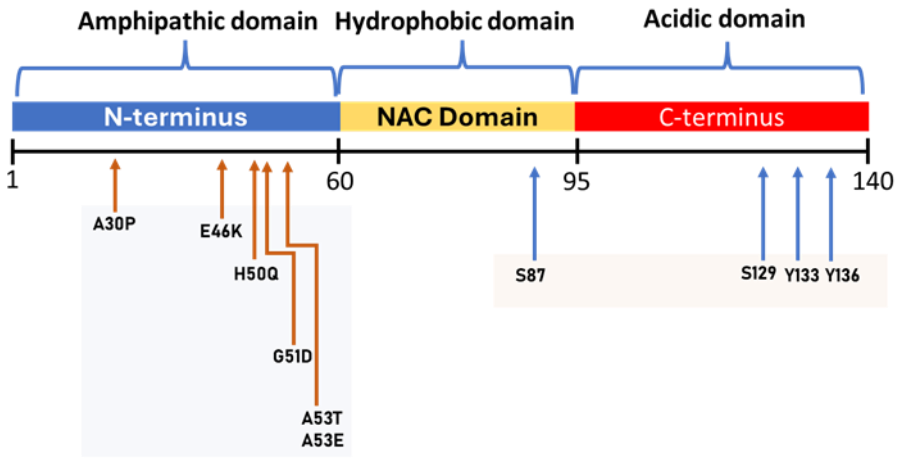

Fig 1.1 Structure of the alpha-synuclein monomer (Schematic depiction). The amino acid residues delimiting the N-terminus, NAC domain and C-terminus have been depicted along with the sites of mutations. The N-terminus is depicting the gene mutations in the α-synuclein associated with autosomal Parkinson's disease. [Redrawn from: (Fan et al., 2021)].

Oxidative stress therefore is a condition when the ROS exceeds the ability of the system/ body to neutralize and eliminate them (Ramalingam and Kim, 2012). As several of the amino acid residues are prone to oxidation (based on the type of ROS involved), oxidative damage results in a variety of oxidative protein modifications. These oxidative alternations may modify the stability, activity, subcellular location or protein - protein interactions of the oxidised proteins (Holmstrom and Finkel, 2014). Molecular mechanistic studies on oxidative studies are less however, *in silico* studies have indicated that ROS could induce β-sheet formation which is prone to aggregation (Korovesis et al., 2023b; Pavlou et al., 2017).

To establish the correlation between oxidative stress and α-synuclein aggregation, *in vivo* mouse models of PD were developed. As SOD2 (superoxide dismutase 2) enzyme is rate limiting factor in the antioxidant machinery of mitochondrion, a partial deficiency of the enzyme induces an oxidative stress in the mice. Thus, to establish a relationship between oxidative stress and α-synuclein aggregation an *in vivo* transgenic murine model of PD with haplodeficiency for SOD2 was generated on the basis of a well characterized (Thy-1)-h (A30P) -α-synuclein transgenic line.

α-synuclein transgenic mice with SOD2 deficiency when compared with littermate controls having normal SOD2 activity exhibited an advanced stage of synucleinopathy in the former after

16 months which was established by higher content of truncated -α-synuclein in the insoluble fraction of the homogenized brains as well as PET (Positron Emission Tomography) plot scores ($p < 0.01$). These results established that reduction in scavenging of free radicals can exacerbate α-synuclein aggregation concluding that elevated levels of oxidative stress can modulate the progression of PD (Scudamore and Ciossek, 2018).

1.3.1 Oxidative stress and PD progression

Now it has been well correlated that during the process of ageing, ROS levels are elevated which results in accumulation of altered/ damaged/ misfolded proteins and which may exhibit an altered structure (physical properties such as aggregation propensity) or both. Several age-related pathologies are largely driven by oxidative stress comprise of neurodegenerative disease such as HD (Huntington's Disease), AD (Alzheimer's disease) and Parkinson's Disease (PD). The major sources of ROS comprise of dysfunctional mitochondrion, Ferroptosis and Parthanatos. Mitochondrial dysfunction (aberrant mitochondrial homeostasis) has already been implicated in pathogenesis of neurodegenerative diseases such as HD, AD and PD (Chakraborty and Ziviani, 2020; Curtis et al., 2022; Franco-Iborra et al., 2018; Srivastava et al., 2021).

Peroxisomes apart from mitochondrion participate in metabolic oxidative process (such as β-oxidation, α-oxidation of fatty acids, polyamines and D-amino acids) and produce ROS as by products which are normally neutralized by anti-oxidant enzymes. Cross talks of peroxisomes with mitochondrion, nucleus, lysosomes, Endoplasmic Reticulum (ER) is the key to maintain normal peroxisome function. Thus, dysfunction of peroxisome generally occurs during aging process as well as in the diseased state due to several cellular alterations thereby highlighting their importance in redox homeostasis (Kim and Bai, 2022).

Excessive oxidative damage to DNA by ROS results in programmed cell death signalling pathway via hyperactivating poly (ADP-ribose) polymerase (PARP). Hyperactivated PARP induces accumulation of large (poly ADP) polymers which in turn promote AIF (apoptosis inducing factor) from outer mitochondrial membrane. AIF complexes with macrophage migration inhibitory factors and this complex moves inside the nucleus where it triggers DNA and cell death. This phenomenon is referred as Parthanatos is responsible for diseases affecting the nervous system such as PD and AD (Huang et al., 2022 a; Koehler et al., 2021). Thus, one can conclude that oxidative stress is the driver of α-synuclein aggregation (misfolding) as well as progression of Parkinson's Disease.

1.4 Misfolded α-synuclein as a Novel drug target

Pathological aggregate formation of α-synuclein (misfolded) in the neuronal and non-neuronal cells of the brain are responsible for a group of neurodegenerative disorders, collectively known as α-synucleinopathies. The pathological aggregates of α-synuclein known as LB have been the hallmark of Parkinson's Diseases (PD) and dementia with Lewy Bodies (DLB) (Goedert et al., 2017; Magalhaes and Lashuel, 2022). Levodopa has been the cornerstone of PD treatment since 1960's and it is still used for sustaining Dopamine, in brain which is lost due to death of dopaminergic neurons in the substantia nigra. Levodopa and Carbidopa provide relief from the classical PD symptoms of Bradykinesia and rigidity. However prolonged use of Levodopa results in dyskinesis (Hansen et al., 2022; Pezzoli and Zini, 2010). Other drugs which are primarily dopamine receptor agonists or inhibitors have been reported to be less effective.

As it is well established now that aggregation and spread of aggregation pathology is responsible for numerous pathogenic mechanisms which cause neurodegeneration or ultimately disease progression. Hence there is need to develop safe and effective disease modifying therapy which limits α-synuclein aggregation (misfolded forms) as well as curtail the spread of α-synuclein aggregation pathology to avert the possible pandemic expected to happen in 2040 where the number of people suffering from PD would touch approximately 14 million (Dorsey and Bloem, 2018). Of the various mechanistic, immunological and peptidomimetic approaches being explored to prevent the aggregation of α-synuclein into pathogenic (misfolded) forms as well as reduce the toxicity of the pre-existing misfolded structures, one of the strategies which is gaining momentum and is extensively being researched is disaggregation or defibrillation of pathogenic forms of α-synuclein (Fields et al., 2019).

Majority of the modern drugs have their origins from plant and plant extracts. These natural products isolated from plants and other biological matrices have helped the medicinal chemists to design novel drugs based on their structure as well as unique mode of action (Rodrigues et al., 2016). Anti-inflammatory and anti-oxidant properties of certain natural products viz. curcumin, resveratrol, ginseng extract, honey, epigallocatechin gallate (EGCG) have attracted much attention for the treatment of neurodegenerative diseases. Resveratrol significantly inhibited the Aβ induced proliferation BV-2 cells as well as release pro-inflammatory cytokines IL6 and TNFα (Feng and Zhang, 2019). EGCG on the other hand has been found to improve the anti-oxidant enzymes glutathione peroxidase and superoxide dismutase thereby reducing the oxidative stress (Zhang et al., 2023; Al-Sayed et al., 2014).

Phytochemicals belonging to terpenoids, flavonoids, lignans, saponins and glycosides have been reported to possess beneficial activities in ameliorating PD (Inoue et al., 2018; Siddique et

al., 2013). Phytochemicals such as geraniol, reynosin, ginkolide A, B and C have found to inhibit α-synuclein aggregation or fibril formation, thereby attenuating toxicity in the experimental models (Cai et al., 2023; Caruana et al., 2011; Harati et al., 2023; Ng et al., 2022; Zhao et al., 2017). Food based plant phytochemicals such as Astaxanthin, Lycopene, Oleuropin, curcumin and resveratrol have also been found to inhibit α-synuclein aggregation and fibrillation (Caruana and Vassallo, 2015; Takahashi-Niki et al., 2015).

One of the major bottlenecks encountered with commercial use of phytochemicals exhibiting promising potential as therapeutic entities for PD is related to their copious supply and cost. Excessive exploitation/annihilation of plants for the recovery of these phytochemicals may lead to ecological disbalance as well as loss of biodiversity (Maria-Scarpa et al., 2022).

1.4.1 Endophytes – novel bioresource and continuum of biodiversity

Plant-microbe interactions may therefore play a crucial role in finding out suitable alternatives for the commercial production of these putative phytochemicals or their congeners. Every plant on earth holds a microbiome within itself and this relationship has been traced back to be as old as 450 million years ago when the first symbiosis was reported between a semi-aquatic green alga and aquatic fungi borne on to land. Thus, by this model the colonization of plants on land was possible due to the intimate association with a filamentous microorganism (Delaux et al., 2015; Wang et al., 2012). These microbes which constitute the endomicrobiome of plants are commonly referred as “Endophytes”. They reside with the plant system without any overt signals of their presence (Bacon and White, 2000). Endophyte exhibits a mutualistic association with the host plant wherein the microorganism resides in the internal plant tissue without causing any adverse effect on the plant. This phenomenon is referred as endophytism (Wani et al., 2015).

As these endophytes co-evolve with their host during the course of development and growth of the plant, they inherit some genes from their host (horizontal gene transfer) which could induce the propensity in these microorganisms to produce putative plant phytochemicals in nature/free fermenting medium without the need of the host plant. This concept was first proved by the discovery of the fungus *Taxomyces andreanae* which was isolated from *Taxus brevifolia*, the taxol tree. 1 kilogram of Taxol recovery required annihilation of 300 taxol trees which is a very slow growing plant (Stierle et al., 1995; Strobel, 2003). As nature is the ultimate chemist, therefore these endophytic microorganisms may also possess the ability to produce congeners of the known phytochemicals which could probably possess better α-synuclein disaggregating properties than their parent compounds. Thus microbial (specifically fungal) endophytes open up avenues for strain improvement using genomic /non-genomic methods to enhance the production of putative phytochemical or a congener. At the same time, it offers commercial

production of the desired phytochemical/congener though fermentative route on the same lines as antibiotic production which is already an established process in the pharma/biotech industry (Cao et al., 2022; Toppo et al., 2023).

Endophytic fungi exhibiting anti-oxidant properties have been isolated from various medicinal plants. Terric acid and 6-methylsalicyclic acid were isolated from *Pseudocercopspora* sp. ESL02 existing as an endophyte in *Elaeocarpus sylverstris*, exhibited an IC_{50} of 0.22 mmol/L and 3.87 mmol/L of DPPH radical scavenging activity (Prihantini and Tachibana, 2017). Eugenol has been isolated from an endophytic fungus *Neopestalotiopsis* sp. which was isolated from *Cinnamomum loureiroi* and exhibited a potent antioxidant activity with IC_{50} of 22.92 ± 0.67 µg/ml. It also holds a promise for commercial production of Eugenol via fermentative route (Tanapichatsakul et al., 2019). Similarly, Physcion was isolated from endophytic *Aspergillus versicolor* SB5 existing as an endophyte in *Juncus rigidus*. Physcion exhibited significant anti-inflammatory activity with an IC_{50} of 43.1 and 17.54 µg / ml for COX-2 and LOX-1, respectively. However, Physcion exhibited a lower radical scavenging activity when compared to the standard (Ascorbic acid) in both DPPH and ABTS radical scavenging assays (Elawady et al., 2023). Another new compound, 5-(1-hydroxybutyryl)-4-methoxy-3 methyl-2H-pyran-2-one(C-HMMP) was isolated from the endophytic fungus *Colletotrichum acutatum* residing in the medicinal plant *Angelica sinensis* via antimicrobial bioassay guided fractionation. C-HMMP exhibited a dose dependent antimalarial and radical scavenging activity with IC_{50} of 0.15 and 131.2 µg/ml (Yehia, 2023). N-acetyltyramine, an alkaloid was isolated from the endophytic fungus *Schizophyllum* sp. HM230 living in the stems of herb, *Vincetoxicum mongolicum* Maxim. This compound exhibited the best anti-oxidant activity among all the alkaloidal compounds isolated from endophytic *Schizophyllum* sp. HM230 (Li et al., 2023).

Hence, from the above data it is clearly evident that endophytic fungi from the medicinal and higher plants are being extensively explored for their anti-oxidant, anti-inflammatory, anti-bacterial properties for development into therapeutic interventions. In the recent past several endophytic fungi have been explored for anti-oxidant and neuroprotective properties (Budiono et al., 2019; Hou et al., 2021; Le et al., 2020; Shen et al., 2023; Vig et al., 2022). However, their evaluation as α-synuclein disaggregators remains underexplored till date.

Camellia sinensis (tea) has catechins, polyphenols and theaflavins which possess strong anti-oxidant activity. Previously one tea polyphenol, EGCG ((-)-Epigallocatechin -3-gallate) has been reported to inhibit α-synuclein aggregation and fibrillation in a dose dependent manner (Zhao et al., 2017). Similarly, *Malus domestica* extracts have also been reported to possess anti-oxidant properties in its methanol extracts. *Malus domestica* has also been reported to be an important

source of dietary phenolic compounds, with potent antioxidant activity as compared to other fruits and is therefore linked with improved health benefits and reduced the risk of degenerative diseases (Boyer and Liu, 2004; Kalinowska et al., 2014; Vasile et al., 2021).

Thus, the present study was oriented to isolate and systematically screen endophytic fungi of *Camellia sinensis* and *Malus domestica* for their potential to disaggregate oligomeric α-synuclein using a recombinant α-synuclein expressing yeast strain. There are no reports till date wherein endophytic fungi have been systematically bioprospected to explore compounds which could disaggregate oligomeric α-synuclein.

CHAPTER-2
PRESENT APPROACH

2.1 Present approach

The endomicrobiome of plants majorly comprise of bacteria, and fungi which reside intracellularly/ intercellularly within the plant tissue without any obvious signatures of their existence (Bacon and White, 2000). Endophytic fungi phylogenetically are a very diverse group of eukaryotes which ubiquitously exist in all plants. The unique disposition of endophytic fungi is their ability to penetrate and colonize in their hosts by exhibiting complex multivariate interactions. The endophytic mycobiome of plants is associated with a plenitude of advantages to the host plant beginning with tolerance to biotic and abiotic stresses to adaptive survival in unique habitats (Chetia et al., 2019; Franken, 2012).

Endophytic fungi have been considered as a goldmine of bioactive compounds as they produce them when cultured axenically. These bioactive compounds exhibit a spectrum of applications in diverse fields such as agriculture, pharmaceuticals, ecology and biotechnology. Fundamental reason of endophytic fungi as the origination of novel bioactive compounds stems from the fact that these secondary metabolites produced by them mediate intra-kingdom and inter-kingdom crosstalks to achieve a balance during their symbiotic existence within the host (Hardoim et al., 2015; Khare et al., 2018; Pun and Joshi, 2023).

Host-dependent colonization by fungal endophytes is based on different strategies adopted by the endophytic fungi to develop and promote a mutualistic association with their respective hosts. Host dependent colonization has also been found to be correlated with the levels of phytohormones such as jasmonic acid and gibberellic acid (Liu et al., 2019). The concept of endophytism has been initially based on the hypothesis of “balanced antagonism” (Schulz et al., 1999) which was later modified to “multiple balanced antagonism” realizing that an endophyte alone in the plant endosymbiotic micro-ecosystem but is accompanied by diverse competitor microbes. Thus, the concept hypothesizes that “a large amount of antimicrobial metabolites are produced by the endophytes to maintain balanced antagonism with the host and the competitor microorganisms resulting in harmonious multifaceted symbiosis” (Schulz et al., 2015).

In the process of co-evolution, endophytic fungi have evolved various abilities similar to their host. One of the survival strategies in host micro-ecosystem is mimicking their host in the production of various secondary metabolites (Zhao et al., 2011). Hence endophytic fungi are regarded as alternative sources of beneficial secondary metabolites which were produced by their hosts only (Singh, 2019). Thus, endophytic fungi are considered as fascinating resources that can be relied upon in quest towards discovery of novel bioactive compounds with less toxicity. Discovery of *Taxomyces andreanae* created a revolution among microbiologists to bioprospect different endophytic fungi for the synthesis of host metabolites. Many endophytic

fungi mimicking the putative host metabolites have been explored such as podophyllotoxin have been produced by two isolates of *Phialocephala fortinii* isolated from the rhizomes of *Podophyllum peltatum* (Eyberger et al., 2006). Other compounds which have been mimicked by endophytic fungi comprise of Camptothecin (Shweta et al., 2014), emodin and hypericin (Kusari et al., 2008) and huperzine A (Cruz-Miranda et al., 2020).

During establishment of mutualistic or symbiotic association between the plant and microbes the first line of defence by the host plant is the synthesis of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), nitric oxide (NO), hydroxyl radical ($OH^{\bullet}$) and diffusible singlet oxygen ($^1O_2$) which act as signals indicating beneficial symbiotic interactions with fungi. Hence endophytic fungi during establishment of a symbiotic association generates signal molecules which can reduce the production of ROS/ oxidative stress (Torres, 2010; Zorov et al., 2014).

2.2 Hypothesis

As it has been well documented that oxidative stress is one of the drivers of α-synuclein aggregation as well as pathological progression of α-synucleinopathies. It becomes imperative to reduce the ROS, thereby reducing the oxidative stress for further α-synuclein aggregation as well as disaggregation of the pre-existing oligomeric α-synuclein aggregates.

As endophytic fungi offer immense possibilities for producing putative phytochemicals exhibiting oligomeric α-synuclein disaggregation properties as well as reduce the generation of ROS burst responsible for the α-synuclein aggregation (misfolding) the present research work is oriented on bioprospecting the endophytic fungi from *Camellia sinensis* and *Malus domestica* with the following objectives;

1. To screen endophytic fungi isolated from plants *Camellia sp*. and *Malus sp*. for α-synuclein inhibition
2. To isolate and characterize the α-synuclein inhibitors from endophytic fungi
3. *In vitro* evaluation of efficacy of α-synuclein inhibitor(s)

CHAPTER-3
REVIEW OF LITERATURE

3.1 Background

The functioning of proteins in living cells can't be overestimated since they are responsible for almost all molecular-related biological processes. Proteins are known as the structural and functional units of living cells. The functions of proteins are unlimited, from building blocks to catalysts and signal transducers (Cheng et al., 2020; Kinoshita, 2020; Seyedabadi et al., 2022). They are known to be the most abundant biopolymer of living cells, constituting ~ 55 % of the dry biomass of the cell (Feijo Delgado et al., 2013).

Proteins may act alone or bind with membranes (Das and Eliezer, 2019), polysaccharides (Schjoldager et al., 2020), small molecules (Li et al., 2013 b), ions (Jing et al., 2017), and other proteins to generate protein complexes (Drew et al., 2021; Luck et al., 2020; Orchard et al., 2014). On the other hand, post-translational modifications increase the polypeptide chain's chemical diversity (Betts et al., 2017; Brodbelt, 2022; Sostaric et al., 2018).

For a century, the scientific community has firmly believed in the induced fit model, which states that binding an enzyme with an appropriate substrate leads to conformational changes in the active site of the protein (Koshland, 1995). Since then, the 3-D form of the protein has been understood as the functional structure prerequisite for the protein structure-function paradigm and an approach toward the "one gene-one enzyme" hypothesis. According to the hypothesis, a single 3-D structure of the proteinous enzyme determines the protein's specific function (Rieger et al., 1968). Various findings do not support this lock and key model theory, which were initially regarded as exceptions. To understand such proteins, the concept of intrinsically disordered proteins existed (Peng et al., 2015).

Intrinsically disordered proteins form a class of proteins that may contain different segments lacking a defined 3D structure (Dunker et al., 2008). Approximately 25 - 30 % of the eukaryotic proteins are IDPs, whereas > 70 % of signalling proteins are intrinsically disordered proteins in nature (Oldfield et al., 2005). They exist as highly dynamic protein bodies that can perform reversible interconversion between secondary and tertiary conformations (Uversky et al., 2000). Intrinsically disordered proteins are further classified as collapsed and extended forms (Uversky, 2019). Functionally, these proteins control various cellular activities such as entropic chain activities (Uversky and Dunker, 2012), protein modifications (Theillet et al., 2014), molecular recognition, and assembly (Yang et al., 2020). Intrinsically disordered proteins act as RNA (Tompa, 2012), protein chaperones (Tompa and Kovacs, 2010), allosteric regulators (Berlow et al., 2018), and enzyme catalysts (Chakrabortee et al., 2010). On the contrary, the protein class is responsible for various disorders such as amyloidogenesis (Bhattacharya et al., 2018), cancer (Santofimia-Castano et al., 2020), cardiovascular diseases (Monti et al., 2022), and

neurodegenerative disorders (Ayyadevara et al., 2022). This research has been conducted to study an Intrinsically disordered protein known as α-synuclein protein. The protein is responsible for the progression of various synucleinopathies such as Parkinson's disease (PD), multiple system atrophy (MSA), Parkinson's disease dementia (PDD), and dementia with Lewy bodies (DLB) (Staerz et al., 2022).

3.2 Alpha-synuclein: the underlying cause of synucleinopathies

Synuclein is a small protein present primarily in neural tissues and certain tumors. The protein study was started in 1988 by Maroteaux et al. (1988) with cloning and identification, followed by genetic information (Polymeropoulos et al., 1997). The synuclein family is the molecular innovation of eukaryotes, as no common protein ancestors exist (Lavedan, 1998). Members of the synuclein family exist only in the vertebrates. The first synuclein probably appeared in the central nervous system (CNS) during sophisticated regulation of synaptic transmission and regulation.

The synuclein family consists of three members in all the vertebrates, α, β, and γ synuclein, with one exception of pufferfish *"Fugu rubripes"* which has four synuclein members, i.e., α, β, γ1, and γ2 in its genome. Fujita et al. (2006) explained the antagonistic properties of different members of α and β synuclein. Interestingly, alpha-synuclein acts as a chaperone and buffers for stress-induced response. At the same time, beta synuclein behaves as an anti-chaperone and provokes the stress effect, which is how the dual protein family regulates the process by stimulating adaptive evolution.

The SNCA gene encodes the alpha-synuclein protein, located on chromosome 4q21.3-q22. Alpha-synuclein is a nerve terminal protein, primarily localized in the synaptic nerves comprises 140 amino acids (Patel and Bordoni, 2023). The protein is organized as seven exons, and five of them are protein coding. Three isoforms of the protein do exist; the predominant one with 140 amino acids, the second isoform is α-synuclein-126 produced due to an inframe deletion of exon 3, and the third isoform α-synuclein-112, by an inframe deletion of exon 5. The most common isoform of α-synuclein-140 consists of three domains (Beyer, 2006). In the native state, the protein is found in the soluble and unfolded cytosol, exerting properties such as heat resistivity. In normal physiological conditions, the protein is in the monomeric form (Bandopadhyay, 2016).

3.2.1 Structural aspect of alpha-synuclein protein

The N-terminus is the positively charged amphipathic region, composed of 60 amino acid residues. The region is dominated by a highly conserved hexameric (KTKEGV) motif in seven series of 11 AA. The region plays an essential role in the α-synuclein lipid interaction via its ability to disrupt lipid bilayers, induce α-synuclein interactions, and reduce β-sheet confirmations (FNE

12). It is a highly conserved domain, and all the possible point mutations in the SNCA gene are discovered in the N-terminus (Torok et al., 2016).

NAC domain is the central domain known as the core region composed of residues 61-95. The domain is encoded by exon 4, which is involved in amyloid formation**.** The non-Aβ-component of the AD amyloid (NAC) domain is a hydrophobic region critical for β-sheet formation. The C-terminus domain of α-synuclein comprises 43 amino-acid residues, containing five glutamate and five aspartate residues. The terminus has a high net negative charge and low hydrophobicity. The C-terminus is responsible for the inhibitory effect of protein aggregation. The structure possesses 129-phosphorylated serine residue, whereas dephosphorylation induces the activity of the NAC domain and causes protein aggregation. The C-terminus shows homology with small heat shock proteins, suggesting protecting α-synuclein protein from degradation (Emamzadeh, 2016).

3.2.2 Physiological activities of protein

The physiological form of α-synuclein protein is primarily present in the presynaptic nerve terminal, ranging in a 5-50 μM concentration. The protein is suggested to play an essential role in synaptic vesicle neurotransmission (Banks et al., 2020; Roman-Vendrell et al., 2021; Soll et al., 2020), promote SNARE (N-ethylmaleimide-sensitive factor activating protein receptor) complex formation, and promote neurotransmitter release (Burre et al., 2010), and helps in regulating the size of presynapse (Vargas et al., 2017). The monomeric protein is non-toxic and is reported to play a crucial role in maintaining body functions. The role of protein is studied in the whole process of regulation of synaptic vesicles, i.e., trafficking, docking, fusion, and recycling (Cheng et al., 2011).

The protein plays a vital role in maintaining the viability of dopamine neurons in the neural area substantia nigra pars compacta (Benskey et al., 2018). In the synaptic vesicles, the physiological protein interacts with vesicular monoamine transporter-2 (VMAT2) and regulates dopamine storage. In favour of the same, VMAT2 further controls the effect of oxidative stress in the cytosol induced by dopamine metabolites. Degeneration of dopaminergic neurons in the synaptic vesicles is considered one of the earliest events caused by irregular forms of α-synuclein protein (Chadchankar et al., 2011; Gonzalez-Hernandez et al., 2004).

Added support comes with the combined study of diabetes and Parkinson's disease. Geng et al. (2011) suggested that the interaction of the physiological form of protein with kir6.2 subunits of ATP channels inhibits dopamine synthesis and thus regulates neurotransmission. More evidence suggests that the interaction promotes brain-derived neurotrophic factor expression and helps relieve PD and diabetes (Avshalumov and Rice, 2003; Vargas-Medrano et al., 2014).

Kowalski et al. (2022) studied the role of monomeric α-synuclein protein in maintaining calcium homeostasis by activating plasma membrane calcium ATPase (PMCA) in the neurons. PMCA is a single polypeptide transmembrane protein recognized as an active transporter. PMCA maintains the concentration of free calcium ions in the cytosol, which is necessary for the proper functioning of the cells.

α-synuclein protein also plays an essential role in mitochondrial activity. It can bind with the negatively charged phospholipids and cross the membrane. The protein may interact with the alpha subunit of ATP synthase in the inner mitochondrial membrane (Cremades et al., 2012; Guardia-Laguarta et al., 2014; Robotta et al., 2014). Ludtmann et al. (2016) analyzed the role of monomeric protein in mitochondria by using knock-out mice and provided evidence that the protein may affect ATP efficiency due to the direct interaction of monomeric α-synuclein protein with ATP synthase present in the mitochondrial membrane. This interaction further regulates the bioenergetic needs of neuronal synapses.

3.2.3 alpha-synucleinopathies

Multiple factors induce oligomerization of the monomeric α-synuclein protein and cause a group of diseases collectively known as α-synucleinopathies (Alafuzoff and Hartikainen, 2018). Synucleinopathies are a group of neurodegenerative diseases that develops due to aggregation of α-synuclein protein in the neurons and glial cells. Primary α-synucleinopathies include PD, DLB, MSA, and PDD (McCann et al., 2014). All forms of α-synucleinopathies have a common universal feature known as intracytoplasmic inclusions of proteins. Different α-synucleinopathies are categorized on the basis of the appearance of various intracytoplasmic inclusions. Intracytoplasmic inclusions are proteinous intracellular entities made up due to the aggregation of α-synuclein protein (Fig 3.1). These inclusions are known as Lewy body in PD and DLB; however, glial cytoplasmic inclusions in MSA (Dickson, 2018; Gilman et al., 2008; Koga et al., 2020).

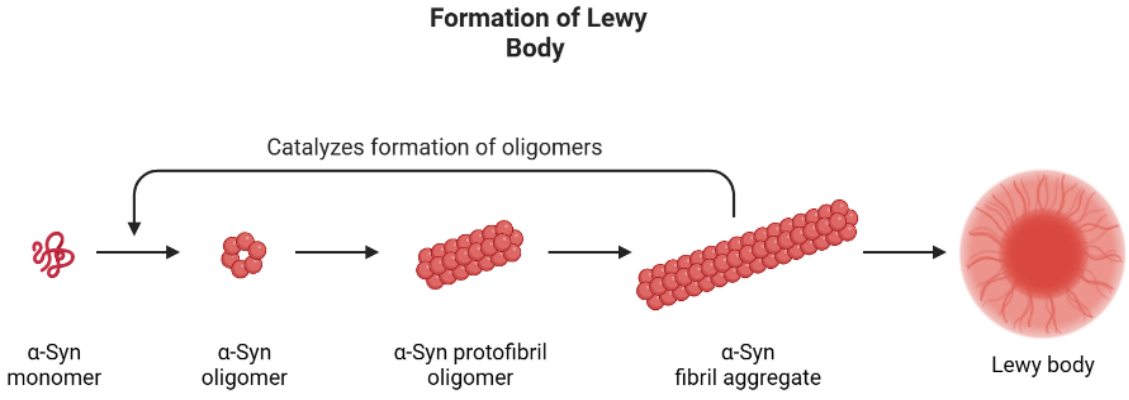

Fig 3.1 Sequential events in formation of Lewy body

3.2.3.1 Parkinson's disease (PD)

PD is the most prevalent α-synucleinopathy. It is known as the second most common neurodegenerative and most common motor neurological disorder worldwide. Epidemiology suggests that PD is an age-related disease that increases steadily with age and is more prevalent in males than females (3: 2) (Kalia and Lang, 2015). Li et al. (2022 b) showed that the number of PD patients older than fifty is expected to double by 2030. Zhang et al. (2022 a) suggested association of physical activity and diet causing PD in adults. The symptoms associated with the disease are categorized as motor symptoms (bradykinesia, resting tremor, rigidity), and the most common non-motor symptoms are recognized as (constipation, sleep behaviour disorder, hypotension, and rapid eye movement) (Kulisevsky, 2022).

The hypothetical signs of PD may vary with the time of progression (Fig 3.2). In normal physiological conditions, the dopaminergic neurons are maintained and started decreasing slowly in the prodromal stage, with a decrease in the nigral neurons survival rate (light blue curve).

Red dots in the prodromal stage indicate an increment of inflammatory markers, which may

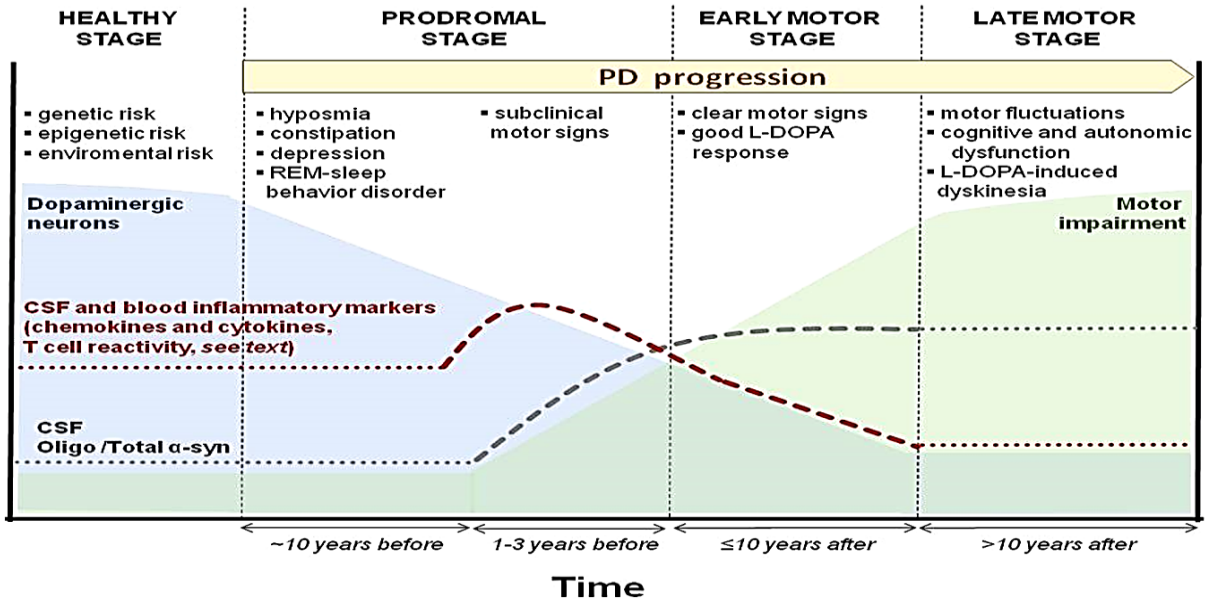

Fig 3.2 Hypothetical signs and biomarkers in Parkinson's disease emphasizing over dopaminergic neurons (light blue), inflammatory markers (red dots), motor impairment (green curve) and concentration of oligomeric alpha-synuclein protein (grey dots) Redrawn from: (Calabresi et al., 2023).

gradually decrease in the early motor stage. The green curve shows motor impairment increasing gradually in the prodromal stage. Grey dots depict elevated oligomeric α-synuclein/ total oligomeric α-synuclein ratio until early motor stage. The fig 3.2 represents signs and biomarkers associated with all the stages (Calabresi et al., 2023).

3.2.3.2 Other synucleinopathies

Other than PD, the oligomeric form of α-synuclein protein is reported in other disorders named dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and Parkinson's disease dementia (PDD). DLB and PDD are characterized as common forms of dementia and share neuropathological and clinical features such as hallucinations, cognitive fluctuations, and, most importantly, dementia (Jellinger and Korczyn, 2018). Both of these forms of dementia are distinguishable based on the onset of symptoms, which may ultimately give rise to clinical/ latent parkinsonism. In DLB, parkinsonian dementia begins within one year of start after cognitive impairment, which may or may not lead to Parkinsonian, whereas in PDD, motor symptoms are followed by cognitive impairment and Parkinsonian (Emre et al., 2007; Kim et al., 2014; McKeith et al., 2005).

MSA is another rare neurodegenerative disorder that takes place due to abnormal accumulation of toxic forms of α-synuclein protein known as glial cytoplasmic inclusions. The disease is categorized based on symptoms such as Parkinsonian as MSA-P and cerebellar as MSA-C. MSA-P is the most common type, with symptoms similar to PD, whereas MSA-C is categorized as ataxia, which means irregular muscle contraction (Kaji et al., 2020).

3.3 Factors responsible for protein oligomerization

The exact sequential mechanism responsible for the induction of neurotoxicity by oligomeric α-synuclein protein is yet to be identified. However, disruption of certain cellular events accountable for disease progression due to genetic or environmental changes is well understood. The most prevalent α-synucleinopathy, i.e., PD, was studied in detail to determine the factors responsible for the progression of the disease (Calabresi et al., 2023). Based on these reasons, PD could be studied as sporadic and familial.

Deng et al., (2018) reviewed the genetics of PD and suggested that among diagnosed patients, 5-10 % of patients had mutations in their genes. Funayama et al. (2023) reviewed all the PD-related genes registered in Online Mendelian Inheritance in Man. At least eleven autosomal dominant and nine autosomal recessive genes have been implicated in the progression of PD.

The effects which are proposed in making the protein toxic and toxic protein causing cell death altogether are recognized as a) disruption of mitochondrial morphology, b) increase in endoplasmic reticulum stress, c) downregulation and inhibition of ubiquitin-mediated proteasomal degradation, d) disruption of calcium ions homeostasis, e) change in membrane potential and f) synaptic dysfunctioning. Post-translational modifications such as ubiquitination and phosphorylation promote toxic species formation of α-synuclein protein. In physiological conditions, less than 5 % of the protein is phosphorylated. Conversely, 90 % phosphorylated protein at serine residue in the toxic species shows a direct relation between post-translational

modification and protein oligomerization (Tenreiro et al., 2014). Calcium ions are also regarded as critical factors inducing α-synuclein neurotoxicity (Angelova et al., 2016).

3.3.1 Oxidative stress and alpha-synucleinopathies

The generation of reactive oxygen species (ROS) occurs due to the enzymatic process, i.e., due to mitochondrial enzymes, NADPH oxidase, and respiration of inflammatory and structural cells (Zorov et al., 2014). Environmental factors such as UV radiation and smoking also increase oxidative stress and produce ROS (Ortiz et al., 2016). ROS are partially reduced and unstable forms of oxygen, which are very active and have a concise life cycle. This reduced form of oxygen may include oxygen radicals ($O_2^{\cdot}$, $OH^{\cdot}$) and non-radicals (H_20_2, O_3, $^1O_2$), easily convertible into the oxygen radicals. In normal physiological conditions, antioxidants such as GSH, catalase, and SOD compensate for oxidative stress. Unfortunately, during the fibrillation and oligomerization of α-synuclein protein, excess oxidative and nitrosative stress production occurs, and antioxidants do not diminish the effect of excessive stress. Elevated oxidative stress levels are driven by the pathology of numerous neurodegenerative disorders such as AD, PD, and other synucleinopathies (Domanskyi and Parlato, 2022).

All forms of the protein, ranging from monomer to oligomer, including intermediates, are intracellular and can cross the plasma membrane (Cremades et al., 2012; Nakamura et al., 2008). Notably, the oligomeric form is toxic because it inhibits the expression of endogenous antioxidant GSH and enhances oxidative stress (Deas et al., 2016). Interestingly, oligomeric α-synuclein protein is not linked with enzymatic production of the ROS and possesses the ability to produce ROS *in vitro* by itself by interacting with transition metals (Chen et al., 2015; Lothian et al., 2019; Ludtmann et al., 2018; Ramis et al., 2018; Reeve et al., 2015). Oligomeric protein disturbs cell physiology in multiple ways (Fig 3.3) (Abramov et al., 2020). The interaction may lead to lipid peroxidation sequentially, followed by an increment in α-synuclein induced channel formation and upregulated calcium signalling (Angelova et al., 2020). Disturbance in calcium homeostasis and superoxides may oxidize proteins of F_0-F_1 ATP synthase, decrease ATP efficiency, and open mitochondrial permeability transition pores (Angelova et al., 2015; Ludtmann et al., 2016). The oligomeric form of protein also interacts with Hsp70 and affects chaperone activity and the protein folding process, and this is enough for mediating apoptosis, necrosis, and ferroptosis (Aprile et al., 2017; Hinault et al., 2010). The insoluble form of protein further leads to the activation of DNA-repairing enzymes, decreasing NAD+ content and thus activating ADP ribose polymerase, mediating cell death (Delgado-Camprubi et al., 2017; Kam et al., 2018).

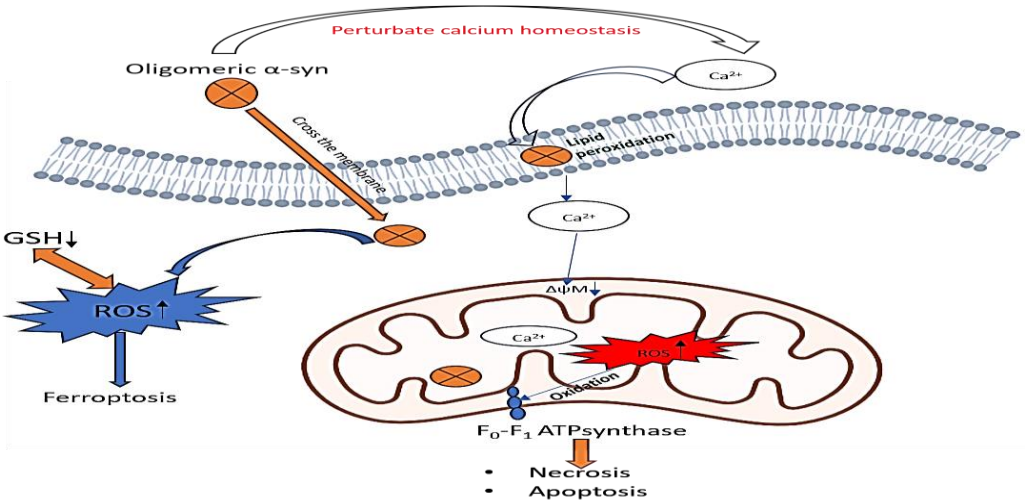

Fig 3.3 Oxidative stress in disturbing the cellular process and progression of disease

3.4 Therapeutic options for treatment of α-synucleinopathies

To date, no curative treatment of PD has been reported that may modify the course of the disease. As we discussed earlier, the condition is growing exponentially, and the number of patients is expected to double in the next 30 years. The annual cost calculated to be spent per patient in Europe is EUR 3910, while in the USA, it is USD 4551 (Gomez-Inhiesto et al., 2020). As a result, we tried to summarize all the possible rectifying therapies ever reported by targeting various mechanisms towards misfolded/ oligomeric α-synuclein protein. The targeted treatment could be categorized as disease-modifying therapy depending on whether the therapy may target the underlying causes and symptoms of disease and non-disease modifying, which is acting by reverting dopamine functioning.

3.4.1 Dopaminergic symptoms relief drug therapy

Numerous options were identified which may interfere with the pathogenesis of synucleinopathies. Primary among all is the treatment of reduced dopaminergic levels in the substantial nigra pars compacta. Over the past few years, L-dopa (levo-dihydroxyphenylalanine) has been considered the primary drug that helps restore the lost dopamine in the striatum (Zahoor et al., 2018). In 1975, the drug was approved by the FDA, and since then, the drug has been the ultimate choice for treating the earlier cardinal symptoms of PD (Mian, 2021). L-dopa is known to be the precursor of dopamine in the striatum and helps in relieving the level of dopamine. The drug's action is restricted due to peripheral tissue and gastrointestinal tract metabolism, which may reduce potency and undesirable side effects. Hence, to compensate for the side effects, the drug is prescribed in a combination of carbidopa (Greig and McKeage, 2016; Hinz et al., 2014).

Table 3.1: Food and Drug administration's approved anti-parkinsonian drugs				
Drug	**Brand name**	**Approval year**	**Site of action**	**Side effects**
Carbidopa and levodopa	Rytary	2015	Dopaminergic neurons	Insomnia, dyskinesia, hypotension, anxiety
	Duopa	2015	Inhibits decarboxylation of levodopa in the peripheral region	Hypertension, respiratory infection, erythema
Pimavanserin	Nuplazid	2016	Target agonist and antagonist activity at serotonin receptors	Dilemma
Amantadine	Gocovri	2017	Uncompetitive antagonist of NMDA receptor to treat dyskinesis	Hypotension, hallucination, non-motor fluctuations
Safinaminde	Xadago	2017	Inhibit MAO-B enzyme	Dyskinesia, nausea, insomnia

Drug combinations and routes of administration may vary depending on the patient's response (Greig and McKeage, 2016; Thakkar et al., 2021; Zibetti et al., 2014). Drug intake of L-dopa was up to 1 g initially until 1988, when the FDA approved 800 mg/ day uptake of L-dopa/ carbidopa formulation (Brodell et al., 2012). Unfortunately, even after these numerous precautions, the drug is associated with motor fluctuations known as L-dopa-induced dyskinesia (Table 3.1). L-dopa could block aromatic amino acid decarboxylase and induce dyskinesia, which may include a wide variety of symptoms, including ballism, chorea, dystonia, and hyperkinesia (Hansen et al., 2022). The study shows that L-dopa-induced dyskinesia may vary in patients, but prospective data suggest the onset of side effects in 10-30 % of patients in one year of treatment. However, 94 % of patients develop these symptoms after 15 years of exposure to the drug (Hely et al., 2005; Manson et al., 2012). The wearing-off effects of the drugs are commonly reported within a very short period. Hence, other drugs have been formulated to combat the side effects for relief of dopaminergic symptoms. Clinical phase trials were studied at clinicaltrial.gov as of January 31st, 2022, to understand the drugs more precisely. The clinical trial III suggested ABBV-951, APL-130277, Apomorphine, IPX203, Levodopa-Carbidopa Intestinal Gel, LY03003, ND0612, Opicapone, P2B001, and Tavapadon to be effective for treatment of PD (McFarthing et al., 2022).

3.4.2 Antioxidants

Oxidative and nitrosative stress are responsible for dysregulating many other enzymes and cause mitochondrial dysfunctioning, lipid peroxidation, dopamine oxidation, and ionic balance perturbance. To prompt the analysis, the brains of PD patients were analysed and confirmed

decreased levels of antioxidants such as glutathione. A variety of such antioxidant molecules was studied via experimental and clinical analysis.

Endogenous molecules melatonin is a natural antioxidant compound studied for its potential in slowing the progression of idiopathic PD by targeting mitochondria and eliminating OH radicals (Paul et al., 2018; Rasheed et al., 2018), improve sleep disorders (Ahn et al., 2020; Belaid et al., 2015; Gilat et al., 2020; Sun et al., 2016; Zhang et al., 2016), reduce side effects of L-dopa (Naskar et al., 2015) and reduce COX-2 activity (Belaid et al., 2015) and thus enhance clinical improvement in PD patients. Another endogenous antioxidant molecule is CoQ_{10}, a mitochondrial carrier for electron transport that helps in the protection of dopaminergic neurons and improves motor performance (Beal et al., 2014). Administration of urate precursor, inosine, helps improve PD symptoms (Huang et al., 2017; Nakashima et al., 2019; Sarukhani et al., 2018 b). Many other endogenous molecules such as kynurenic acid (Ferreira et al., 2018), L-Carnitine (Gill et al., 2018), Glutamine (Zhao et al., 2019), n-3 polyunsaturated fatty acid (Hernando et al., 2019), sulfur containing antioxidants such as lipoic acid, hydrogen sulfide and N-acetylcysteine (Sarukhani et al., 2018 a; Virel et al., 2019; Zhou et al., 2018; Zhou and Cheng, 2019) are reported in replenishing the enzymatic activity and relieving symptoms of PD.

Schirinzi et al., (2019) suggested the role of high dietary vitamin E intake in the neuroprotection and treatment of PD. Phenols/ polyphenols and terpenes are known for their antioxidant potential. The compounds were studied in animal models to analyze their function in PD therapy (Percario et al., 2020). The potent class of compounds exhibited potential benefits towards reducing oxidative stress and thus modulating a lot more activities towards PD protection, as shown in Table 3.2.

Table 3.2: Potential benefits of antioxidants in Parkinson's disease

Compound class	Compound name	Neuroprotective effect	Reference
Phenols	Tyrosol	Reduce ROS level, Promote expression of chaperones	(Garcia-Moreno et al., 2019)
	Tricetin	Induce expression of Nrf-2 Reduce 6-OHDA induced oxidative stress	(Ren et al., 2019)
	Chrysin	Reverse neurochemical deficits, Treat behavioural abnormalities	(Goes et al., 2018; Krishnamoorthy et al., 2019)
	Acetoside	Upregulate Nrf-2 signaling pathway,	(Li et al., 2018 b)

		Prevent neural damage	
	Pinostrobin	Upregulate Nrf-2 signaling pathway, Upregulate heme-oxygenase activity	(Li et al., 2018 a)
	Curcumin	Decrease oxidative stress markers, Improve motor disabilities	(Nguyen et al., 2018; Rajasankar, 2019; Wang et al., 2017)
	Hesperidin	Reduce iron content, Restore dopamine levels, Treat mitochondrial dysfunctioning	(Poetini et al., 2018)
	Naringenin	Decrease α-synuclein expression	(Mani et al., 2018; Sugumar et al., 2019)
	Resveratrol	Activate SIR/ Akt1 signalling pathway, Inhibit α-synuclein aggregation	(Wang et al., 2018; Zhang et al., 2018)
	Genistein	Prevent mitochondrial oxidative damage	(Wu et al., 2018 a)
	Rosmarinic acid	Upregulate heme-oxygenase activity, Inhibit α-synuclein expression	(Qu et al., 2019)
	Salidroside	Regulate Wnt/ β catenin signalling pathway, Maintain Calcium ions homeostasis	(Wu et al., 2018 b)
	Anacardic acid	Prevent lipid peroxidation, Repair motor complications, Modulate SOD gene expression	(Medeiros-Linard et al., 2018)
Terpene	Thymol	Reduce dopaminergic neuronal loss, Attenuate inflammatory mediators	(Babazadeh et al., 2023)
	Astragaloside IV	Target pro-inflammatory cytokines, Inhibit α-synuclein expression	(Yao et al., 2023)
	Carvacrol	Promote memory deficit improvements	(Javed et al., 2023)

	β-Amirin	Protect effect on dopaminergic neurons, Inhibit α-synuclein aggregation	(Wei et al., 2017)
	Asiatic acid	Regulate mitochondrial dysfunctioning	(Ding et al., 2023)

Three antioxidant compounds were studied until the clinical phase trial deferiprone, sulforaphane, and tocovid suprabio (McFarthing et al., 2022). Many other drugs are reported that may alleviate the pathogenesis of PD by reducing oxidative stress. The medicines that help in neurotransmission by restoring dopamine are pramipexole (Dong et al., 2023) and paroxetine (Talebi et al., 2023). Rubio-Osornio et al. (2023) suggested the role of the antioxidant drug simvastatin in treating PD by modulating neuroinflammatory response in the substantia nigra pars compacta. Ebselen, Lactoferrin, hydralazine, and phenothiazine are other antioxidant drugs that significantly improve the symptoms and show promising effects in the treatment of PD (Amoroso et al., 2023; Jiang et al., 2023; Vrijsen et al., 2023; Yong et al., 2023).

3.4.3 Deep brain stimulation

Deep brain stimulation is a neurosurgical approach in which brain-implanted electrodes are used to treat neurological conditions and motor complications in PD patients. In PD patients, the electrode placed near subthalamic regions is responsible for a significant contribution to the occurrence of movement disorder (Lozano et al., 2019). Deep brain stimulation therapy may cause prolonged neuronal membrane depolarization, inhibiting action potential and releasing inhibitory neurotransmitters. Recent studies present evidence about the mechanism of action of deep brain stimulation therapy in motor and non-motor complications as measured with the Unified Parkinson's disease Rating Scale (Hartmann et al., 2019). The most crucial factor behind the success of deep brain simulation therapy is known as its direct effect on levodopa-related motor complications. However, the therapy is effective in treating motor complications but may result in adverse neuropsychiatric effects in substantial regions (Mosley et al., 2020).

3.4.4 Advanced Therapies

3.4.4.1 Immunotherapy

After accounting for the side effects of above-mentioned therapies, other treatments were discovered to potentially suppress CNS immunological activation and eliminate exogenous α-synuclein that helps recover mitochondrial abnormalities. Immunotherapy of anti-α-synuclein oligomerization could be divided into two categories, i.e., active and passive immunization. Active and Passive immunization may treat the disease by reducing the release of

proinflammatory cytokines. Active immunization targets host genome oligomeric α-synuclein protein over a long period after administrating with active antibodies and thus helps in long-term prevention against neurodegenerative conditions (Vijayakumar and Jankovic, 2022). On the other hand, passive immunization targets antigen's epitope specificity. Cimpanemab and prasinezumab are two antibodies that successfully completed pre-clinical trials by targeting oligomeric α-synuclein N-terminus and C-terminus, respectively (Jensen et al., 2023). Furthermore, the antibodies cannot effectively cross the blood-brain barrier which is the primary concern regarding their clinical application (Benskey et al., 2018).

3.4.4.2 Gene therapy

Adeno-associated viral vectors are the most common vectors used for gene therapy to treat PD patients. The vector may target aromatic amino acid decarboxylase (Malaquias et al., 2022), glutamic acid decarboxylase (Shalaby and El-Agnaf, 2022), neurturin and glial cell line-derived neurotrophic factors (Serva et al., 2022). This treatment benefits patients with significant side effects due to consumption of levodopa. By consuming levodopa in a suitable quantity, an adequate amount of dopamine can be maintained by using this therapy (da Silva Oliveira et al., 2020). Glutamic acid decarboxylase enzyme-associated vectors have proven beneficial in the treatment of PD patients by alleviating GABA synthesis and attenuating motor dysfunctioning (Nashid and Kethar, 2022). Neurturin is another gene to be targeted for the treatment of PD. Neurturin is a neurotrophic factor that helps dopaminergic neurons proliferate and survive (Buttery and Barker, 2020). The therapy has been proven safe and effective after pre-clinical trials (Barker et al., 2017; Marks et al., 2008).

3.4.4.3 Cell-based therapy

In stem cell-based therapy, mesenchymal stem cells are used due to their risk-free properties in the human body. Mesenchymal stem cells are non-hematopoietic and multipotent cells with low immunogenicity, lower risk of tumor formation, and little ethical concern (Xiong et al., 2010). They have been proven highly beneficial for PD treatment (Gugliandolo et al., 2017). They may improve motor complications as they could be developed into tyrosine hydroxylase and possess anti-inflammatory and paracrine actions (Harrell et al., 2019). Although, the treatment is promising, and many clinical trials are underway (Hoang et al., 2022). Unfortunately, graft enlargement and gene alteration may suppress tumor suppressor genes in the transplanted gene, resulting in tumor formation. Hence, thorough supervision is required to ensure the minimal danger of generating the tumor to make the therapy beneficial.

3.5 Plant-derived Molecules in the treatment of PD

Human civilization has significantly depended on plant sources for herbal drugs for centuries. According to the World Health Organization (WHO), more than 80 % of the global population depends upon conventional medication in the form of pharmaceutics and diet, which is easy, safe, and provides effective primary health care. However, FDA-approved small molecules from 1981 to 2014 were studied, which depicts that 51% of molecules were isolated from natural sources (Chen et al., 2014). Natural compounds are isolated from biological activity possessing natural sources, i.e., plants, animals, and microorganisms (Baker et al., 2007). Newman and Cragg (2016) assessed the role of United States Food and Drug Administration (USFDA)-approved natural products in drug discovery and concluded that among all 38.1 %, natural drugs prevailed for the treatment of various diseases, such as anti-lipidemic drugs (simvastatin) (Gupta et al., 2020), hypertensive drug (captopril); immunosuppressive agent (cyclosporin and rapamycin); antitumor (taxol and campothecin); antibiotic (penicillin) (Li and Vederas, 2009).

Table 3.3: Phytochemicals targeting alpha-synuclein protein

Plant name	Common name	Compound name	Model organism	Activity of compound	Reference
Camellia sinensis	Tea	Epigallocatechin Gallate	*In vitro*	Prevent fibrillization	(Zhao et al., 2017)
Mallotus philippinensis	Kamala tree	Quercetin	*In vitro*	Inhibits α-synuclein aggregation	(Harati et al., 2023)
Scutellaria baicalensis	Chinese skullcap	Baicalein	*In vivo*	Inhibits apoptosis and inflammasome activation, Attenuates α-synuclein aggregation	(Yao et al., 2022)
Tripterygium wilfordii	Thunder god vine	Celastrol	*In vitro*	Downmodulate α-synuclein specific T-cell response and promote clearance of toxic structure	(Ng et al., 2022)
Curcuma longa	Turmeric	Curcumin	*In vitro*	Increase α-synuclein solubility and prevent oligomerization	(Lei et al., 2023)
Coffee arabica	Coffee	Chlorogenic acid	*In vitro*	Target GLP-1 and reduce oligomerization rate	(Angeloni et al., 2022)
Asteraceae	Sunflower family	Acacetin	*In vitro*	Treat motor functions, Inhibit α-synuclein oligomerization	(Zhang, et al., 2022 b)
Cistanche deserticola	Desert broomrape	Acetoside	*In vivo*	Downregulates caspase-3 activity, Inhibit α-synuclein oligomerization	(Yuan et al., 2016)
Vitaceae	Grape family	Resveratrol	*In vitro*	Disaggregate fibrils	(Wu et al., 2011)

Plants have been extensively explored for their potential for α-synuclein disaggregation. Different compounds have been analysed from various plants to study their mechanism of action towards inhibiting the structural changes and preventing α-synucleinopathies (Table 3.3). The activity of the compounds was confirmed via *In vitro* (PC12 cells, SH-SY5Y cells) and *In vivo* model organisms *Caenorhabditis elegans*, *Saccharomyces cerevisiae,* and transgenic mice (Kim et al., 2022; Minyan et al., 2023; Muhammad et al., 2023; Vats and Saxena, 2023; Zhong et al., 2022). Further, IC_{50} of different compounds was reviewed to understand the potential of compounds. Among all, black tea extract has the highest inhibition potential, followed by (−)-Epigallocatechin gallate (EGCG), a phenolic compound isolated from *Camellia sinensis* (Table 3.4).

Table 3.4: Naturally available alpha-synuclein inhibitors

Compound name	Class of compound	IC_{50} value	Reference
Tannic acid	Phenolic	61 nM	(Caruana et al., 2011)
Nordihydroguaiaretic acid	Phenolic	20 nM	(Caruana et al., 2011)
(−)-Epigallocatechin gallate (EGCG)	Phenolic	1.06 μM ± 0.05 μM	(Jiang et al., 2022)
Black tea extract	Phenolic	0.79 μM	(Caruana et al., 2011)
Baicalein	Phenolic	37±2.7 μM	(Bhatia et al., 2020)
Myricetin	Phenolic	2.52 μM	(Caruana et al., 2011)
Morin	Phenolic	4.24 μM	(Najib et al., 2022)
Rosmarinic acid	Phenolic	4.8 μM	(Najib et al., 2022)
Quercetin	Phenolic	15.4 ± 0.3 μM	(Bhatia et al., 2020)

3.6 Microbiological therapy for the treatment of PD

There are few reports of anti PD compounds derived from marine-based natural products. The marine-based natural compounds may target oligomeric α-synuclein and various other targets, such as oxidative stress and monoamine oxidase B (Huang et al., 2019). The sources for isolating these natural compounds are Archaea, Bacteria, fungi, algae and coral (Table 3.5).

Table 3.5: Alpha-synuclein disaggregators from microbial source

Source	Compound	Mechanism of action	Reference
Algae	Astaxanthin	Scavenge ROS production, Inhibit lipid peroxidation	(Galasso et al., 2018; Grimmig et al., 2017)
	Fucoidan	Improve antioxidant level and dopamine synthesis	(Meenakshi et al., 2016)
	Fucoxanthin	Modulate signalling pathway and enhance neuroprotection	(Lin et al., 2017)

	Spirulina platensis	Neuroprotection in 6-OHDA induced model	(Lima et al., 2017)
Archaea	Mannosylglycerate	Prevent α-synuclein misfolding	(Huang et al., 2019)
Bacteria	NP7	Promote antioxidant activity	(Koppula et al., 2012)
	Piloquinone	Inhibit monoamine oxidase	(Lee et al., 2017)
Coral	11-dehydrosinulariolide	Upregulate DJ-1 protein expression	(Feng et al., 2016)
Fungi	Neoechinulin A	Neuroprotection against MPP^+ neurotoxicity in PC12 cells	(Akashi et al., 2011)

3.7 Endophytic fungi as a novel source of α-synuclein disaggregation

Endophytic fungi constitute microorganisms living within the plant's core without exhibiting any overt symptoms. These exist symbiotically with their host plant and complete their partial or complete life cycle, co-evolving with them. They show a continuum in physiological states, colonization patterns, and secondary metabolism (Ancheeva et al., 2020; Rodriguez et al., 2009). These symbionts reportedly acquire the ability to produce putative phytochemicals of their host plant, possibly during co-evolution with the host (Venugopalan and Srivastava, 2015; Bielecka et al., 2022).

The role of nature-oriented products in drug discovery cannot be overestimated. Chen et al. (2014) reviewed FDA-approved small molecules from 1981-2014, among which 51% of molecules were isolated from natural sources. The high demand for plant-derived small molecules is responsible for plant species extinction. The class of endophytic fungi was then studied as an alternative to minimize the exploitation of plants and the high cost of production of secondary metabolites.

Endophytic fungi exist in a symbiotic relationship with the host plant while secreting novel bioactive metabolites that help in strengthening the host defence mechanism, i.e., tolerance to abiotic stress, pathogens, and herbivore deterrence (Hamayun et al., 2017; Mousa et al., 2016). The era of endophytic fungi came into existence after the discovery of paclitaxel, an anticancer drug isolated from *Taxomyces andreanae,* in 1993. The Discovery of Taxol established a new paradigm for the therapeutic potential of endophytic fungi. The discovery of this 3-billion-dollar drug influenced research on endophytic biology and discovered numerous compounds belonging to different chemical classes. These diversified bioactive compounds include alkaloids, terpenes, lactones, anthraquinones, quinone, steroids, phenols, and flavonoids which act on numerous diseases (Azhari et al., 2023; Dantas et al., 2023; Wen et al., 2022; Wu et al.,

2022). Therapeutic significant yet less explored endophytic fungal entities facilitate the discovery of secondary metabolites due to their impact on the pharmaceutical industry. Bioprospecting endophytic fungi leads to production of commercially significant metabolites including antimicrobial (mullein) (Fan et al., 2014); anticancer (Camptothecin) (Shweta et al., 2010); antitumor (vinblastine, vincristine) (Kuriakose et al., 2016); antioxidants (curcumin) (Yan et al., 2014); anti-inflammatory (aconitine) (Yang et al., 2013); antidiabetic (kaempferol) (Huang et al., 2014); antineoplastics (hypericin) (Jendželovská et al., 2016), anti-neurodegenerative (Table 3.6) (huperzine A) (Le et al., 2020) and HIV-1 protease inhibitor (Singh et al., 2004). Multifaceted applications of endophytic fungal isolates in the environment are also recommendable (Gao et al., 2020; Ghaffari et al., 2019; Soldi et al., 2020).

To the best of our knowledge, the area of discovery of endophytic fungal secondary metabolites as α-synuclein disaggregation is not explored yet and we reported the disaggregation potential of endophytic fungi for the first time (Vats and Saxena, 2023).

Table 3.6: Neuroprotective effects of endophytic fungal compounds

Endophytic fungi	**Source of endophytic fungi**	**Compound**	**Neuroprotective effects**	**Reference**
Alternaria alternata	*Salvia przewalskii*	Dibenzopyrone phenolic derivatives	Activate Nrf-2 and cellular antioxidant defence system	(Hou et al., 2021)
Nigrospora oryzae	*Tinosopra cardifolia*	Quercetin	Alzheimer's disease	(Vig et al., 2022)
Chaetomium globosum	*Imperata cylindrica*	10-indonyl cytochalasans	Antioxidative role	(Shen et al., 2020)
Phomopsis sp.	*Vatica mangachapoi*	10-phenyl cytochalasans	Promote antioxidant activity	(Shen et al., 2020)
Fusarium sp.	*Euphorbia* sp.	OQ-Fus-2-F	Anti Acetylcholinesterase activity	(Al-Qaralleh et al, 2021)
Fusarium lateritium	*Cornus officinalis*	Tricyclic pyridine alkaloids	Activate Nrf-2, maintain Ca^{2+} homeostasis, reduce ROS	(Lee et al., 2020)
Fusarium solani	*Morus alba*	Fusarubin, γ-pyrone	Protective effects against glutamate induced cell death	(Choi et al., 2020)
Alternaria brassicae	*Huperzia serrata*	Huperzine A	Acetylcholinesterase inhibitor	(Zaki et al., 2019)
Neosartorya fischeri	*Glehnia littoralis*	Meroditerpenoid compounds	Reduce oxidative radicals, elevate intracellular Ca^{2+}	(Bang, et al., 2019 b)
Collectotrichum gloeosporioides	*Suaeda japonica*	Collectotrichamide	Preventive effects against glutamate induced cytotoxicity	(Bang, et al., 2019 a)

CHAPTER-4
MATERIALS AND METHODS

4.0 Materials and Method:

4.1 Plant Sample Collection

Asymptomatic stem, roots and leaf samples of *Camellia sinensis* was collected from Assam (27°31'10"N 95°53'18"E) and Palampur (32.109722°N 76.536641°E), whereas, similar samples for *Malus domestica* were collected from Shimla (31°6'12"N 77°10'20"E). The collected plant samples were independently packaged into polypropylene sterile zip packets and stored at 4 °C till further use.

4.2 Isolation of endophytic fungi

Endophytic fungi were isolated from the disease-free stem, leaves and roots. Surface sterilization was carried out to remove the epiphytes. All the samples were washed under running tap water and air dried, followed by surface disinfection aseptically under a biosafety cabinet. The isolation of fungi was performed as per the method of (Katoch and Pull, 2017) with minor modifications. For aseptic sterilization, all the samples were dipped in 70 % (v/v) ethanol for 2 min followed by 0.5 % (v/v) Sodium hypochlorite (NaOCl) for 2 min in case of leaf and 5 min for stems and the followed by 90 % (v/v) ethanol treatment for 30 sec. Sterilized plant samples were rinsed with autoclaved distilled water six times and air dried (Verma et al., 2007). To confirm the efficacy of surface sterilization, imprints of sterile samples were taken on the Potato dextrose agar (PDA) plate for 48 hr to ensure the absence of epiphytic microflora. Subsequently, the leaf and stem samples were cut into 3 mm and 5 mm pieces, respectively, with the help of a sterile blade and placed on half-strength PDA plates using a sterile scalpel.

For the growth of endophytic fungi, the plates were inoculated with different samples were incubated for 10 days with 12 hr light and dark photoperiod at 26 ± 2 °C. The plates were observed after every 24 hr and the hyphae that emerged were picked using a sterile needle and plated individually over full-strength PDA to obtain axenic cultures of the endophytic fungi (Chen et al., 2010).

The pure cultures of endophytic fungal isolates were designated based on the segment number followed by plant details (Botanical name of the plant, plant part and place of collection). As shown in Fig 4.1, in 1CSSTAS culture code, 1 stand for segment number, CS stands for *Camellia sinensis*, ST for stem plant part and AS for Assam.

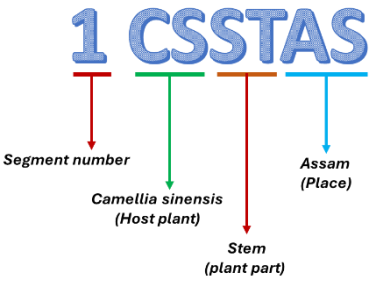

Fig 4.1 Coding outline of pure isolates of endophytic fungi

4.3 Fungal diversity studies

4.3.1 Isolation rate and colonization frequency of endophytic fungi

In a given sample of plant tissue, colonization frequency and isolation rate are measured to understand the fungal richness in the given sample (Sun et al., 2008). Samples were incubated for 6 weeks under daily examination. Colonization frequency and isolation rate is calculated as:

$$Colonization\ frequency = \frac{No.of\ individual\ fungi\ recorded}{Total\ no.of\ segment\ screened} \times 100$$

$$Isolation\ rate = \frac{No.of\ isolates\ obtained\ from\ segment/\ pieces}{Total\ no.of\ segment/\ pieces}$$

4.3.2: Shannon-Wiener diversity index

The species diversity in both plant samples were compared by Shannon's index (Fernandes et al., 2015). The formula used for calculation of Shannon-wiener index is as follows:

$$H = -\sum_{i=1}^{s} Pi\ ln\ Pi$$

Where, H= Diversity in a sample of species,

Pi= relative abundance of i^{th} species or kinds and measured by n_i / N,

N = total no. of individuals of all kinds,

n_i = number of individuals of i^{th} species,

ln = natural log

4.3.3: Species evenness

Species evenness was calculated by using the following formula (Whittaker, 1972):

$$E = \frac{H'}{\ln S}$$

Where, E= species evenness,

H' = Shannon-Wiener index

ln S= Natural log of total no. of species

4.3.4: Simpson's diversity index

The Simpson's diversity Index (1-Dominance) was calculated by using the formula (Simpson, 1951):

$$D = 1\left(\frac{\sum n\,(n-1)}{N(N-1)}\right)$$

Where, N = Total no. of organisms of all species,

n = Total no. of organisms of a species

4.4 Preservation of endophytic fungi

Pure endophytic fungal isolates were preserved in the repository for subsequent use. Glycerol-PDA slants were prepared by dissolving 39 g of PDA (Hi Media) and 100 ml of glycerol in 1000 ml of double distilled lukewarm water. The glycerol slants were autoclaved at 121 °C for 15 min at 15 psi. Axenic cultures of endophytic fungi so obtained were aseptically inoculated on the PDA slant and incubated at 26 ± 2 °C for 10 days with 12 hr light and dark period. For each isolate, 2 slants and 2 vials were prepared and stored at 4 °C until further use (Zhang et al., 2009; Ababutain et al., 2021).

4.5 Production of culture filtrate for assaying α-synuclein disaggregation

The endophytic fungi were used for the production of culture filtrate in the Potato Dextrose Broth (PDB, Hi Media, India) to confirm the extracellular potential towards α-synuclein disaggregation. Briefly, the method comprised inoculation of 5 mm mycelial plug of 7 days old fungal culture in 30 ml sterile PDB (Santos et al., 2015). The culture was incubated at 26 ± 2 °C, 120 rpm for 10 days with 12 hr light and dark period (Huang et al., 2001). After completion of the incubation period, the fungal biomass was separated using Whatman filter paper (grade I) followed by centrifugation (Thermoscientific) at 5000 rpm for 10 min at 30 ± 2 °C. The cell free filtrates were stored at - 20 °C to till further use (Ezra, 2004).

4.6 Optimization of α-synuclein inducing recombinant strain

4.6.1 Phenotypic growth assay of Yeast

This assay was designed to establish the growth pattern of a recombinant yeast *Saccharomyces cerevisiae* "SY246". The genotype of *Saccharomyces cerevisiae* "SY246" is Mat a, D5A2, lys- ssa1 :: Kan, ssa2 :: HIS3, ssa3 :: syn-GFP-TRP1, ssa4 :: syn-GFP-URA3/pRS317-PSSA1-SSA1 and was expressing α-synuclein. This was received as a generous gift by Dr. Deepak Sharma (Principal Scientist, Yeast Biology and Protein Biophysics group, CSIR - Institute of Microbial Technology, Chandigarh). A growth phenotypic assay was performed to understand the mid-logarithmic growth pattern of the strain. The culture was streaked onto the Yeast peptone dextrose agar (YPDA, Hi Media, India) plate followed by incubation at 30 °C for 2-3 days. The colonies so obtained were re-inoculated in sterile Yeast peptone dextrose broth at 200 rpm (YPDB, Hi Media, India). To optimize the mid-logarithmic phase, the optical destiny (O.D.) was recorded up to 14 hr at 600 nm using spectrophotometer (BioTek Powerwave 340, USA) (Abdul-Mumeen et al., 2020; Ghaemmaghami et al., 2003).

4.6.2 α-synuclein induction by *Saccharomyces cerevisiae* SY246

The α-synuclein protein in *S. cerevisiae* SY246 was under galactose inducible promoter region. Thus, to induce α-synuclein production, the yeast culture was grown on YPDB medium till mid-logarithmic phase. Subsequently the cells were pelleted down by centrifugation at 6000 rpm for

5 min. The supernatant was discarded and cell pellet so obtained was washed thrice with sterile milli-Q water. For protein induction, the washed pellet was inoculated in the 2 % (w/v) Galactose (Hi Media, India) and 2 % (w/v) Raffinose (Hi Media, India) containing Yeast peptone galactose raffinose broth (YPGRB) media and incubated at 30° C for 10 hr at 600 rpm (Dixon et al., 2005).

4.6.3 GFP assay to confirm α-synuclein induction

In the fusion protein cassette, induction of α-synuclein protein was confirmed via GFP assay. Briefly, 1 ml sample comprising of induced cell pellet lysate, obtained by ultrasonication was loaded into a cuvette at room temperature and placed into a spectrophotometer (Perkin Elmer). Excitation and emission spectra were recorded with emission slit width of 10 mm at wavelength 395 nm and 509 nm, respectively (Savchuk et al., 2019). The fluorescence spectra of the buffer were subtracted from the protein. Emission spectra of 3 individual preparations were recorded to confirm the GFP quenching (Eremenko et al., 2013).

4.6.4 Oxidative stress quantification via DCFH-DA assay

This assay was performed to quantify the oxidative stress in *S. cerevisiae* SY246. DCFH-DA (2',7' dichlorodihydrofluorescein diacetate, Hi Media, India) is a cell permeable dye, which in the presence of ROS (reactive oxygen species), rapidly oxidized to highly fluorescent 2',7' dichlorodfluorescein (DCF) (Eruslanov and Kusmartsev, 2010). Briefly, 0.5×10^7 cells/ ml of 10 hr old galactose-induced cells were taken for the oxidative stress quantification. A 25 µM solution of DCFH-DA was freshly prepared from a stock solution (5 mM) and was incubated with the galactose-induced cells of SY246 for 30 min at RT under dark conditions (Esteves et al., 2009). Excitation and emission spectra of the samples was recorded at 485 nm (excitation) and 535 nm (emission) using fluorescence spectrophotometer (Parkin Elmer) (Beckmen and Ames, 1998).

4.7 Preliminary screening assay for α-synuclein disaggregation

Nitroblue tetrazolium (NBT, Sigma Aldrich) assay was designed to screen culture filtrate extracts of endophytic fungi based on scavenging of ROS. ROS are produced by yeast strain during the formation of α-synuclein protein (Javvaji et al., 2020). The NBT is reduced to blue coloured formazan after interacting with superoxide radicals generated during formation of protein. The reduction in the intensity of the blue coloured product indicated lower oxidative stress. The protein disaggregation assay was carried out in a 96 well microtiter plate. Briefly, the test wells (S1-S26) comprised of 25 µl (1 mg/ml) of cell-free culture filtrates were incubated with 150 µl of protein expressing media, i.e., YPGRB. Subsequently the plates were incubated at 30 °C for 18 hr, 600 rpm. After the culmination of the incubation period 20 µl of NBT (0.5 mg/ml) was added, and the plates were incubated at 30 °C for 2 h. The amount of formazan produced as a result of reduction by the ROS was measured by recording the absorbance at 575 nm (BioTek Powerwave

340, USA) (Kapoor and Saxena, 2016). The positive control comprised of plant based disaggregators viz. Piceatannol, resveratrol and quercetin have been designated as PC1, PC2 and PC3 respectively. The negative wells comprised all reagents except culture filtrates (Fig 4.2). Each test was carried out in triplicate and the data was represented as mean ± SD values. The percentage reduction in superoxide anions is calculated by using the formulae:

Formulae: [(A-B) - (C-D)] / (A-B) × 100

Where, A is OD at 575 nm with protein but without sample, B is OD at 575 nm without protein and sample, C is OD at 575 nm with protein and sample and D is OD at 575 nm with sample but without protein. Sample refers to the cell- free culture filtrate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC1	PC1	PC1	PC2	PC2	PC2	PC3	PC3	PC3	S1	S1	S1
B	S2	S2	S2	S3	S3	S3	S4	S4	S4	S5	S5	S5
C	S6	S6	S6	S7	S7	S7	S8	S8	S8	S9	S9	S9
D	S10	S10	S10	S11	S11	S11	S12	S12	S12	S13	S13	S13
E	S14	S14	S14	S15	S15	S15	S16	S16	S16	S17	S17	S17
F	S18	S18	S18	S19	S19	S19	S20	S20	S20	S21	S21	S21
G	S22	S22	S22	S23	S23	S23	S24	S24	S24	S25	S25	S25
H	S26	S26	S26	NC	NC	NC	NC	NC	NC	NC	NC	NC

Fig 4.2 Microtiter plate template for screening α-synuclein protein disaggregation activity. PC1, PC2 and PC3 refers to the positive controls, NC- negative control while S1-S26 are test extracts / culture filtrate.

4.8 Thioflavin T assay

4.8.1 Protein aggregation kinetics

Thioflavin T (ThT) is a benzothiazole dye that has been used for staining cross-β-architecture of amyloid fibrils (Sulatskaya et al., 2018). This leads to change in the spectral characteristics after binding and exhibits a stronger fluorescence emission. The conversion of the monomeric α-synuclein to oligomeric form was done by using 70 µM α-synuclein protein (Sigma-Aldrich). Briefly, the monomeric α-synuclein protein was dissolved in Phosphate buffer saline (PBS) and incubated at 37 °C, 800 rpm for 168 hr. To assess the conversion from a monomeric to oligomeric state ThT fluorescence assay was carried out at regular intervals of 24 hr. Hence, monomeric and oligomeric forms of α-synuclein was evaluated by using 1 mM ThT dye. The excitation and emission spectra were recorded at wavelength 450 nm and 482 nm respectively (Wordehoff and

Hoyer, 2018). Emission spectra of triplicate samples were recorded to confirm the ThT cross β-spectral analysis.

4.8.2 Protein disaggregation kinetics

To assess the protein disaggregation kinetics, 70 µM oligomeric protein was incubated with and without disaggregators in this assay. The cell – free culture filtrates (1 mg/ml) of endophytic fungi having ≥ 85 % activity were incubated with fibril samples until 168 hr at 800 rpm, 37 °C to optimise the disaggregation kinetics (Murray et al., 2023). The presence of cross β-sheet structure of the protein was investigated via ThT assay as explained in section 4.8.1.

4.8.3 Effect of culture filtrates on aggregation and disaggregation of α-synuclein

The endophytic fungi showing ≥ 85 % reduction in oxidative stress via NBT assay were further screened for their protein disaggregation potential by using a fluorescence spectrophotometer (Parkin Elmer). The analysis used oligomeric protein in the absence (control) and presence of cell free culture filtrates designated as test samples at a concentration of 25 µg/ml to assess inhibition or disaggregation. Absorbance for the cross-β-architecture study were recorded at 487.5 nm (Marzano et al., 2020).

4.9 Sandwich ELISA

Sandwich ELISA was carried out using "Human Alpha Synuclein oligomer ELISA Kit" (AlphaSYNo ELISA kit, Catalogue No.: MBS730762) as per manufacturer's instructions. The standard analysis was performed by using six known concentrations of oligomeric α-synuclein protein provided along with the kit, i.e., 0, 2.5, 5, 10, 25 and 50 ng/ml. Briefly, 50 µl standard solutions/ PBS and sample solutions were added in the primary antibody-coated wells to determine the standard, blank, and conc. of the protein in the sample (Pagan et al., 2019). The kit was incubated at 4 °C for 12 hr followed by washing with 1X wash buffer. 100 µl conjugate solution was mixed appropriately to the standard and sample wells. The plate was incubated at 37 °C for 1 hr in the dark conditions, followed by manual washing with 1X wash buffer. The washing step was performed for five times to remove all the liquid. 50 µl Substrate A and B were added in the wells and the plate was incubated under dark conditions for 30 min at 37 °C. Finally, 50 µl stop solution was added in all the wells, mixed well, and OD was quantified immediately at 450 nm using a microplate reader (BioTek Powerwave 340, USA).

4.10 Identification of potential endophytic fungi

The culture that exhibited maximum disaggregation potential was identified by morphotaxonomic features including cultural as well as microscopic characteristics and further confirmed via molecular phylogenetic methods.

4.10.1 Morphotaxonomy

The potential endophytic fungus was grown over 6 different media: Potato dextrose agar (PDA, Hi Media, India), Corn leaf agar (CLA, Hi Media, India), Pine leaf agar 1.5 % (w/v) (PLA), Saborauds dextrose agar (SDA, Hi Media, India), Water agar 1.5 % (w/v) (WA), and Cooke rose Bengal agar (CRBA, Hi Media, India) to assess its sporulation potential under different growth conditions. The culture plate was incubated at 26 ± 2 °C for 5-7 days under 12 hr light/dark cycles. The morphological properties such as growth rate, colony colour, colony diameter, texture, pigmentation, odour/ volatile fumes were observed to identify the fungus (Misra et al., 2021; Dwibedi and Saxena, 2018).

4.10.2 Microscopic identification

Mycelial mass was picked with a fine tip needle and placed over a clean glass slide containing a drop of water for examination of microscopic characteristics. The mycelial mass was properly teased with the help of sharp needles. The mounting of the culture was done in lactophenol cotton blue (Leck, 1999) with minimal modifications. The teased fractions were carefully covered with clean cover slip and sealed using DPX (Hi Media, India) mountant, subsequently the slide was observed under a compound microscope within a magnification range of 10x-1000x (Nikon Eclipse ε100). Image J software was used to measure the microscopic measurement of the culture for at least 50 observations. Various macroscopic aspects include upper surface colony colour, lower surface pigmentation, growth rate (cm) as well as microscopic aspects such as conidial morphology, apical cell morphology, mean length and width of conidia (µm) and their septation were observed.

4.10.3 Molecular taxonomy

Most of the endophytic fungi were identified to genus level on the basis of morphological features. Following morphological studies, molecular phylogenetic studies were performed to ascertain their genus as well as species (Tedersoo and Nilsson, 2016).

4.10.3.1 Isolation of Genomic DNA

Isolation of DNA was performed with the help of Fungal/ Bacterial Miniprep Kit (Cat. No.: D6005; Quick-DNA™; Zymo Research) as per instructions of manufacturer (Odetunde et al., 2020). For isolation of the genomic DNA of the selected fungal endophyte 50-100 mg of fresh fungal mycelia was added to the ZRBashingBead™ lysis tube (0.1 and 0.5 mm) and suspended in 200 µl of the isotonic buffer. Subsequently 750 µl of the BashingBead™ Buffer was added to the tube and these tubes were then secured with a 2 ml tube holding assembly and processed for 5 min at maximum speed.

Thereafter the ZRBashingBead™ lysis tubes were centrifuged at 10,000g for 1 min in a microcentrifuge. Subsequently 400 µl of supernatant was withdrawn and transferred to a Zymo-

Spin™ III-F filter in a collection tube and centrifuged at 8000 g for 1min. Then 1200 µl of Genomic lysis buffer was added to the filtrate in the collection tube. 800 µl was taken from the collection tube and transferred to a ZymoSpin™ IICR column in a collection tube and centrifuged at 10,000g for a minute. The flow in the collection tube was discarded and the process was repeated again. Now add 200 µl of the DNA pre-wash buffer to ZymoSpin™ IICR column in a new collection tube and centrifuge at 10,000 g for 1 minute. Thereafter add 500 µl of g-DNA wash buffer to ZymoSpin™ IICR column and centrifuged at 10,000 g for 1 minute. Finally, the ZymoSpin™ IICR column was transferred to 1.5 ml microcentrifuge tube and 100 µl of DNA elution buffer added directly to the column matrix. It was then centrifuged at 10,000 g for 30 sec to elute the DNA. The DNA so obtained was stored at 4 °C till further use.

4.10.3.2 Qualitative and quantitative estimation of genomic DNA

For qualitative estimation of isolated DNA, agarose gel electrophoresis was performed. 1 % (w/v) agarose gel (Hi Media, India) was prepared in 0.5X Tris Borate EDTA (TBE) buffer. Ethidium bromide (0.5 µg/ml) was used for visualization. The agarose was mixed uniformly in the TBE buffer, casted in the comb containing the casting tray, and solidified at RT followed by the removal of the comb. The gel was then placed in the 0.5X TBE buffer containing an electrophoretic tank (Tarson, India). The gel was placed carefully so that it immersed fully for charge conduction. The DNA sample was mixed properly with 5X DNA loading buffer (Thermo Fisher Scientific). A 100 bp DNA ladder ranging from 100 bp- 1000 bp (GeneDireX) was added to the respective walls along with the DNA samples. During electrophoretic separation, the voltage was maintained at 65 volts until the tracking dye reached 3/4th distance of the gel. For the qualitative estimation, UV-transilluminator (Bio-imaging System) was used to confirm the presence of DNA. UV light-fitted gel documentation (Bio-Rad) system was used for gel imaging.

For quantitative estimation, spectrophotometer (SHIMADZU “A124256”) analysis at 260 nm was performed (Teare et al., 1997; Khare et al., 2014). The DNA concentration was determined by using formulae,

DNA Concentration (µg/ml) = OD 260 nm × 50 µg/ml × Dilution factor

Nanodrop 2000 (Jenway™ Genova) was used to confirm the purity and quantity of the DNA sample at 260 and 280 nm.

4.10.3.3 Gene amplification

Internal transcribed spacer region (ITS), translation elongation factor 1-α (EF1α) and beta-tubulin (TUB) were amplified. Primers ITS1 (TCCGTAGGTGAACCTGCGG), ITS4 (TCCTCCGCTTATTGATATGC) (Zarrin et al., 2016), and EF1-728F (CATCGAGAAGTTCGAGAAGG), EF2-(GGAGGTACCAGTCATCATGTT) (Le et al., 2022) and T1 (AACATGCGTGAGATTGTAAGT), T22

(TCTGGATGTTGTTGGGAATCC) (Nosratabadi et al., 2018) were used for amplification of ITS, EF-1α and TUB gene respectively (Eurofins Labs, India). Briefly, 25 µl of reaction mixture contained 20 ng extracted DNA, 10 µM primers, 2.5 mM dNTP and 1.5 U of Taq DNA polymerase (Thermo scientific™) in 10X Taq buffer. Thermal cycling parameters were optimized for PCR amplification as; initial denaturation at 96 °C for 5 min followed by 39 cycles at 95 °C for 45 sec. Annealing temp. was optimized as 56 °C for 45 sec, followed by extending at 72 °C for 45 sec, and ultimately the final extension was performed at 72 °C for 5 min. 1.5 % (w/v) agarose gel electrophoresis, and gel imaging by using Bio-Rad gel documentation system were performed to confirm the amplification of DNA sample. The amplified PCR product was delivered to SoIResLab, Haryana for PCR product purification and sequencing.

4.10.3.4 Sequence assembly and phylogenetic tree construction

The purity of the obtained chromatograms of forward and reverse sequences of three loci viz. ITS, EF1α and TUB were checked using Sequencher version 5.4.6 (Gene Codes Corporation, Ann Arbor, MI 48108 USA). The consensus sequences of ITS, EF1α and TUB loci were then submitted to Genbank, followed by NCBI nBLAST search to search similar sequences. The selection criteria for similarity of sequences of closely related organisms was based on the E-value, and query coverage. Approximately 25 - 30 sequences were selected from the nBLAST sequences search and their FASTA files were downloaded. These sequences were aligned with the query sequence using ClustalW in MEGA X. The alignment of the sequences was subjected to phylogenetic distance-based analysis using maximum likelihood probability method and the model used was Kimura - 2. The phylogeny was tested by bootstrap method for 1000 replications.

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories). The rate variation model allowed for some sites to be evolutionarily invariable. All positions containing gaps and missing data were eliminated (complete deletion option).

4.11 Docking and molecular dynamics simulations

4.11.1 Compounds selection and preparation

Twenty compounds from the tea plant, *Camellia sinensis* were chosen for the molecular docking. The name of the chosen compounds was Theaflavin-3-digallate, Theaflavin, Theaflavin-3'

gallate, Theaflavin-3-gallate, Gallocatechin gallate, Catechin gallate, Luteolin, Quercetin, Gallocatechin, Epigallocatechin, Myricetin, Apigenin, Kaempferol, Epigallocatechin gallate, Epicatechin, Caffeic acid, Caffeine, Theophylline, Gallic acid, and Adenine. The compounds were drawn using a 2-D sketch tool of maestro software (Tahlan et al., 2019) . All the compounds were converted into 3-D structures and minimized. The minimized 3-D structures of compounds were prepared using a ligand preparation module of MGL tools (Rauf et al., 2015).

4.11.2 α-synuclein structure preparation

The 3-D crystallized structure of α-synuclein was retrieved from the protein databank database with PDB -ID 2N0A (Zhang et al., 2021). The structure consists of 10 α- synuclein monomers interacting with each other and creating amyloid. α- synuclein with 27 to 97 amino acids are interacting during the formation of amyloid structure. Whereas all other regions of the protein are unstable and do not interact with the adjacent α-synuclein. All the chains in the amyloid are identical. Therefore, a single chain was used for the molecular docking approach. A protein preparation module in MGL tools was used to prepare the α- synuclein structure. The α-synuclein is a linear protein without any active or drug pockets. Therefore, the amino acids from 27 to 97 were selected as the binding site, and another structure region was removed.

4.11.3 α-synuclein structure-based virtual screening

Molecular docking based virtual screening of a library of 20 compounds with 2N0A was performed to predict their docking score and detailed interactions. The prepared α-synuclein and twenty compounds were subjected to AutoDock Vina (Trott and Olson, 2010) software by selecting ten possible poses. The approach was used to identify the top hit compounds that could be developed as a potential disaggregator of misfolded α-synuclein.

4.11.4 Molecular Dynamics Simulations

The α-synuclein and hit compound's complex structures were used for molecular dynamics simulations using DESMOND (Bowers et al., 2006) software to understand the interaction of compounds with α-synuclein in its dynamics state and the conformational changes in the structure of a protein. The simulation system was built with a TIP3P solvent model in 10 A°, distended as X, Y, and Z coordinates of Orthorhombic periodic boundary conditions. The simulation system was neutralized by adding oppositely charged ions respective to the system's total charge, and additionally, 0.15 M NaCl salt was added to the system. The atomic parameters of the molecules were produced by the OPLS_2005 forcefield (Banks et al., 2005; Jorgensen et al., 1996). Further, the energy minimization on the simulation system was performed with a simulation time scale of 10 nanoseconds (ns). The energy-minimized simulation system was equilibrated through NVT (Number, Volume, and Temperature) and NPT (Numbers, Pressure, and

Temperature) protocols with a 10 ns simulation time scale for every restricted and unrestricted process. Finally, the 100 ns time scale simulation was performed by recording trajectory intervals at every 10 picoseconds (ps) under 300 °K temperature and 1 bar pressure. The root means square deviation (RMSD) and root mean square fluctuation (RMSF) were used for the analysis.

RMSD measures the average change in selected residue or atoms for a particular frame concerning a reference frame. RMSD was calculated for all the frames in the trajectory of the simulation.

$$RMSD = \frac{1}{N}\sum_{i=1}^{N}\left(r_i'(t_x)\right) - \left(r_i(t_{ref})\right)^2$$

Where N is the number of atoms in the selected residue or group. t_{ref} is the simulation time scale of the reference molecule, typically the time at first frame regarded as t = 0 and r' is the position of the selected atoms in frame x. Frame x is recorded at time t_x. This procedure is repeated for every frame in the simulation trajectory.

The RMSF is used to characterize the local changes in each residue's side chain. The RMSF for a residue i is:

$$RMSF = \sqrt{\frac{1}{T}\sum_{t=1}^{T}\langle\left(r_i'(t)\right) - \left(r_i(t_{ref})\right)^2\rangle}$$

Where T is the trajectory time for RMSF calculations, t_{ref} is the reference time, r_i is the residue I position. r^i is residue i atom position after superposition on the reference. The angle brackets represent the average square distance of atoms in the residue.

4.12 Isolation of bioactive moiety using liquid-liquid extraction

The cell-free culture filtrate which exhibited maximum disaggregation potential of misfolded α-synuclein and was subjected to filtration using Whatman 1 filter paper and subsequently centrifuged at 10000 rpm, 10 min to make it cell free. This cell free culture filtrate was further fractionated using liquid - liquid extraction using solvents of different polarities. The extraction was performed in increasing order of polarity, i.e., Hexane, dichloromethane (DCM), chloroform followed by ethyl acetate having polar index 0.1, 3.1, 4.1, and 4.4 respectively.

Extraction procedure comprised of using cell free culture filtrate with solvent in a ratio of 1:2 in a separating funnel (Borosil, India). The mixture was vigorously shaken for 15 minutes and allowed to settle down to separate the aqueous and the solvent (non-aqueous) phase. The polar or non-aqueous layer was separated and stored while the aqueous layer was again extracted with the same solvent. In this way the cell free culture filtrate was extracted three times and all the solvent fractions were pooled (Abubakar and Haque, 2020). Subsequently the residual aqueous layer was extracted with series of solvent as per the method described above (Fig. 4.3). The organic

layers of each solvent were pooled and dehydrated using anhydrous sodium sulphate (Loba Chemie) and evaporated until dried crude fraction residue is obtained. The dried fraction residue was weighed and reconstituted in the methanol. The fractions were stored at 4 °C until next use.

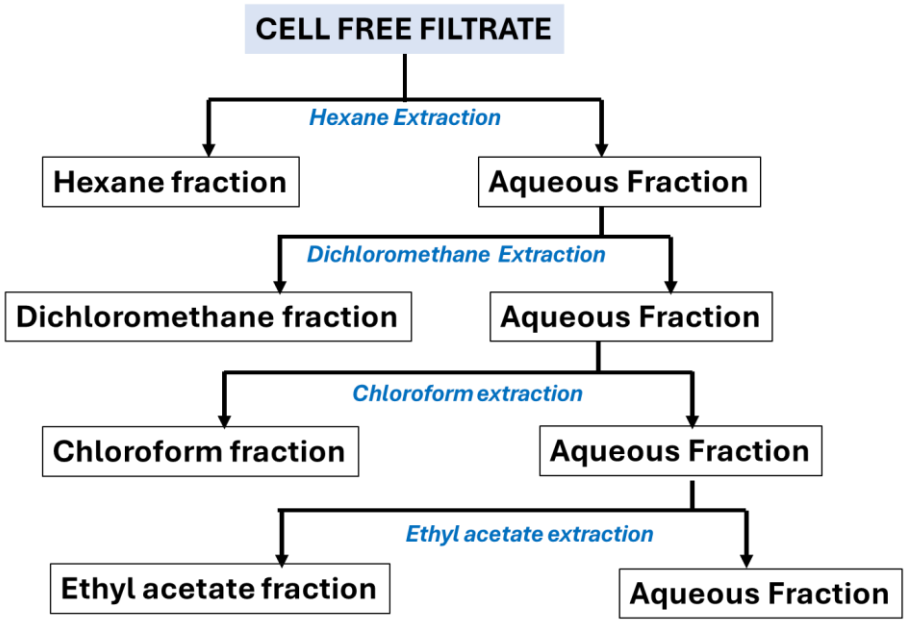

Fig 4.3 Schematic representation of the liquid-liquid, solvent based extraction for isolation of the misfolded alpha-synuclein disaggregator from the cell free filtrate of the selected endophytic fungus.

4.13 Phytochemical analysis of crude bioactive residue

The crude bioactive fraction was analysed to understand the broad chemical class of the compounds. Phytochemical tests were performed to understand the phytoconstituents present in the sample.

4.13.1 Test for alkaloid

Dragandroff's reagent (CDH; 025595) was used to confirm the presence of alkaloids. Briefly, 150 µl of Dragandroff's reagent was added to 1 ml (0.1 mg/10 ml) of residue. Appearance of the he orange red precipitates indicated the presence of alkaloids.

4.13.2 Test for amino acid

To estimate presence of free amino groups in the sample, 100 µl (0.1 mg/10 ml) of residue was incubated with 200 µl of ninhydrin solution. The mixture was incubated for 5 min in the boiling water bath. Purple coloration of the reaction mixture confirms the presence of amino acids (Sachin et al., 2010).

4.13.3 Test for anthraquinones

The presence of anthraquinones in the test sample was ascertained as per Chakraborty et al. (2021). Briefly 1 ml of test sample was first mixed with 5 ml of benzene (C_6H_6) in a test tube, and

vortexed and then filtered. Subsequently 2.5 ml of 10 % (v/v) ammonium hydroxide (NH_4OH) solution was added to the filtrate. The presence of reddish pink colour in the ammoniacal phase suggests the presence of anthraquinones.

4.13.4 Test for carbohydrates

Benedict's test was performed to confirm the presence of carbohydrates. In 1 ml of crude extract, few drops of Benedict's reagent (Composition: 1 L of Benedict's reagent can be prepared by mixing 17.3 g $CuSo_4.H_2O$, 100 g Na_2Co_3, 173 g $Na_3C_6H_5O_7$) were added followed by heating for 5 min in a boiling water bath. The appearance of yellow red precipitates indicates the presence of carbohydrates.

4.13.5 Test for flavonoids

The flavonoids were qualitatively confirmed in the residue by mixing 100 µl (0.1 mg / 10 ml) residue with 500 µl ethanol (CH_3CH_2OH) and 500 µl water, followed by vortexing the mixture. Subsequently 500 µl of concentrated HCl and 90 mg zinc turnings (Zn) were added give rise to pink colour, indicating the presence of flavonoids which could be extracted with butanol (Aynehchi et al., 1982).

4.13.6 Test for saponins

Frothing test was performed to confirm the presence of saponins in the test sample. Briefly, 100 µl (0.1 mg/10 ml) residue was mixed with 200 µl of water followed by vigorous shaking. Formation of stable froths represents the presence of saponins (Hossain et al., 2013).

4.13.7 Test for tannins

Presence of tannins was confirmed with the help of a ferric chloride ($FeCl_3$) reagent. In 100 µl (0.1 mg/10 ml) residue, a few drops of ferric chloride were added followed by the addition of sulphuric acid. The appearance of yellow to reddish-brown precipitate indicates the presence of tannins in the sample (KC et al., 2020).

4.13.8 Test for terpenoids

Briefly, the 1 mg/ml residue was treated with 1 ml chloroform ($CHCl_3$) (LOBA) followed by 0.5 ml acetic anhydride (CH_3COOH). A few drops of conc. sulphuric acid (H_2SO_4) were added along the walls. The pinkish colour indicates the terpenoid's presence (Zuo, 2002).

4.13.9 Determination of total phenolic content

The amount of total phenolic content in the sample was determined with the help of Folin-coicalteu's (FC) reagent. Fifty microlitres of gallic acid (standard) and samples were mixed with 250 µl FC reagent and 200 µl sodium bicarbonate ($NaHCO_3$) in a conc. of 7.5 % (w/v), followed by a 90 min incubation at 30 °C. The data was analysed in triplicates. The absorbance of the sample and standard was measured at 765 nm using a UV-visible spectrophotometer (Powerwave 340,

BioTek, USA). Results were expressed in milligrams Gallic Acid Equivalents (mg GAE / g extract) (Baptista et al., 2019).

4.13.10 Determination of total flavonoid content

The aluminium calorimetric method was used to calculate the total flavonoids in the sample solvent. Standard (rutin) and sample solvent were mixed with aluminium chloride (NH_4Cl) in a ratio of 1:1 followed by 10 min incubation at 30 ℃. The test was performed in triplicates. The absorbance of the sample and standard was measured at 435 nm using medium throughput reader (Powerwave 340, BioTek, USA). Results were expressed in milligrams rutin equivalents (mg RE/g extract) (Paiva et al., 2020).

Assay Type	TPC						TFC					
	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	B	GA6	GA6	GA6	B	B	B	R6	R6	R6
B	B	B	B	GA7	GA7	GA7	B	B	B	R7	R7	R7
C	NC	NC	NC	GA8	GA8	GA8	NC	NC	NC	R8	R8	R8
D	GA1	GA1	GA1	GA9	GA9	GA9	R1	R1	R1	R9	R9	R9
E	GA2	GA2	GA2	GA2	GA2	GA2	R2	R2	R2	R2	R2	R2
F	GA3	GA3	GA3	S1	S1	S1	R3	R3	R3	S1	S1	S1
G	GA4	GA4	GA4	S1	S1	S1	R4	R4	R4	S1	S1	S1
H	GA5	GA5	GA5	S1	S1	S1	R5	R5	R5	S1	S1	S1

Fig 4.4 Template for alpha-synuclein protein disaggregation activity. B= Blanks, GA= Gallic Acid (Standard); S= Sample; R-Rutin

4.14 Purification and characterization of bioactive residue

4.14.1 Mass production of the crude bioactive residue via submerged fermentation

The most potent culture exhibited maximum disaggregation potential was further subjected to submerged fermentation. The culture was grown in 45 L PDB to obtain crude bioactive residue through Liquid-liquid extraction using chloroform (LOBA). The crude bioactive residue was further subjected to thin-layer chromatography (TLC) and column chromatography for compound purification. Purified compounds were analysed for disaggregation potential.

4.14.2 Thin layer chromatography of the bioactive residue

The analytical tool was used to purify the bioactive residue compounds. Partially purified fraction of the bioactive residue was subjected to preparative TLC (20 × 20 cm). In order to achieve maximum separation, different binary and tertiary solvent combinations were optimized. Briefly,

the TLC plates were preactivated by incubating the silica gel plates (Sigma Aldrich) at 100 °C for 12 h. 2 µl bioactive fraction in a concentration of 0.5 mg/ml was spotted on the plate 1 cm above the edge using a capillary and allowed to develop in the mobile phase pre-saturated TLC chamber until the solvent front travelled a distance of 16 cm.

Further, the plates were taken out and solvent was allowed to evaporate. The plates were visualized under short and long wavelength to confirm the presence of bands number. The retention factor (R_f) of each band was calculated by the formula:

R_f = Distance travelled by solute/ Distance travelled by solvent

4.14.3 Purification of the bioactive residue by column chromatography

Silica-based column chromatography was performed for the purification of compounds present in the bioactive residue. The wet column was prepared by using silica (60-120 mesh, Merck, Millipore) suspended in chloroform (LOBA) and packed in a glass column (90 × 5 cm) with a G_0 filter. The column was allowed to be equilibrated for 2 hr. Partially purified extract (~ 3 g) was absorbed on a silica bed (bed height = 2.5 cm) and was loaded on the pre-packed column (length = 80 cm). The TLC-optimized mobile phase was used in a gradient of (100:0 → 0:100). Void volume of the column was calculated to quantify the mobile phase. Analysis of obtained fractions was performed on the basis of TLC, and purity was confirmed based on UV-Spectral analysis. The fractions were pooled on the basis of R_f value. The disaggregation potential of compounds dissolved in methanol was confirmed on the basis of quantitative analysis (refer section 4.9). The fraction with maximum disaggregation potential was further analysed for antioxidants and protein disaggregation studies.

4.15 DPPH (2,2-diphenyl-1 picrylhydrazyl) scavenging activity

The DPPH free radical scavenging property of the pure compound was determined according to Novilla et al. (2022) with minimal modifications. Briefly, 50 µl compound in different conc. (0.1 mg/ml- 0.8 mg/ml) was mixed with 200 µl DPPH (0.1 mmol/L in methanol) in a microtiter plate. The mixture was shaken vigorously and incubated in dark conditions at room temperature for 30 min. The antioxidant activity of the compound was analysed via DPPH assay and was determined at 517 nm using Biotek throughput reader, Powerwave 340. Ascorbic acid was used as a standard, and DPPH was used as a control. Methanol was used as a negative control. IC_{50} of the free radical scavenging compound was calculated using the software “GraphPad Prism, version 9.0.0” by using the formulae:

Free radical scavenging (%) = $OD_{control}$ -OD_{sample}/ $OD_{control}$ * 100

The test was performed in triplicates, and data were represented as mean ± SD.

4.16 Structure elucidation of compound

4.16.1 Liquid chromatography-mass spectroscopy (LC-MS)

To establish the mass of the bioactive compounds, liquid chromatograph hyphenated with mass spectroscopy (LC-MS) was used. A stock solution of 1 mg/ml of the bioactive fraction was prepared with methanol (LOBA) was analysed in an LC-MS system (Waters Micromass, O-Tof Micro). The equipment was first equilibrated using methanol, followed by sample analysis using mobile phase methanol: water (9:1). Briefly, 20 µl compound was injected into LC-MS system using a Waters-symmetry C_{18} Unisol chromatographic column (4.6 mm × 250 mm × 5 µm).

Time of Flight-Mass spectrometry was used to analyze the peaks in the sample. The Mass Spectrometer is coupled with Waters 2795 HPLC with quaternary pumping configured for flow rates from 0.05 to 5.0 ml / min. The ion optics used was capillary with voltage of 3000 V and cone voltage of 30 V. The desolvation and cone gas flow was adjusted to 750 L/hr and 30 L/h. The source block temperature was 110 °C, and the desolvation temperature was 300 °C. The autosampler is configured with a 100 µl syringe.

4.16.2 IR spectroscopy of the bioactive fraction

The functional groups in the compound were identified via IR (infra -red) spectrum obtained on Perkin Elmer-spectrum RX IFTIR fitted with a beam-condensing ATR accessory. The peaks were obtained within the resolution of 1 cm^{-1} and scan range of 4000 cm^{-1} to 250 cm^{-1}. For each spectrum, 32 scans were captured. 100 µl of bioactive fraction (1 mg/ml) dissolved in chloroform (LOBA) was loaded onto the cell. The spectral resolution containing the temperature control stage was 0.2 cm^{-1} (Dahiya et al., 2018).

4.16.3 Nuclear Magnetic Resonance (NMR)

For NMR studies, 7 mg compound was dissolved in 0.5 ml deuterated DMSO (Sigma Aldrich). The NMR spectra were recorded at 30 °C in the phase-sensitive mode using a Bruker Avance II-400 NMR spectrometer instrument operating at a proton NMR frequency of 400.131 MHz and carbon frequencies of 100.525 MHz at 18 °C. Based on the phytochemical analysis of the cell free extract the proton and carbon 90 °C pulse widths were set to 10.9 sec and 8.37 µsec, respectively (Yuan et al., 2014).

4.17 DC_{50} determination

To assess the *in vitro* efficacy of misfolded α-synuclein disaggregator, half maximal degradation concentration (DC_{50}) of the pure compound was established in a dose-dependent manner by using different concentrations of the pure compound. The dose ranged from 0 - 2.5 µM. The method adopted to establish DC_{50} was Sandwich ELISA (Refer Section 4.9). DC_{50} of compound was calculated using the software “GraphPad Prism, version 9.0.0” by using the formulae:

$DC_{50} = OD_{control} - OD_{sample} / OD_{control} * 100$

The test was performed in biological triplicates and data were represented as mean ± SD.

4.18 Disaggregation assay

The assay was carried out to ascertain the changes in the fluorescence emission spectrum of the ThT dye when it binds to oligomeric structures as well as understand the changes caused by the compound (disaggregator) in these oligomeric structure by tracking the fluorescence emission (Kindly refer 4.8). Briefly, the compound was investigated in a dose-dependent manner by using a concentration range from 0.1- 2.0 μM. The test was performed in triplicates, and data were represented as mean ± SD.

4.19 Statistical analysis

All the assays were performed in biological triplicates, and the data was represented in terms of mean ± standard deviation. The results were analysed by ANOVA followed by Tukey's post hoc test ($p < 0.05$) was considered as significant.

CHAPTER-5
RESULTS

5.1 Isolation of endophytic fungi from plant samples

Seventy-nine endophytic fungal isolates were isolated from the stem and leaf parts of *Camellia sinensis* and *Malus domestica*. The culture code was assigned to the cultures based on plant name, plant part, and sampling location (Refer Table 5.1 Appendix). The cultures were tentatively identified on the basis of morphological and microscopic characteristics (Fig 5.1 and Fig. 5.3).

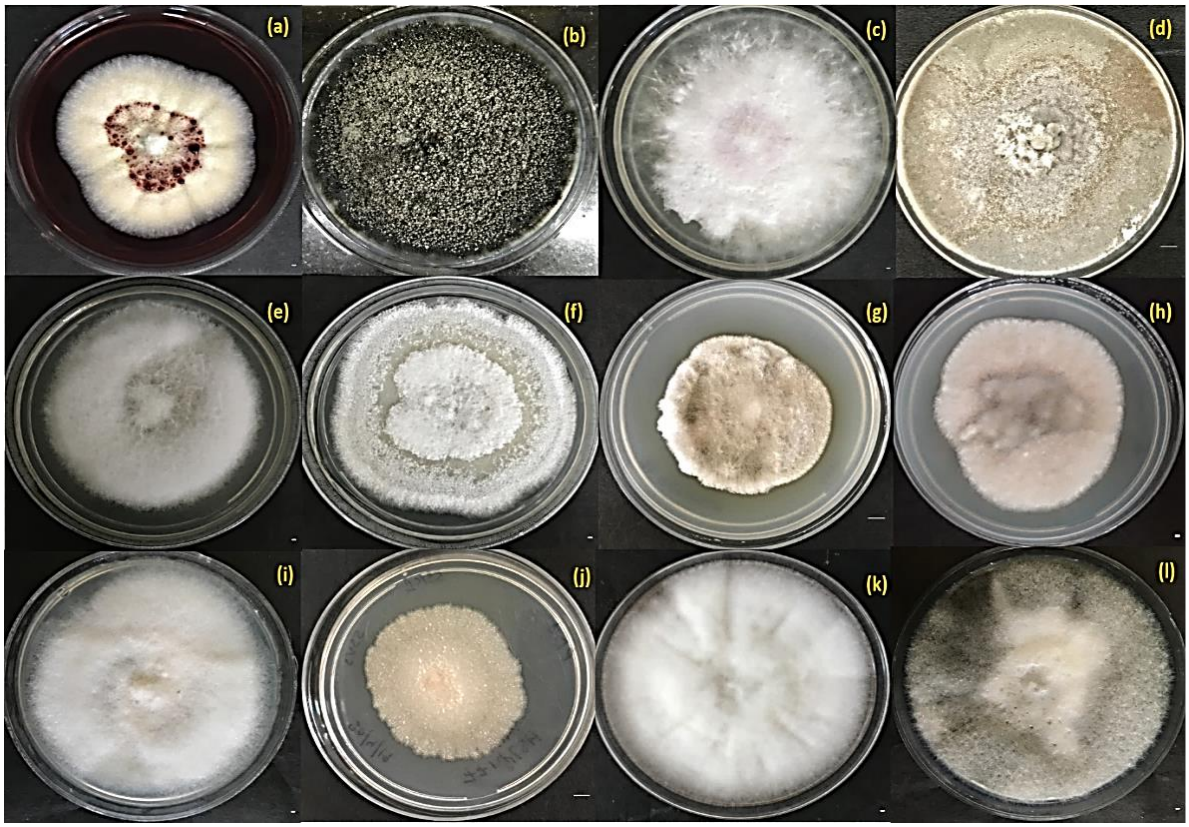

Fig 5.1 Endophytic fungal isolates from *Camellia sinensis* and *Malus domestica* (a): *Aspergillus* sp. (b): *Botryosphaeria* sp. (c,e,g,i,j): *Fusarium* sp. (d): *Lasiodiplodia* sp. (f): unidentified (h): *Fusarium oxysporum* (k): *Penicillium* sp. (l): *Nigrospora* sp. (Bar: 10mm)

Endophytic isolates recovered from *Camellia sinensis* and *Malus domestica* altogether belonged to fourteen genera. The most prevalent species were tentatively identified as *Fusarium* sp. in the tea samples; however, apple isolates showed an abundance of *Colletotrichum* sp. 4.3 % of cultures were non-sporulating type in *Camellia sinensis* and none in *Malus domestica*. *Nigrospora* sp., *Epicoccum* sp., *Curvularia* sp., *Xylaria* sp. and *Colletotrichum* sp. were exclusively reported from *Malus domestica*. On the other hand, *Melanconiella* sp., *Cladosporium* sp., and *Chaetomium* species exhibited exclusive existence as endophytes in *Camellia sinensis* (Fig. 5.2).

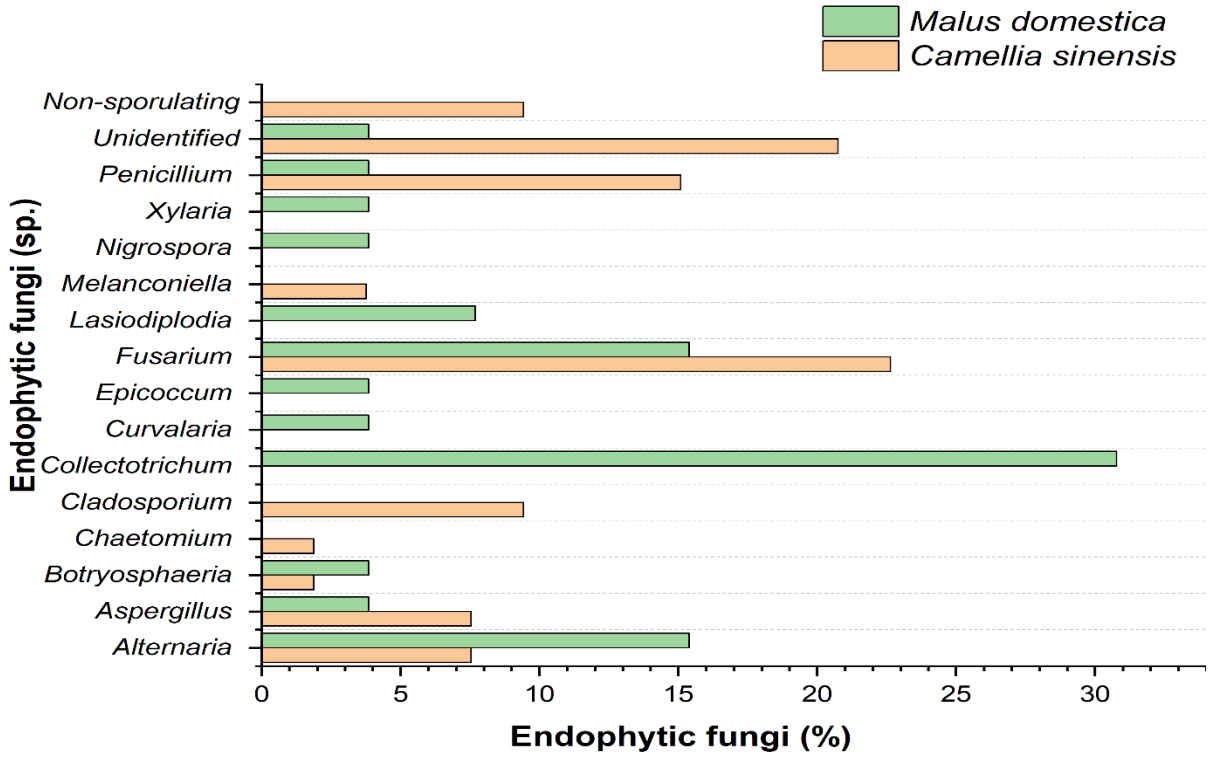

Fig 5.2 Relative recovery of endophytic fungal isolates belonging to different genera

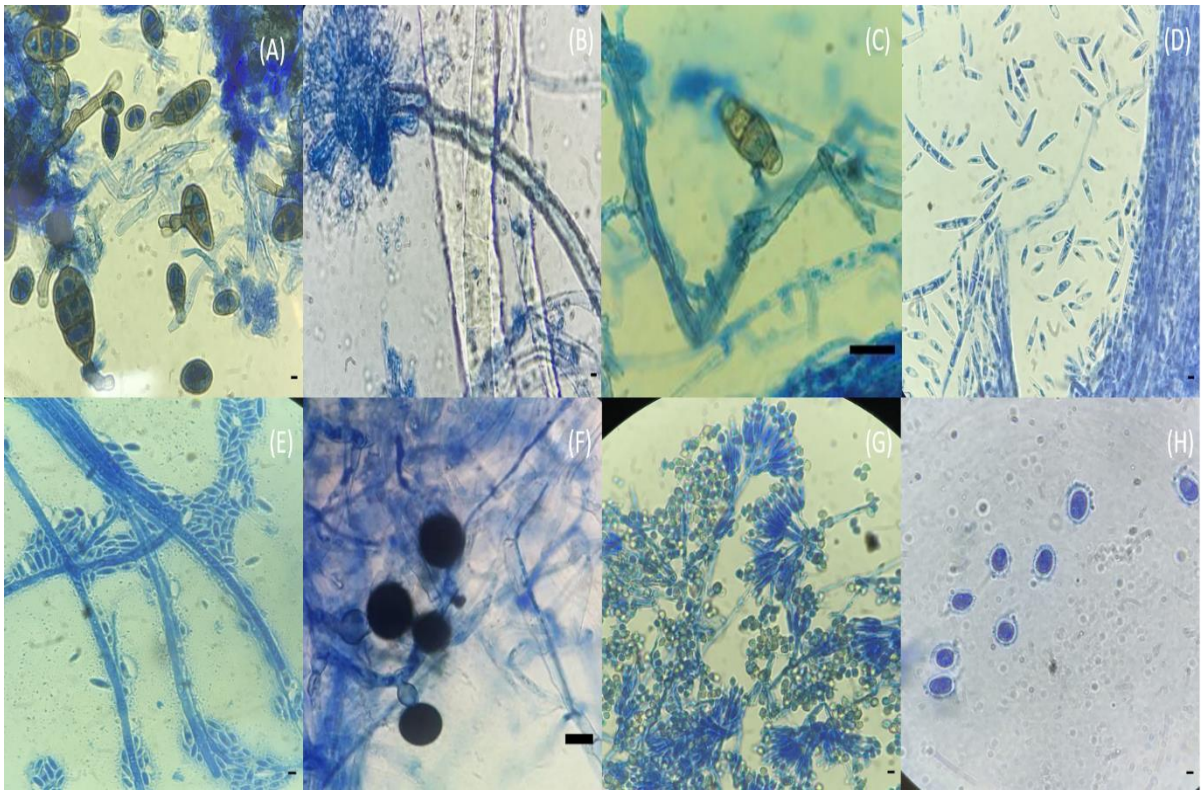

Fig 5.3 Microscopic features of some endophytic fungal isolates during their tentative identification (a) Conidia of *Alternaria* sp. (b) Phialide, vesicle, conidia and conidiophore of *Aspergillus* sp. (c) Conidia of *Curvalaria* sp. (d, e) Macro and micro conidia of *Fusarium* sp. (f) *Nigrospora* sp. conidia attached with conidiophores (g) stipe, metulae and phialides of *Penicillium* sp. (h) immature condidia of *Lasiodiplodia* sp. (Bar: 10mm)

The diversity of fungal isolates was compared by using the Shannon-Wiener and Simpson's index and their component (Species evenness). The diversity index was found higher in *Malus domestica* than *Camellia sinensis* (Table 5.2). Some of the endophytic fungi could not be identified due to their non- sporulating nature. The host tissues of both plant samples exhibited different colonization of endophytic mycoflora. A maximum of isolation rate of 25 % was observed in *Camellia sinensis* isolated from Palampur which was higher than *Camellia sinensis* collected from Assam (18.66 %) and *Malus domestica* (18.57 %) (Table 5.3).

Table 5.2: Diversity index of endophytic fungi isolated from *Camellia sinensis* and *Malus domestica*

Plant sample	Shannon's wiener index (H')	Species evenness I	Simpson's index
Camellia sinensis	2.06	0.52	0.87
Malus domestica	2.14	0.66	0.87

Table 5.3: Isolation rate (IR) of different plant samples

Plant	Location	Isolation parts	No. of segments	Total isolates (79)	IR (%) (Sample wise)	IR (%) (Location wise)
Malus domestica	Shimla	Leaf	125	25	20	18.57
		Stem	15	1	6.66	
Camellia sinensis	Assam	Leaf	100	20	20	18.66
		Stem	50	8	16	
Camellia sinensis	Palampur	Leaf	80	25	17.85	25
		Stem	20	0	0	

5.2 Optimization of α-synuclein inducing recombinant strain

5.2.1 Growth phenotypic assay of *Saccharomyces cerevisiae* "SY246"

The mid-log phase of the strain SY246 was determined by performing growth phenotypic assay in a 96 well microtiter plate. Optical density (OD) was taken at 600 nm in a time interval of 10 min to find out the mid-log phase. The mid-log phase was found between 10-12 hr (Fig 5.4). Further, protein induction in the recombinant strain was confirmed via GFP assay (Fig 5.5). YPGRB media was used as control, whereas, protein induction was confirmed at 10^{th} hr by recording emission at wavelength 509 nm.

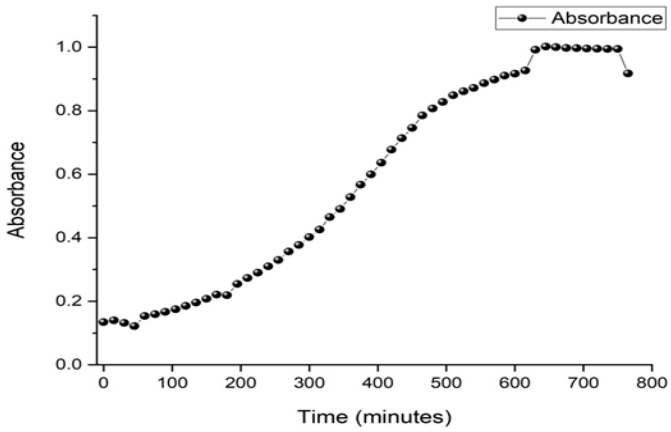

Fig 5.4 Growth curve of *Saccharomyces cerevisiae* "SY246" strain determined at wavelength 600 nm

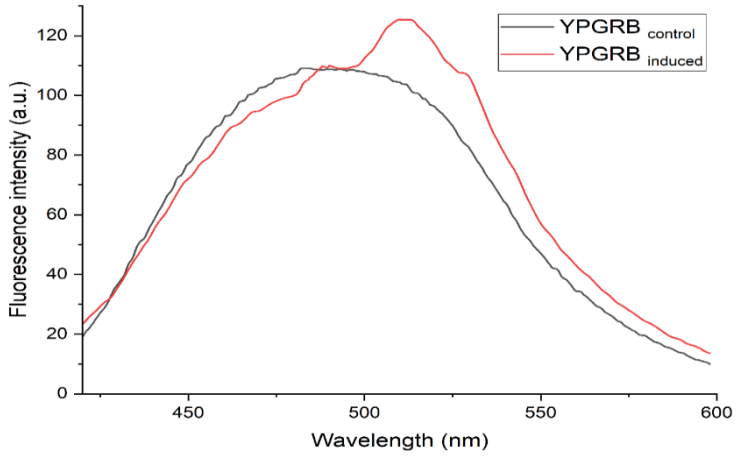

Fig 5.5 GFP fluorescence emission spectra after alpha-synuclein induction in YPGRB at emission wavelength 509 nm

5.3 Preliminary screening of endophytic fungal isolates for alpha-synuclein disaggregation

5.3.1 *In vitro* Nitroblue Tetrazolium microtiter plate assay

A total of 79 endophytic fungal cultures were screened using a preliminary NBT assay. The range of reduction in the formation of blue formazan varied from 0 to 92.4 % (Fig 5.6) by following the layout (Fig 4.2). Among 79, only 12 cultures exhibited > 40 % reduction in oxidative stress, major factor responsible for protein aggregation. Based upon the analysis, disaggregation potential of *Camellia sinensis* (85.7 %) is reported higher as compared to *Malus domestica* (14.3 %). In the microtiter plate assay, the highest disaggregation potential was confirmed by 59CSLEAS, which is comparable to positive control Piceatannol (Table 5.4). Only 7.6 % of cultures expressed strong disaggregation potential to disaggregate oligomeric protein up to the extent of 75 % and more. Based on Tukey's post-hoc statistical analysis, we decided to perform further analysis with four

cultures designated as 59CSLEAS, 9CSSTAS, 108CSSTAS and 10CSLEAS, which showed statistically similar disaggregation potential, i.e., equivalent to the positive control, piceatannol ranges from 92.4 %, 90.6 %, 90.1 % and 86.9 % respectively.

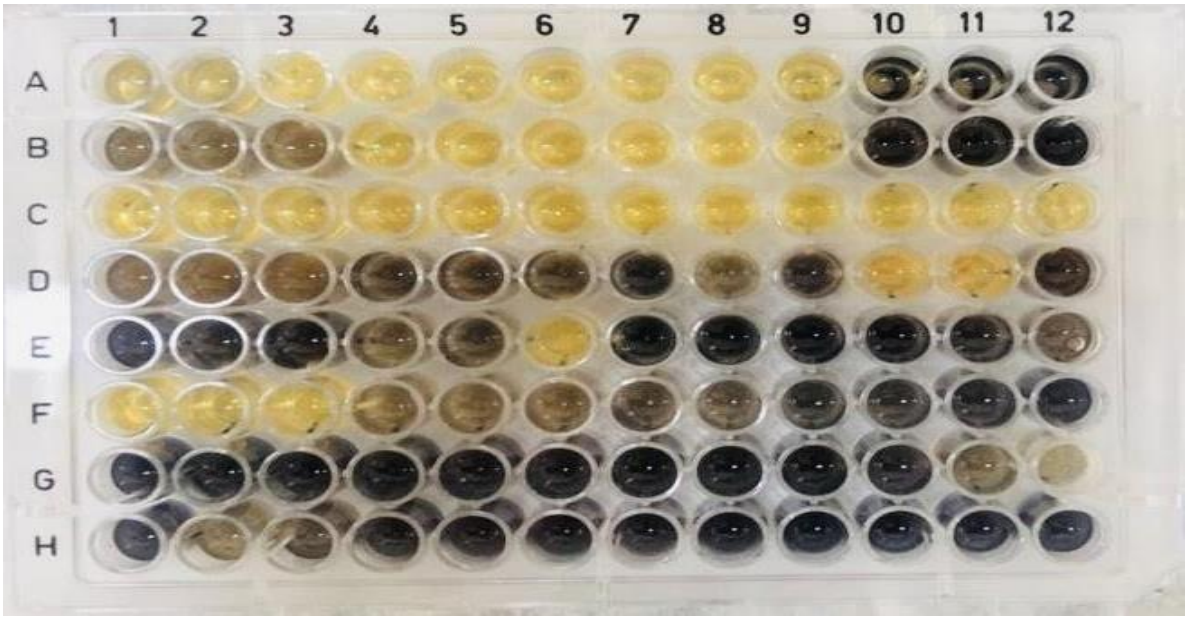

Fig 5.6 Template for NBT assay to understand reduction in oxidative stress and induction in disaggregation of oligomeric alpha-synuclein protein

Table 5.4: *In vitro* alpha-synuclein disaggregation activity of fungal endophytes using *in vitro* Nitroblue tetrazolium (NBT) microtiter plate assay

S. No.	Culture code	α-synuclein disaggregation (%)	S. No.	Culture code	α-synuclein disaggregation (%)
1	**Piceatannol**	**92.9^{a}**	22	179MPLSH	34^{ghijklmn}
2	**59CSLEAS**	**92.4^{a}**	23	21CSSTAS	34^{hijklmn}
3	**9CSSTAS**	**90.6^{a}**	24	34CSSTAS	34^{hijklmn}
4	**108CSSTAS**	**90.1^{a}**	25	07CSLEPL	33.7^{hijklmn}
5	**10CSLEAS**	**86.9^{a}**	26	96MPLSH	32.3^{hijklmno}
6	**50CSLEAS**	**84.2^{ab}**	27	92CSLEPL	31.9^{hijklmnop}
7	**101CSSTAS**	**75.2^{bc}**	28	114MPLSH	31^{hijklmnopq}
8	159MPLSH	66.1^{c}	29	73MPLSH	31^{hijklmnopq}
9	21CSLEPL	49.6^{d}	30	12CSSTAS	31^{hijklmnopq}
10	65CSLEPL	45.7^{de}	31	22CSLEPL	30.7^{hijklmnopq}
11	59CSSTAS	45.5^{def}	32	70CSLEAS	30.3^{ijklmnopq}
12	43CSLEPL	44.8^{defg}	33	50MPLSH	30.1^{ijklmnopq}
13	40CSSTAS	44.8^{defg}	34	139MPLSH	29.3^{jklmnopqr}
14	36CSLEPL	41.3^{defgh}	35	14CSSTAS	29.1^{klmnopqrs}

15	93MPLSH	40.9^{defghi}	36	188MPLSH	$28.8^{klmnopqrs}$
16	35CSSTAS	$39.8^{defghij}$	37	19CSLEPL	$28.8^{klmnopqrs}$
17	96CSLEPL	$39.8^{defghij}$	38	3CSLEPL	$27.6^{klmnopqrst}$
18	78CSLEPL	$37.7^{efghijk}$	39	180MPLSH	$27.4^{klmnopqrst}$
19	98MPLSH	$36.6^{efghijk}$	40	25CSSTAS	$27.2^{klmnopqrst}$
20	117CSLEAS	$36.5^{efghijkl}$	41	7CSSTAS	$25.8^{lmnopqrstu}$
21	207MPSSH	$34.9^{fghijklm}$	42	32MPLSH	$25.2^{mnopqrstuv}$

*Data presented are means of three replicates. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

5.3.2 Protein aggregation kinetics

To understand the aggregation kinetics of alpha-synuclein protein, monomeric form of the protein was incubated until 168 hrs. It was found that 70 µM monomeric protein showed maximum changes in the spectral characteristics and exhibited maximum fluorescence emission at 96th hr. The emission spectra were reported to be constant after further incubations. Hence, for further analysis, the monomeric protein was incubated for 96 hrs where maximum conformational changes were reported. Fig 5.7 represents normalised fluorescence at emission wavelength 487.5 A.U.

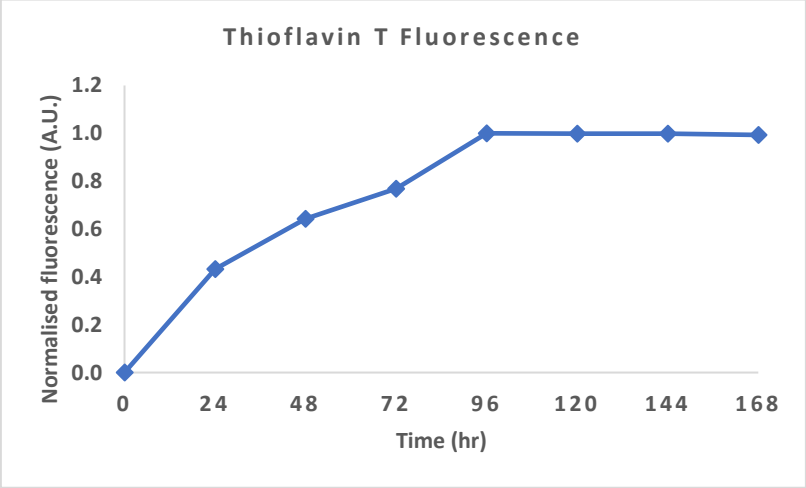

Fig 5.7 Thioflavin T fluorescence assay representing aggregation kinetics of 70 µM monomeric protein via shift in fluorescence intensity at wavelength 487.5 nm

5.3.3 Thioflavin T assay

Samples exhibited > 86 % activity were tested for Thioflavin T analysis to assess the disaggregation potential of culture filtrates. To confirm the spectral changes in the oligomerized protein, cell-free filtrate of the cultures viz. 59CSLEAS, 9CSSTAS, 108CSSTAS, and 10CSLEAS was used as disaggregator. Briefly the disaggregation kinetics was established by incubating the

culture filtrates with the aggregated alpha-synuclein for 120 hrs (Fig 5.8). Disaggregation of the protein after treatment exhibits similar kinetics and found to be disaggregated until 72 hrs and constant beyond that. Maximum disaggregation was observed at 72nd hr. Hence, for further disaggregation analysis, incubation period was optimised as 72 hr.

Among 4 top cultures, maximum reduction in the β-coil conformation was observed in culture filtrate of isolate 59CSLEAS. The endophytic cell-free filtrate, 59CSLEAS disaggregated the oligomeric protein and decreased the emission spectrum by 1.63-fold followed by 9CSSTAS (1.56-fold), 108CSSTAS (1.53-fold) and 10 CSLEAS (1.12-fold) (Fig 5.9). PBS and alpha synuclein were used as control.

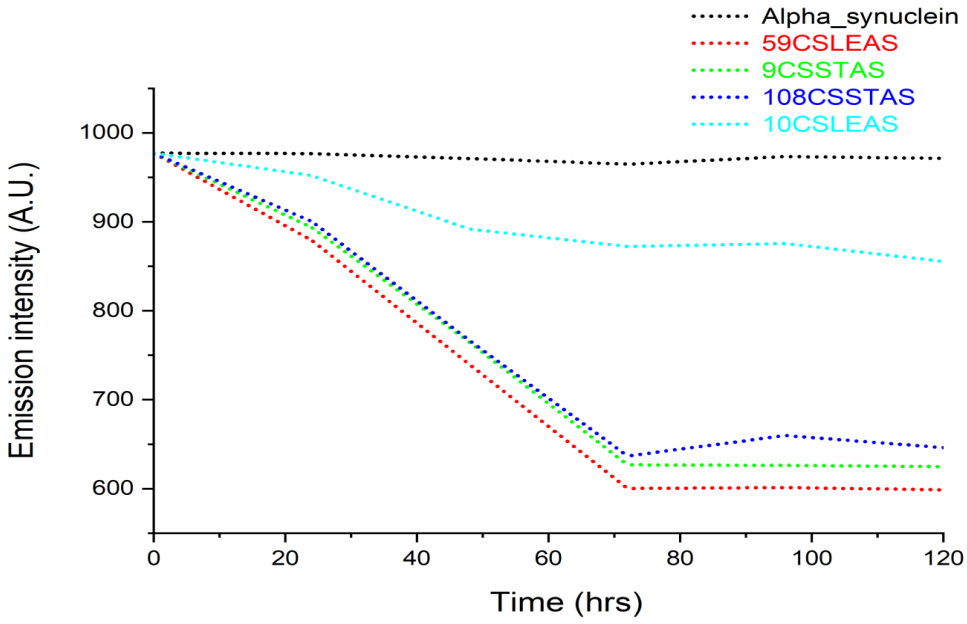

Fig 5.8 Disaggregation kinetics of oligomeric protein after treating with 59CSLEAS, 9CSSTAS, 108CSSTAS, and 10CSLEAS endophytic cell- free fungal culture filtrate at wavelength 487.5 nm

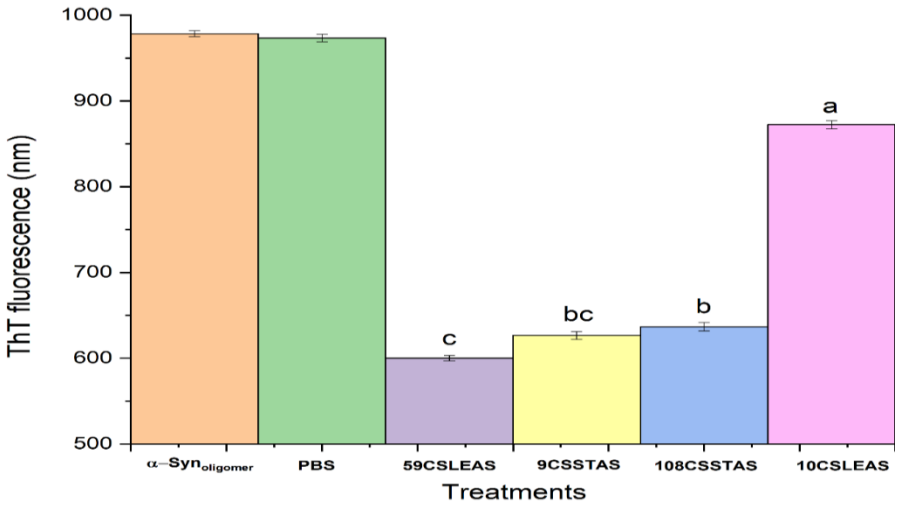

Fig 5.9 Thioflavin T fluorescence assay representing shift in fluorescence intensity at wavelength 487.5 nm of oligomeric α-synuclein before and after treated with PBS, endophytic fungal filtrates of 59CSLEAS, 9CSSTAS, 108CSSTAS, 10CSLEAS; letters a, b, c are analysed as significant letters by Tukey's post hoc test ($p < 0.05$)

5.3.4 DCFH-DA assay

The generation of ROS is related to oxidative effect which may be opined to be enhanced during the production / misfolding of α-synuclein. Hence, by DCFH-DA assay, it could be ascertained that decrease in the oxidative stress in the recombinant strain may be due to disaggregation of α-synuclein. Based on the ThT analysis, culture filtrates of 59CSLEAS, 9CSSTAS, and 108CSSTAS were further subjected to DCFH-DA assay (Fig 5.10). It was found that the culture filtrate of 59CSLEAS exhibited a reduction in ROS generation i.e., oxidative stress by 64.6 %, whereas 9CSSTAS and 108CSSTAS reduced the stress by 40.22 and 35.75 %, respectively. The activity of 59CSLEAS is presumably due to disaggregation of the α-synuclein compared to the control, where no disaggregation was performed. Hence, sample 59CSLEAS was selected for further evaluation to understand the disaggregation efficacy.

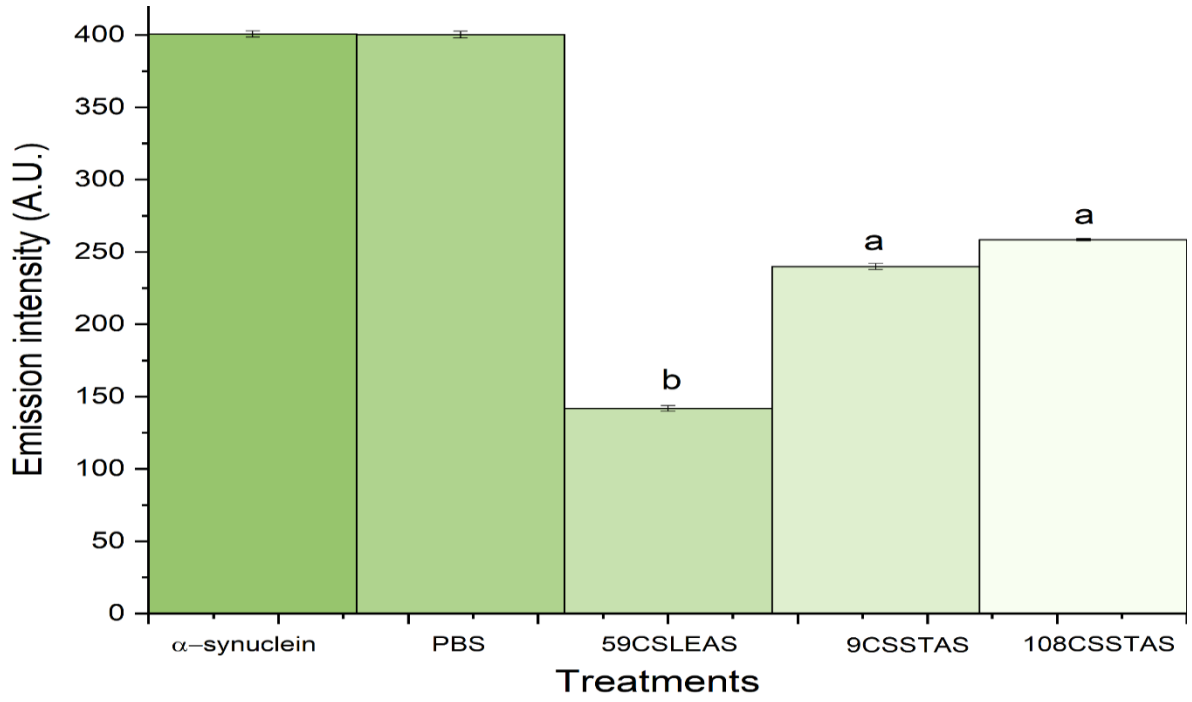

Fig 5.10 Dichloro-dihydro-fluorescein diacetate analysis of α-synuclein before and after treating with disaggregators; letters a, b are analysed as significant letters by Tukey's post hoc test ($p < 0.05$)

5.3.5 Sandwich ELISA

Sandwich ELISA assay was performed to confirm the quantitative disaggregation potential of cell-free culture filtrates. In the Sandwich ELISA assay, each sample was treated in different wells and the results were compared with the standard to determine concentration of α-Synuclein$_{oligomers}$. Only three cell free culture filtrates of endophytic isolates viz. 59CSLEAS, 9CSSTAS, 108CSSTAS exhibited the potential to disaggregate oligomeric protein were analysed quantitatively by Sandwich ELISA method. 59CSLEAS was found to disaggregate the misfolded

protein maximally up to 46.5 % followed by 9CSSTAS and 108CSSTAS with 16.5 % and 10.6 % respectively (Fig 5.11). Hence, to find out a promising entity with disaggregation potential, compound(s) were purified from 59CSLEAS cell free extract.

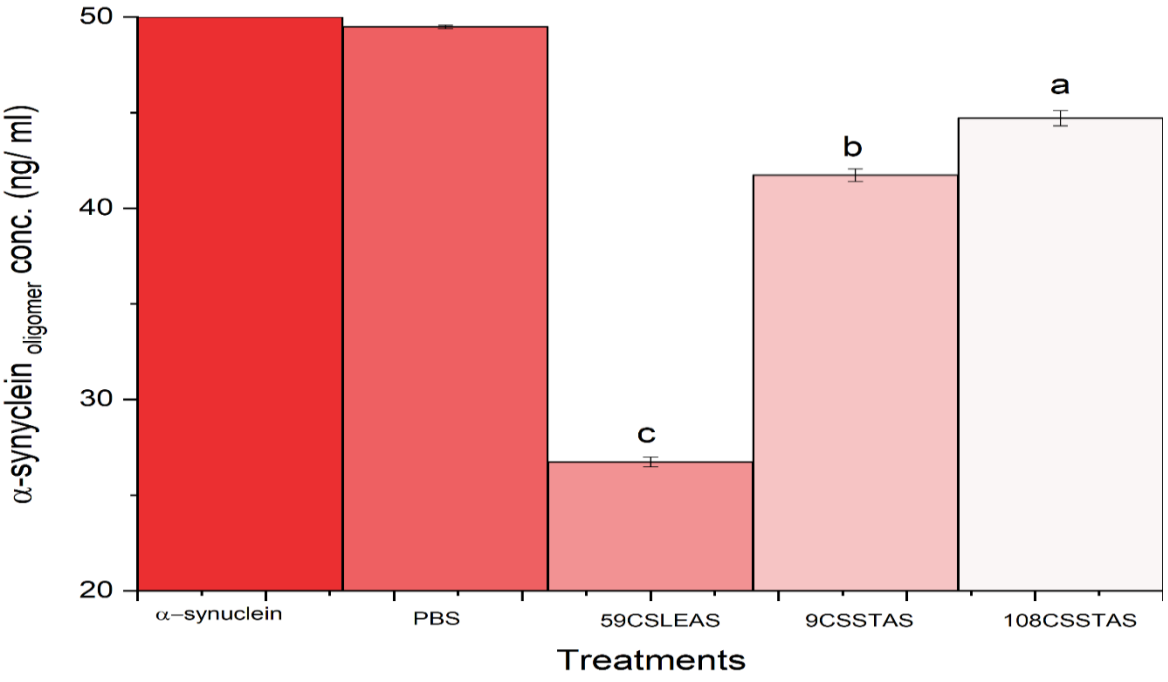

Fig 5.11: Disaggregation effect of endophytic fungal filtrates of #59CSLEAS, #9CSSTAS, #108CSSTAS on oligomeric alpha-synuclein by Sandwich ELISA; letters a, b, c are analysed as significant letters by Tukey's post hoc test ($p < 0.05$)

5.4 Identification of potential endophytic fungus

The selected α-synuclein disaggregating endophytic fungus 59CSLEAS, was identified using morphotaxonomic and molecular techniques.

5.4.1 Morphotaxonomy

The identification of most potent culture 59CSLEAS was done on the basis of macroscopic and microscopic characteristics. The culture was grown on 6 different media, CMA, CRBA, PDA, PLA, SNA and WA, to understand the culture's reproductive characteristics (Fig 5.12). The endophytic fungus produced white coloured growth on SNA and CMA, white to peach-violet growth on PDA and CRBA while little to no growth was observed on PLA and WA, respectively. 5 days old PDA grown culture was studied for macroscopic and microscopic characteristics. Lower surface of 59CSLEAS culture produced white pigmentation having growth diameter 5.5 ± 0.5 cm. The mean length 38.1 ± 4.2 µm and width 7.2 ± 0.3 µm of macroconidia were studied for 50 observations.

The culture showed curved and thick macroconidia morphology and pointed basal morphology. 2-3 macroconidia septations were observed.

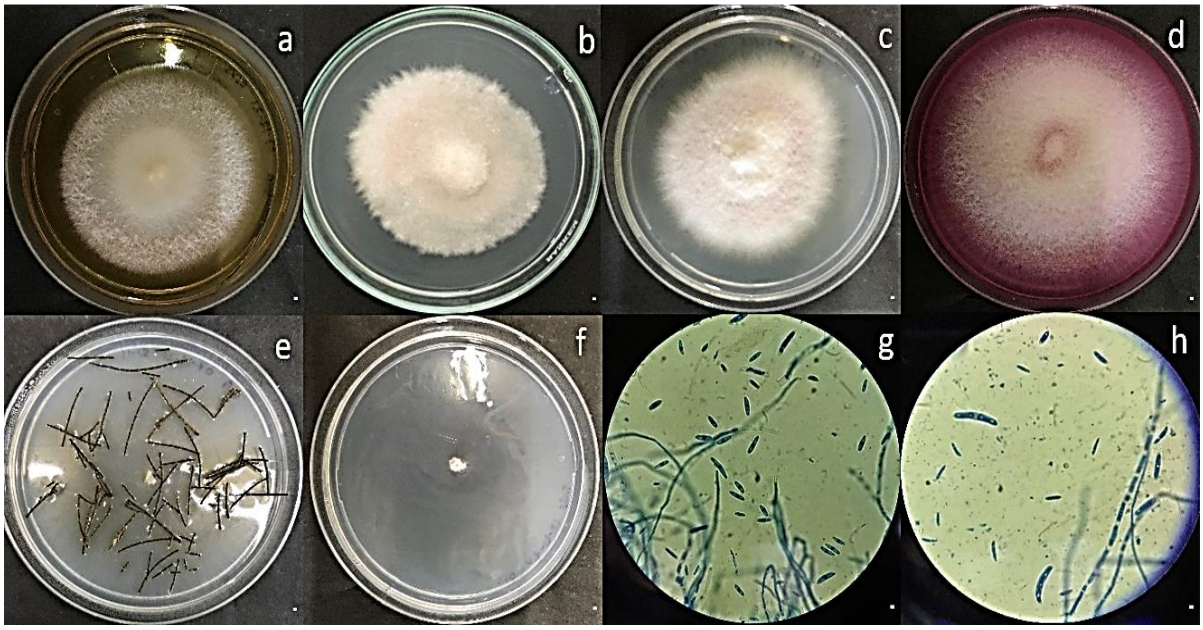

Fig 5.12 Endophytic fungal isolate 59CSLEAS grown on different media. a) SNA, b) CMA, c) PDA, d) CRBA, e) PLA, f) WA, g) conidia stained with lactophenol cotton blue on SNA, h) on PDA media. (Bar: 10mm)

5.4.2 Molecular identification

The endophytic fungus was exhibiting characteristics features of *Fusarium* species when identified via morphological and key taxonomic identification. Hence, to confirm the species, molecular identification was performed. Fungal genomic DNA was isolated and purified. 20 ng/ml concentration of DNA was used for ITS, TEF1α and TUB region amplification. The isolate was deposited in the NFCCI (National Fungal Culture Collection of India, Pune) with accession number NFCCI-4191. Size of amplicon of ITS, TEF and β-TUB regions were found to be ranging in between 550 - 600 bp, 650-750 bp and ~ 1400 bp respectively (Fig 5.13).

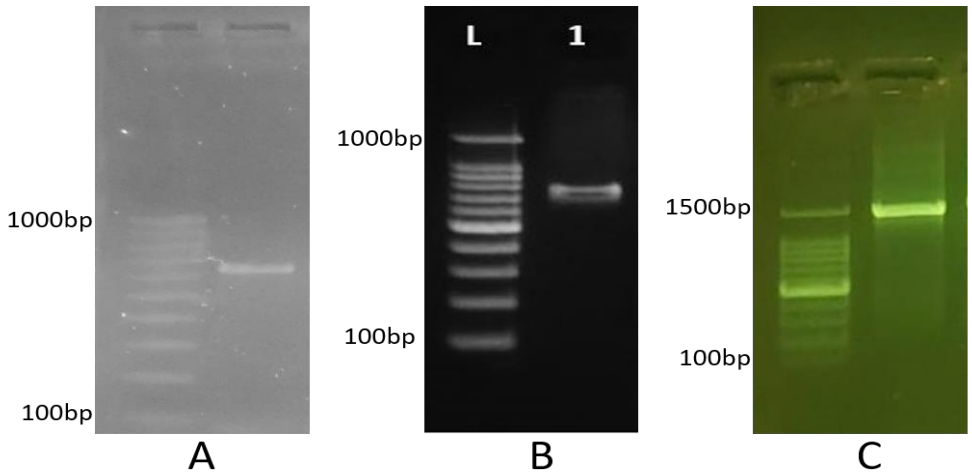

Fig 5.13 Amplified DNA products (A) ITS region (L: 100 bp -1000 bp DNA ladder; lane 1-3: amplified product); (B) TEF1α region (L: 100 bp -1000 bp DNA ladder; lane 1: amplified product), and (C) TUB rDNA amplification of 59 CSLEAS (L: 100 bp – 1500 bp DNA ladder; lane 1: amplified product)

5.4.3 Phylogenetic tree construction

The ITS, TEF1α and TUB sequence of 59CSLEAS were aligned with the type and non-type sequences to establish the phylogenetic placement (Table 5.6). The isolate 59CSLEAS clearly aligned with *Fusarium oxysporum* strain (MZ156849.1) and was clearly demarcated from other species of *Fusarium* (Fig 5.14) when ITS region was analysed. Thus, 59CSLEAS is designated as *Fusarium* sp. However, the types species was further confirmed by amplifying TEF and TUB region (Fig 5.15; 5.16). BLAST analysis of ITS, EF1α and TUB sequence exhibited close homology with *Fusarium oxysporum*. This strain of ribosomal DNA (rDNA) - ITS, TEF and TUB has been deposited in GenBank with accession number "ON226971.1", "OR050538" and "OR522547" respectively.

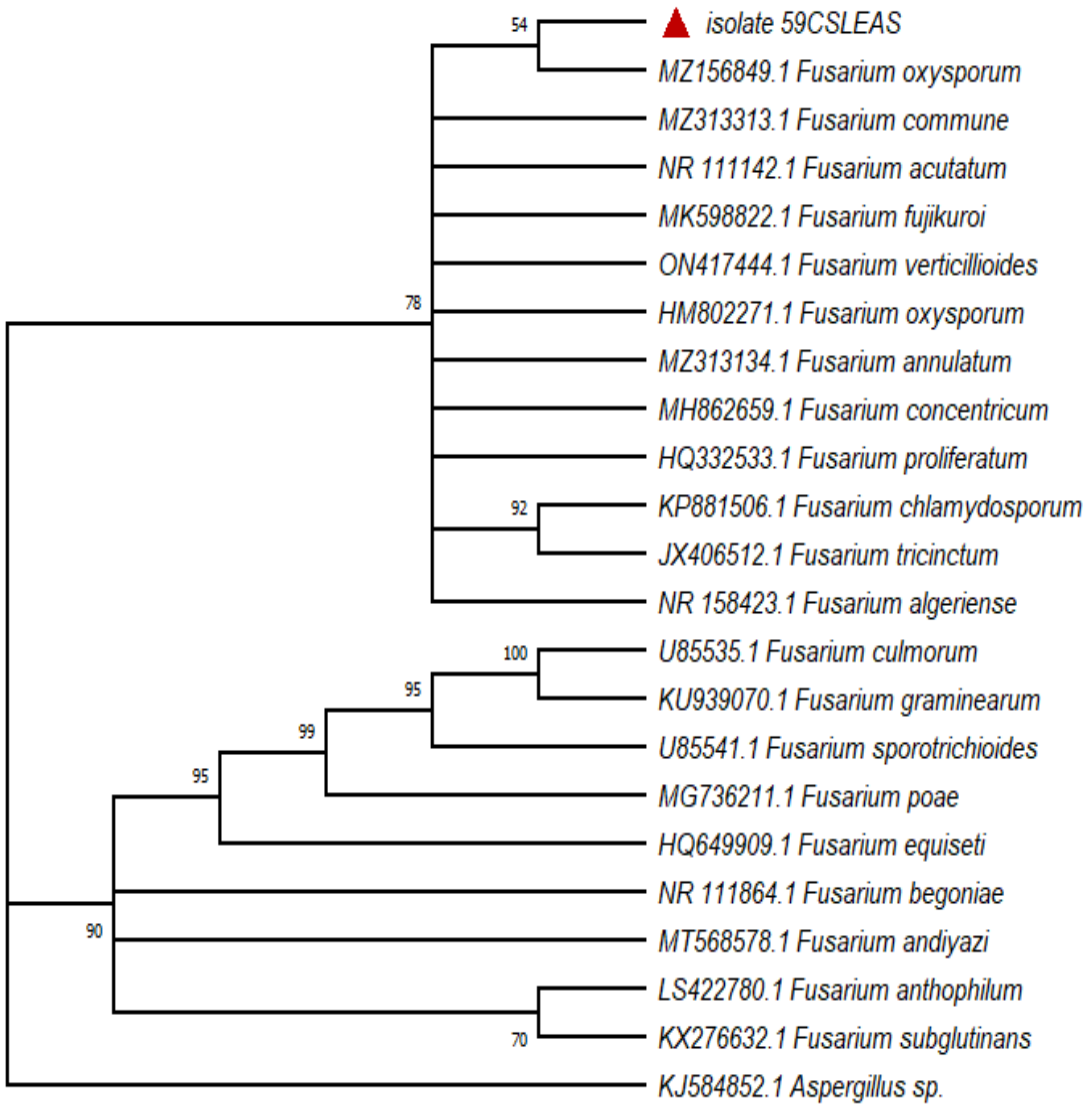

Fig 5.14 Maximum likelihood tree based on ITS representing molecular taxonomy and phylogenetic analysis of culture 59CSLEAS (bootstrap test: 1000 replicates; Outgroup: *Aspergillus* sp.). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA X.

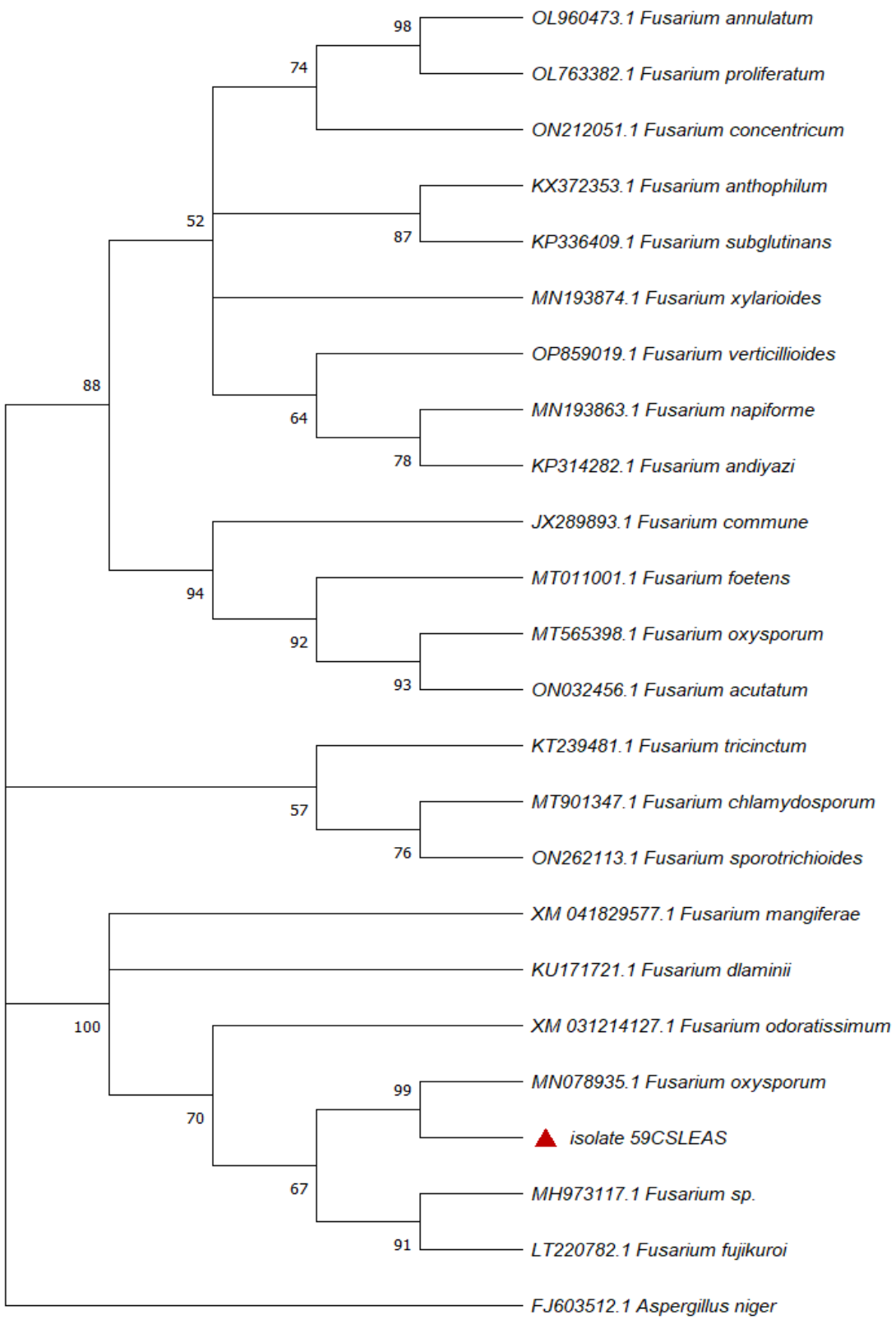

Fig 5.15 Maximum likelihood tree based on TEF representing molecular taxonomy and phylogenetic analysis of culture 59CSLEAS (bootstrap test: 1000 replicates; Outgroup: *Aspergillus niger*). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura-2 parameter model. Evolutionary analyses were conducted in MEGA X.

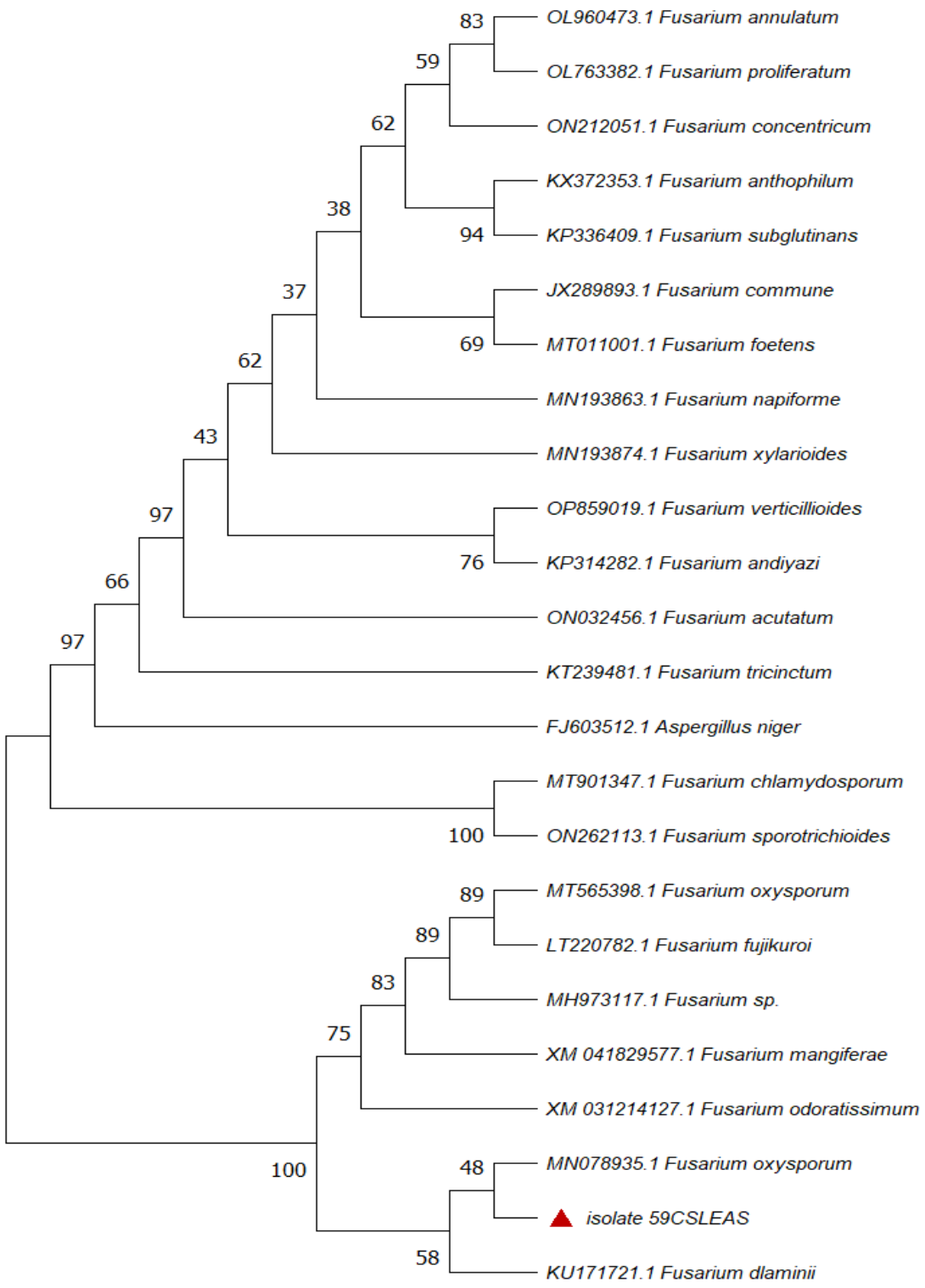

Fig 5.16 Maximum likelihood tree based on TUB representing molecular taxonomy and phylogenetic analysis of culture "#59CSLEAS" (bootstrap test: 1000 replicates; Outgroup: *Fusarium dlaminii*). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA X.

Thus, the potential endophytic isolate based on molecular and morphological taxonomy was identified as *Fusarium oxysporum*.

5.5 *In silico* analysis

5.5.1 Structure-based Virtual Screening

Twenty antioxidant compounds present in *Camellia sinensis* were subjected to docking analysis and presented a docking score in the form of binding energy within the range of -3.4 to -7.1 kcal/mol towards the structure of α-synuclein with PDB.ID: 2N0A. Table 5.5 shows the binding affinities (docking score) and other docking parameters of top 10 compounds used in this study. Interaction analysis of all possible docked conformers of all 20 compounds was carried out to investigate their binding pattern and possible interactions towards the 2N0A binding pocket. Among 20 compounds, the highest binding energy is observed in the case of Theaflavin-3-3-digallate (T33DG) with -7.1 kcal/mol followed by Theaflavin (T) and Theaflavin-3'-gallate (T3G) with -6.7 and -6.4 kcal/mol, respectively. The top three hit compounds are selected for further investigation using molecular dynamics simulations. From molecular docking analysis, it is observed that the ligand Theaflavin-3-3-digallate interacts with Lys80, Ala91, Gly93, Glu57, and Phe3 through approximately 7 hydrogen bonds. Theaflavin binds with Val77, Val48, Glu46, Lys45, and Thr44 through 5 hydrogen bonds, and Theaflavin-3'-gallate binds with 4 hydrogen bonds, i.e., Ile88, Val74, Ala76. The intermolecular interactions between the α-synuclein protein and the two hit compounds are shown in Fig. 5.17.

Table 5.5 Molecular docking results from virtual screening approach

Name of the ligand	Docking Score (kcal/mol)	Ligand Efficiency (kcal/mol)	Torsional Energy (kcal/mol)
Theaflavin-3-3-digallate	-7.1	0.1	4.6695
Theaflavin	-6.7	0.0753	6.5373
Theaflavin-3'gallate	-6.4	0.1123	3.4243
Theaflavin-3-gallate	-6.2	0.0849	4.9808
Gallocatechin gallate	-6.1	0.122	3.7356
Catechin gallate	-5.4	0.1317	1.2452
Luteolin	-5.3	0.171	1.5565
Quercetin	-5.2	0.1576	1.8678
Gallocatechin	-5.2	0.1529	2.1791
Epigallocatechin	-5.1	0.15	2.1791

5.5.2 Molecular dynamics simulation

Seventy amino acids are involved in amyloid formation from α-synuclein in structure PDB.ID: 2N0A. Therefore, we have considered that only 70 amino acids possess domain for molecular dynamics (MD) simulation studies. The other structure part is the loop region that shows higher conformational changes. The stability, conformational changes, and intermolecular interactions of the hit compounds with α-synuclein are found through MD simulations. The α-synuclein has a

+ 3 charge. To neutralize the simulation system, we have added a - 3 Cl charge. The root mean square deviations (RMSD) of a single α-synuclein at its unbound state with another α-synuclein or with a compound is shifted from 2.5 to 19.5 Å, which is unusual in the globular proteins. The globular proteins preferred RMSD change in a range of 1-3 Å. The larger changes in the RMSD of α-synuclein indicate the protein is undergoing enormous conformational changes during the simulation (Fig 5.18).

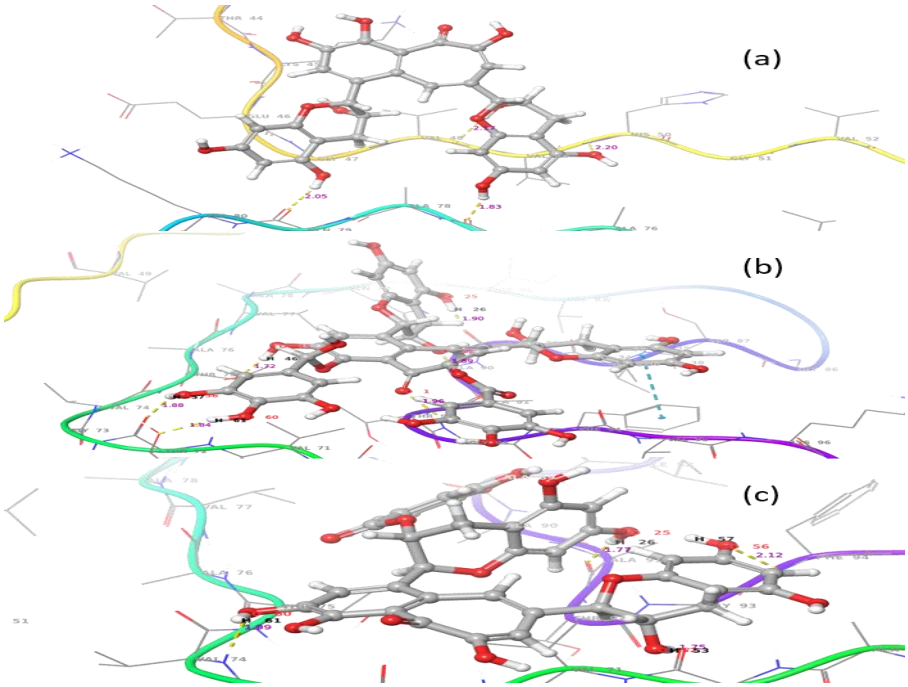

Fig 5.17 Intermolecular interactions between (a) Theaflavin-3-3-digallate (T33DG), (b) Theaflavin (T) and (c) Theaflavin-3'gallate (T3G) with alpha-synuclein protein

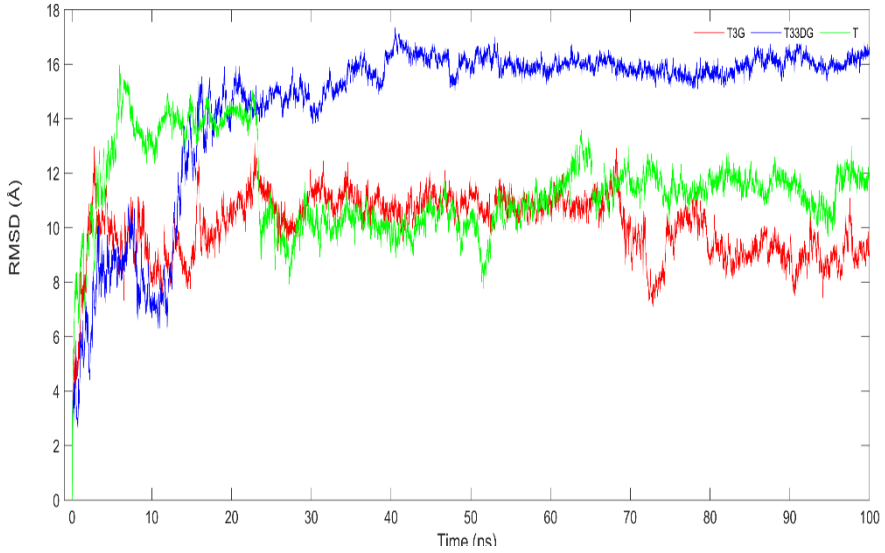

Fig. 5.18 RMSD plot of alpha-synuclein bound with Theaflavin-3-3-digallate (T33DG), Theaflavin (T), and Theaflavin-3'gallate (T3G) respectively

In the case of the root mean square fluctuations (RMSF) analysis, the protein has more fluctuations in the loop region, where the beta-strand region remains rigid. The RMSF plot shows that the region with amino acid index 22 to 24 and 47 to 49 represents the beta-strands. The secondary structure of the α-synuclein protein is monitored throughout the simulation. The SSE (secondary structure elements) analysis summarizes that the protein consists of a 10.33 % beta-strand region, 0.21% helix region, and 89.46 % volatile loop region. The charge of the α-synuclein and Theaflavin-3-gallate complex simulation system is 21 Na and 24 Cl by adding 0.15 M NaCl to the system. The total charge of the simulation system by neutralizing protein with -3 Cl and adding 0.05 M NaCl is 24 Na and 24 Cl. The average RMSD of the α-synuclein bound with Theaflavin-3-gallate is 4.563 Å. Initially, the RMSD is 16 Å which is later stabilized at 11 Å. The secondary structure of the α-synuclein protein is monitored throughout the simulation. The SSE of Theaflavin-3-gallate bound α-synuclein analysis summarizes the protein consists of 0.99 % beta-strand region, 0.01 % helix region, and 99 % region is volatile loop region. The charge of the α- synuclein and Theaflavin-3-3-digallate complex simulation system is 19 Na and 22 Cl by adding 0.15 M NaCl to the system. The total charge of the simulation system by neutralizing protein with - 3 Cl and adding 0.05 M NaCl is 22 Na and 22 Cl. The average RMSD of the α-synuclein bound with Theaflavin-3-3-digallate is 4.563 Å. Initially, the RMSD is 9 Å, stabilizing at a 10-nanosecond time scale with 16 Å.

The secondary structure of the α-synuclein protein is monitored throughout the simulation. The SSE of Theaflavin-3-3-digallate bound α-synuclein analysis summarizes that the protein has a 0.38 % β-strand region, 0.04 % helix region, and 99.58 % region is volatile loop region. The charge of the α-synuclein and Theaflavin complex simulation system is 21 Na and 24 Cl after adding 0.15 M NaCl. The total charges of the simulation system after neutralizing protein with -3 Cl and by adding 0.05 M NaCl are 24 Na and 24 Cl. The average RMSD of the α-synuclein bound with Theaflavin is 5.942 Å. Initially, the RMSD is 15 Å which is reduced to 10 Å after 20 ns time scale. The secondary structure of the α-synuclein protein is monitored throughout the simulation. The SSE of Theaflavin bound α-synuclein analysis summarizes the protein consists of 0.99 % beta-strand region, 0.01 % helix region, and 99 % region is volatile loop region. The RMSD analysis confirmed that the Theaflavin-3-3-digallate (T33DG) showed structural stability during the MD simulations compared with Theaflavin (T) and Theaflavin-3'-gallate (T3G). The fluctuation of protein residue side chains upon binding with ligands is investigated using RMSF in the MD simulation. The protein's C terminal (6 Å) has low fluctuation compared to the N terminal region (10.5 Å). It is o observed that the ligand-binding sites (40-70 residues) show lower fluctuations in

protein-ligand complexes due to the intermolecular interactions. The comparative RMSF of nonbounded protein and complexes as shown in Fig 5.19.

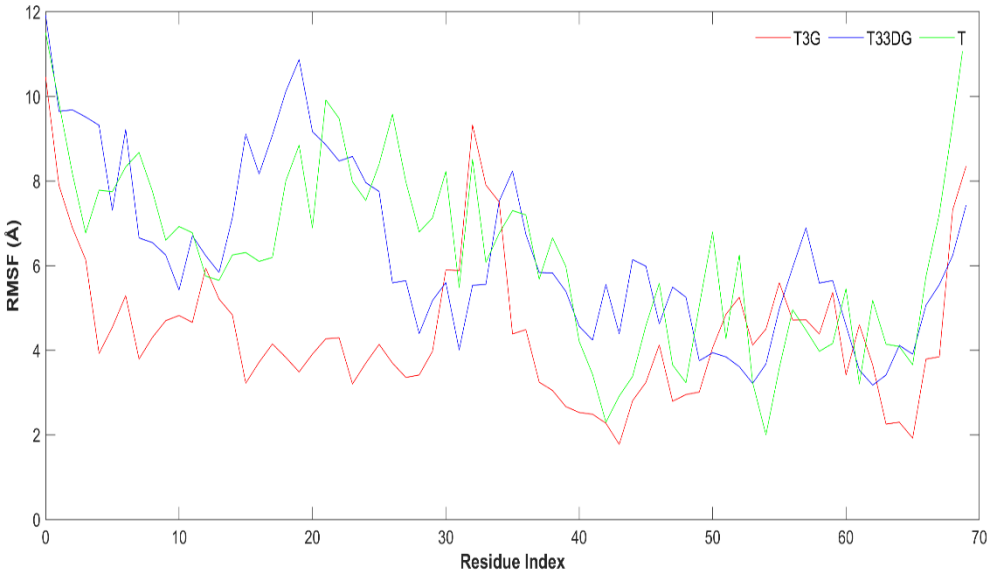

Fig 5.19 RMSF plot of alpha -synuclein bound with Theaflavin-3-3-digallate (T33DG), Theaflavin (T), and Theaflavin-3'gallate (T3G) respectively

From secondary structure analysis of the protein upon binding with T33DG, T, and T3G compounds, it is found that the β-sheet region is converted into the loop. In the T33DG complexed structure, the β-sheet region almost completely disappeared compared to the other two

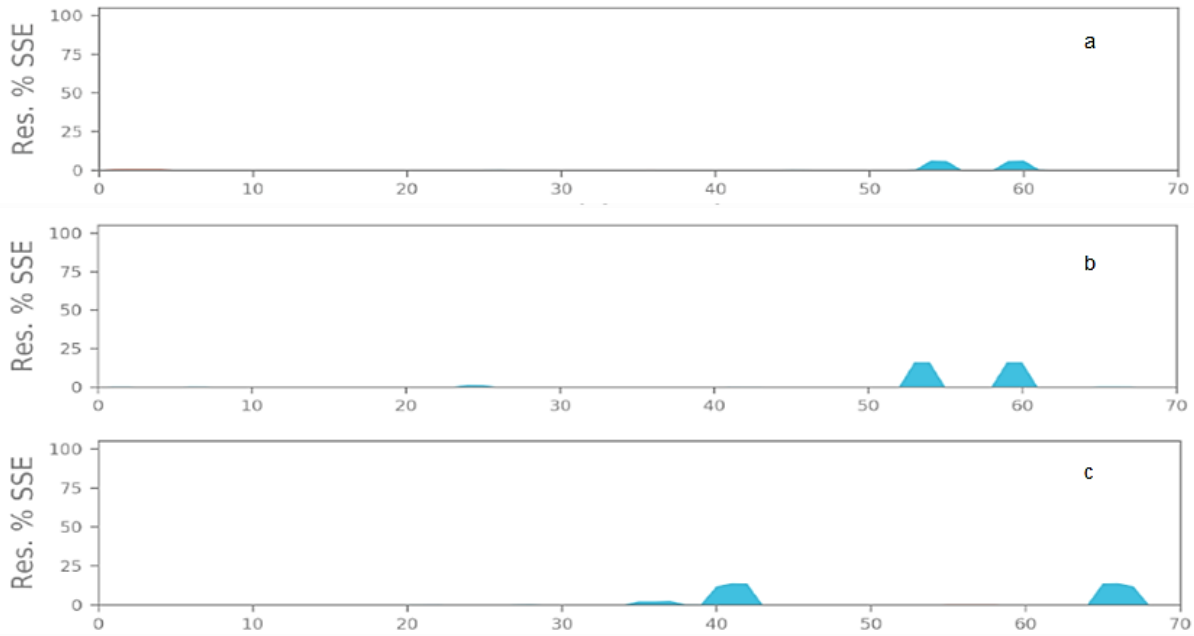

Fig 5.20 The secondary structure alpha-synuclein complexed with(a) Theaflavin-3-3-digallate (T33DG), (b) Theaflavin (T) and (c) Theaflavin-3'gallate (T3G) compounds

This analysis supports that the compound T33DG can disaggregate the α-synuclein amyloids. The secondary structure comparison of the complex structure (Fig 5.20). The molecular dynamics simulations showed that the intermolecular interactions such as hydrogen bond, hydrophobic contact, ionic contact, and salt bridge are analyzed over 100 ns (Table 5.6).

The analysis states that the regions crucial for aggregation have strongly interacted with the ligands. It is observed that the π-π bond is found only between T33DG and Phe94 residue of α-synuclein. The compound T33DG shows a greater number of interactions crossing the threshold strength of the hydrogen bonding, which is over 30 %.

5.6 Partial purification of bioactive residue of selected endophyte by liquid-liquid extraction

The culture filtrate (100 ml) of most potent endophytic fungal isolate, i.e., *Fusarium oxysporum* 59CSLEAS, was partially purified using solvents of increasing polarity. The maximum yield was obtained in aqueous residue resided in crude fraction. Weight of extracted organic residue was calculated after rotary evaporation. The extract weight was found highest in chloroform (6.05 mg) followed by ethyl acetate (4.22 mg), hexane (4.12 mg) and dichloromethane organic layers (2.17 mg) (Table 5.7). Aqueous extract and methanol reconstituted organic residues were further screened to establish *in vitro* disaggregation capacity by performing sandwich ELISA.

Table 5.7: Percentage yield of extracted residues in different solvents

Organic layer	Extract weight (mg)
Hexane	4.12
Dichloromethane	2.17
Chloroform	6.05
Ethyl acetate	4.22
Aqueous fraction	11.61

5.7 Sandwich ELISA to confirm the efficacy of extracts

In the Sandwich ELISA assay, each solvent extract so obtained was treated in different wells and the results were compared with the standard i.e., known concentrations of oligomeric protein to determine concentration of α-$S_{oligomers}$. Among all, chloroform extract exhibited highest disaggregation potential i.e., 56.5 % followed by ethyl acetate with 46 %. On the contrary, dichloromethane and hexane fractions did not exhibit significant disaggregation potential i.e., 20.54 % and 0.32 % respectively (Fig 5.21). Mere disaggregation i.e., 2.49 % was reported by using aqueous extract. Methanol was used as negative control to analyse the study more precisely, where no change in the oligomeric protein concentration was observed.

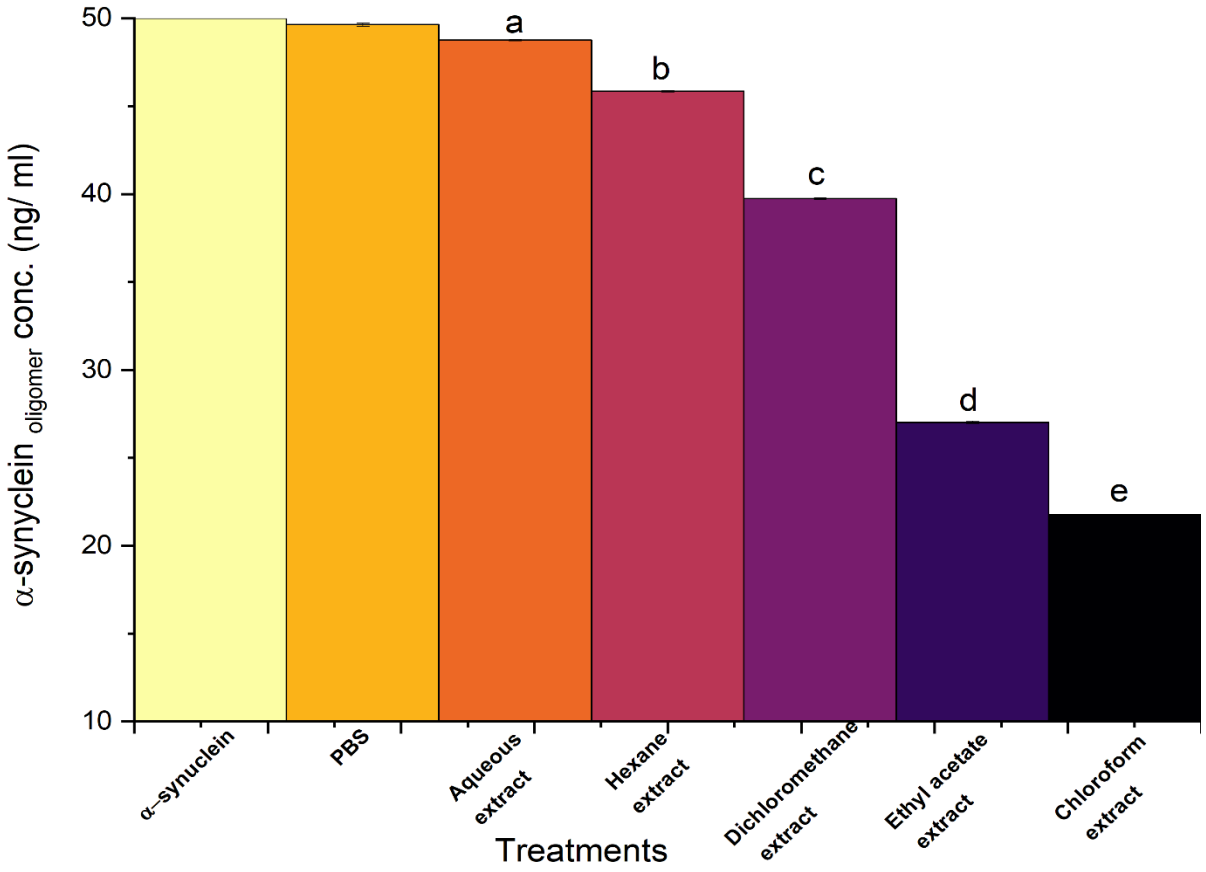

Fig 5.21 Effect of different solvent fractions of 59CSLEAS on disaggregation effect of oligomeric alpha-synuclein using Sandwich ELISA kit; a, b, c, d and e are analysed as significant letters by Tukey's post hoc test ($p < 0.05$).

5.8 Phytochemical testing of bioactive residue

The phytochemical qualitative testing of crude residue revealed the presence of alkaloid, amino acids, carbohydrates, and terpenoids (Table 5.8). Total phenolic content (TPC) and total flavonoid content (TFC) of the extract was also determined (Fig 5.22) by using layout as mentioned in Fig 4.4. The TPC and TFC of the crude residue were calculated as 54.033 mg GAE / g extract and 20.28 mg RE / g extract.

Table 5.8: Phytochemical nature of the extract	
Alkaloid	+
Amino acid	+
Anthocyanin	-
Carbohydrates	+
Flavonoids	+
Phenolics	+
Saponins	-

Tannins	-
Terpenoids	+
(+) Present; (-) absent	

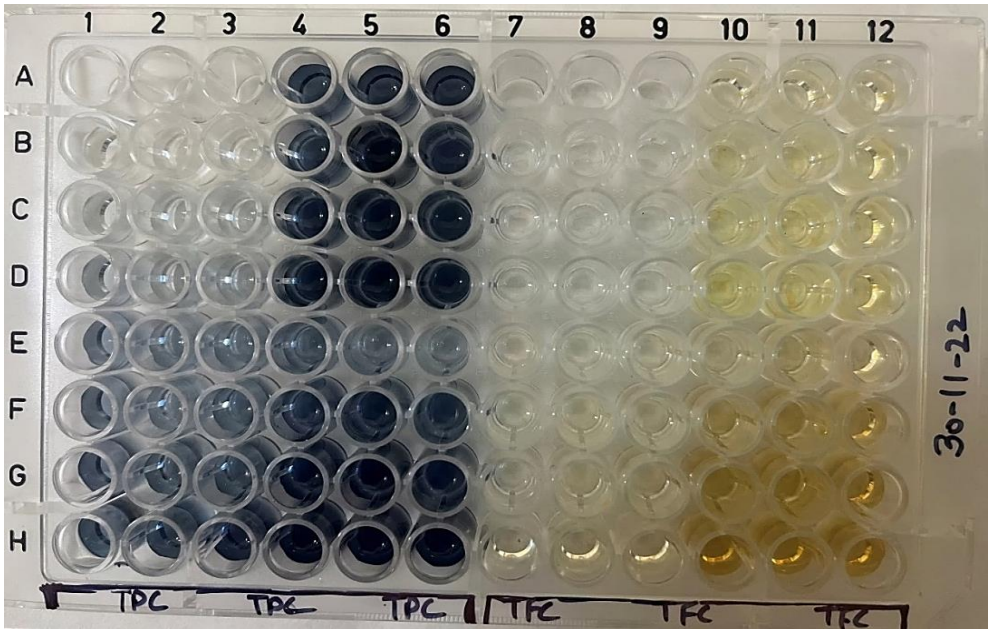

Fig 5.22 96-well microtiter plate Template for TPC in wells 1- 6 and Total Flavonoid content (TFC) assay in wells 7- 12

5.9 Purification and characterization of bioactive residue

5.9.1 TLC fractionation of bioactive residue

The crude chloroform residue of *Fusarium oxysporum* (59CSLEAS) was subjected to TLC using different combinations of mobile phases as mentioned in the Table 5.9. The best mobile phase which resolved the extract in to 11 bands with R_f ranging from 0.2 -0.9 under UV light (365 nm) was Chloroform: Methanol: Ethyl acetate: Acetic acid in the ratio of 1.55 : 0.35 : 0.15 : 2 drops using dropper.

Table 5.9: The crude fraction of *Fusarium oxysporum* 59CSLEAS_chloroform extract was subjected to thin layer chromatography (TLC) using different solvents

S. No.	Solvents	Ratio	Number of bands
1	Hexane: Ethyl acetate	1 : 1	3
2	Hexane: Ethyl acetate: Acetic acid	1.5 : 0.5 : 2 drops	4
3	Hexane: Methanol	1.8 : 0.2	3
4	Chloroform: Ethyl acetate	1.7 : 0.3	5
5	Chloroform: Ethyl acetate: Acetic acid	1.8 : 0.2 : 2 drops	6
6	Chloroform: Methanol	1.7 : 0.3	5
7	Chloroform: Acetic acid	2 : 2 drops	3
8	Chloroform: Methanol: Acetic acid	1.7 : 0.3 : 2 drops	5
9	Chloroform: Methanol: Ethyl acetate: Acetic acid	1.55 : 0.35 : 0.15 :2 drops	11

5.9.2 Purification by column chromatography

For silica gel column chromatography, mobile phase with maximum number of bands was chosen for fractionation of crude chloroform residue of *Fusarium oxysporum* (59CSLEAS). Approximately 2 g residual extract was obtained from large scale fermentation. Different gradients of Chloroform: Methanol: Ethyl acetate: Acetic acid was run starting from 100:0 to 0:100 (Table 5.10). A total of 910 fractions were collected of volume 10 ml. The fractions were pooled into eleven fractions based on TLC profile referred as FC1-FC11 (Fig 5.23). R_f values of the compounds were calculated as 0.24, 0.29, 0.38, 0.43, 0.46, 0.65, 0.63, 0.78, 0.75, 0.83, 0.92.

Table 5.10: Purified compounds on the basis of Retention factor (R_f) value

Fraction group	Fraction No.	Fraction colour	Solvent (chl : methanol : EA : Acetic acid)	Approximate quantity (mg)	R_f value	Nomenclature
1	71-120	White	1 : 0 : 0 : 0	9	0.92	FC1
1 - 2	121-140	White	0.92 : 0.02 : 0.09 : 0.0006	6	0.91	FC1
					0.82	FC 2
2	141-200	White	0.84 : 0.14 : 0.09 : 0.0006	11	0.83	FC 2
2 - 3	201-212	White	0.76 : 0.21 : 0.09 : 0.0006	4	0.82	FC 2
					0.76	FC 3
3	213-260	White	0.69 : 0.29 : 0.09 : 0.0006	8	0.75	FC 3
4 - 5	261-272	Yellow	0.61 : 0.37 : 0.09 : 0.0006	7	0.78	FC 4
					0.64	FC 5
5	273-320	Brown	0.53 : 0.45 : 0.09 : 0.0006	10	0.63	FC 5
6 - 7	321-335	Pale Yellow	0.46 : 0.52 : 0.09 : 0.0006	5	0.65	FC 6
					0.46	FC 7
8	336-385	Red	0.38 : 0.6 : 0.09 : 0.0006	11	0.43	FC 8
8 - 9	386-401	Yellow	0.3 : 0.68 : 0.09 : 0.0006	8	0.44	FC 8
					0.39	FC 9
9	402-578	White	0.23 : 0.75 : 0.09 : 0.0006	14	0.38	FC 9
10	579-790	White	0.153 : 0.83 : 0.09 : 0.0006	14	0.29	FC 10
10 - 11	791-810	White	0.076 : 0.91 : 0.09 : 0.0006	4	0.28	FC 10
					0.23	FC 11
11	811-890	White	0 : 0.98 : 0.09 : 0.0006	12	0.24	FC 11

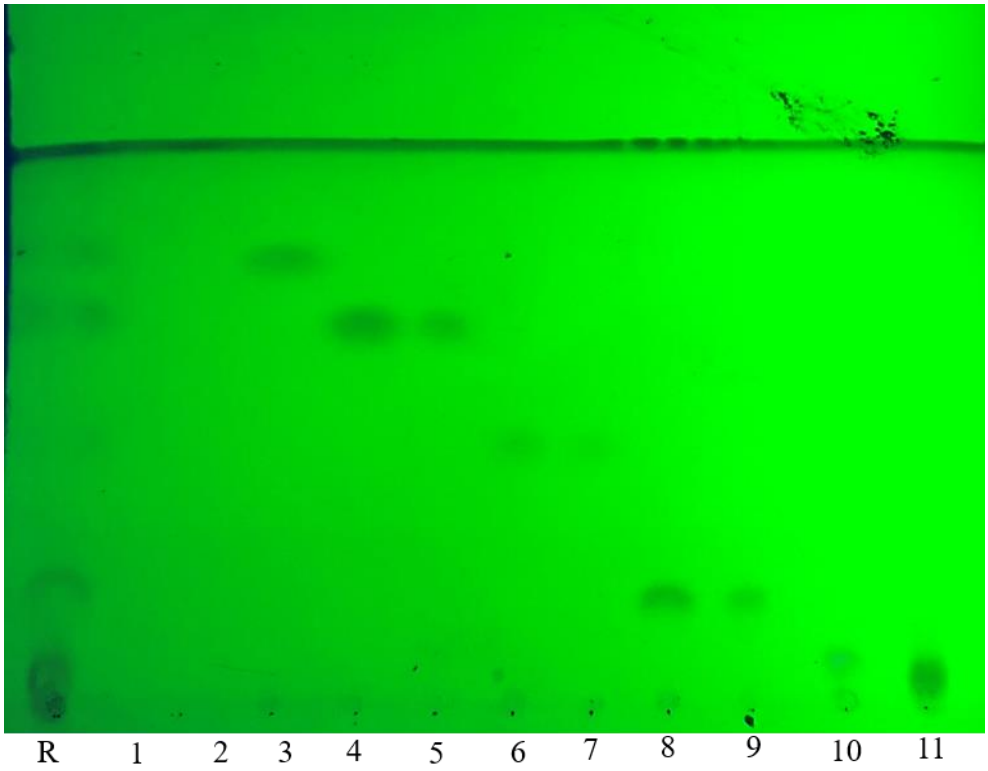

Fig 5.23 Pooled fractions TLC (R=reference); Lane 1-11 represents TLC of 11 different fractions after R_f value-based clubbing

5.10 Sandwich ELISA to reconfirm the disaggregation potential of column fractions

All the fractions so obtained were reconfirmed for their disaggregation potential of misfolded/ oligomeric α-synuclein protein using sandwich ELISA assay. Based on this it was concluded that 50 ng of oligomeric α-synuclein protein disaggregated in a range of 48 ng -15.06 ng (Fig. 5.24)

Among 11 fractions, FC10 possessed the ability to disaggregate protein with maximum potential and reduced concentration of oligomeric protein by 69.8 %. On the other hand, FC1 seems to be capable of decrease the protein concentration by 4 % only. Based on One way-ANOVA and Tukey's post-hoc analysis, FC10 fraction (16 mg) was found to be statistically significant and hence assumed as the most potent compound responsible for disaggregation of oligomeric protein. Before establishing the detailed analysis of the compound, structural elucidation was performed.

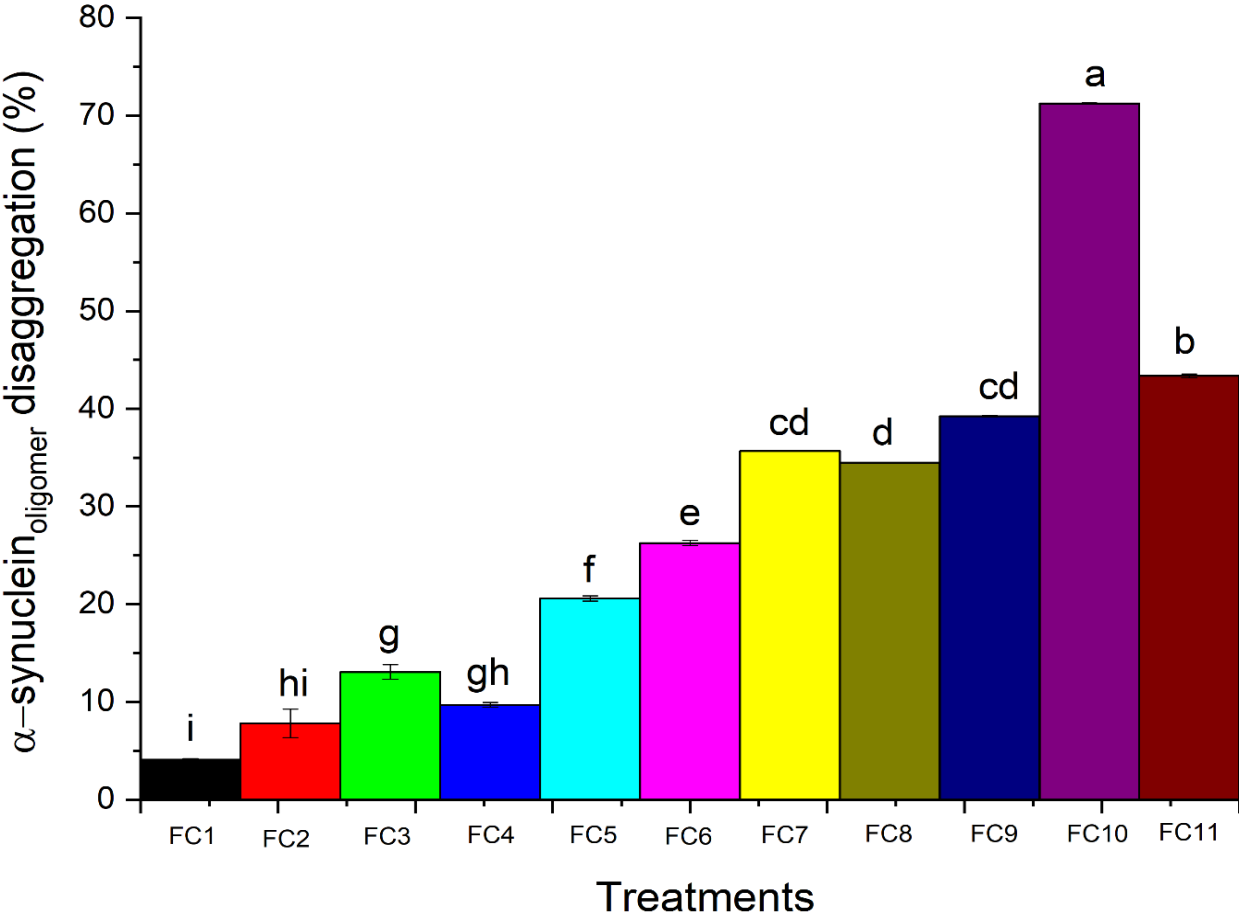

Fig 5.24 Oligomeric α-synuclein disaggregation effect of FC1-FC11 by using Sandwich ELISA kit; letters (a-i) are analysed as significant letters by Tukey's post hoc test ($p < 0.05$); FC (Fractionated compound)

5.11 Antioxidant assay

In order to evaluate antioxidant potential of FC10 compound, DPPH assay was performed. Half maximal inhibitory (IC_{50}) concentration of the fraction FC10 was found to be 0.120 ± 0.02 mg/ ml (Fig 5.25). Ascorbic acid was used as a standard.

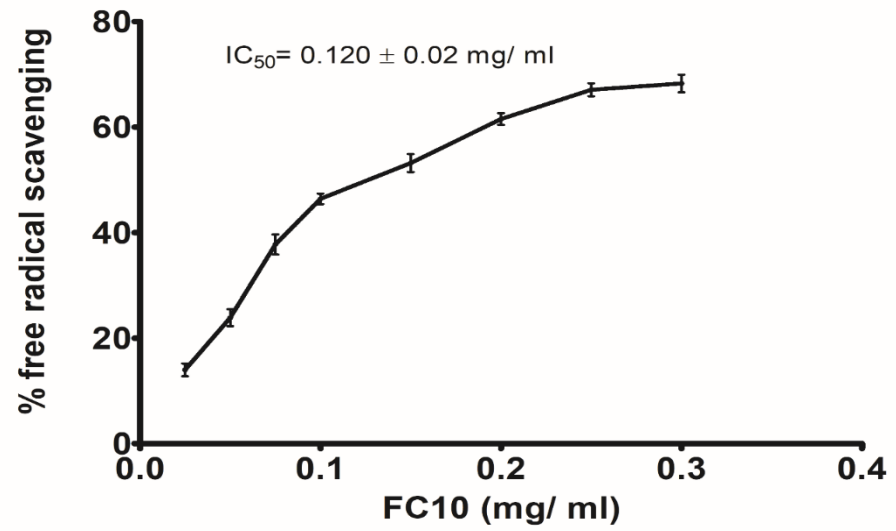

Fig 5.25 Graph representing percentage free radical scavenging after treatment with different conc. of FC10 by using DPPH assay; FC (Fractionated compound). The values of IC_{50} are expressed as mean ± standard deviation.

5.12 Structure elucidation of the compound

The structure of the FC10 was elucidated using LC/ ESI-MS, ^{13}C NMR, ^{1}H NMR, FTIR spectroscopic techniques. The peak obtained with retention time at 4.61 min in mass spectral information was further studied and analysed (Fig 5.26). The ESI-MS spectrum of the fraction was observed in the positive ion mode and the molecular mass of the bioactive fraction was found to be 459.0873 g / mol and this corresponds to the molecular weight of EGCG i.e., 458.372 g / mol (Fig 5.27) (Li et al., 2022 a). A series of peaks were observed at m / z 139.03 were observed due to $C_7H_7O_3$, whereas peaks at m/z 289.07 is due to $C_{15}H_{15}O_7$. Peaks at m / z 444.82 is due to cleavage between the conjugated double bond (Spacil et al., 2010). However, peak at 481.10 m / z marks the presence of epigallocatechin glucuronide which is known as conjugated metabolite of EGCG (Chen et al., 2018; Huang et al., 2022 b; Yassin et al., 2014). FTIR spectra exhibited several characteristics peaks i.e., O-H group (3474, 3253, 3356 cm^{-1}), C=O group (1690, 2168 cm^{-1}). Peak at wavenumber 1628 cm^{-1} may shows aromatic C=O/ C=C in aromatic ring, however peaks at 1527-1534 cm^{-1} wavenumber exhibited the presence of aromatic C=O group (Table 5.11). The spectrum shows the existence of amide bonds I and II at 1690 cm^{-1} (Fig 5.28) (Dahiya et al., 2018).

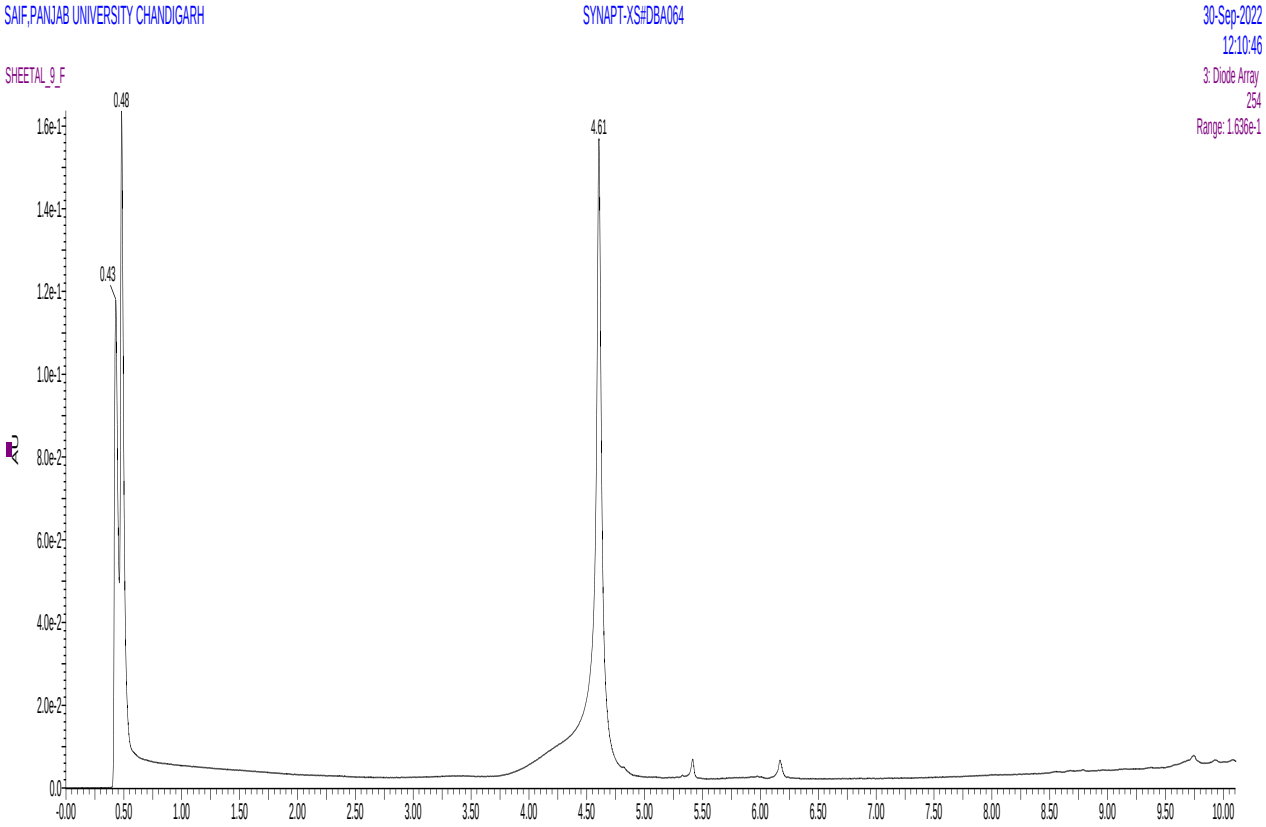

Fig 5.26 LC-MS spectrum of FC10; FC10 (Fractionated compound)

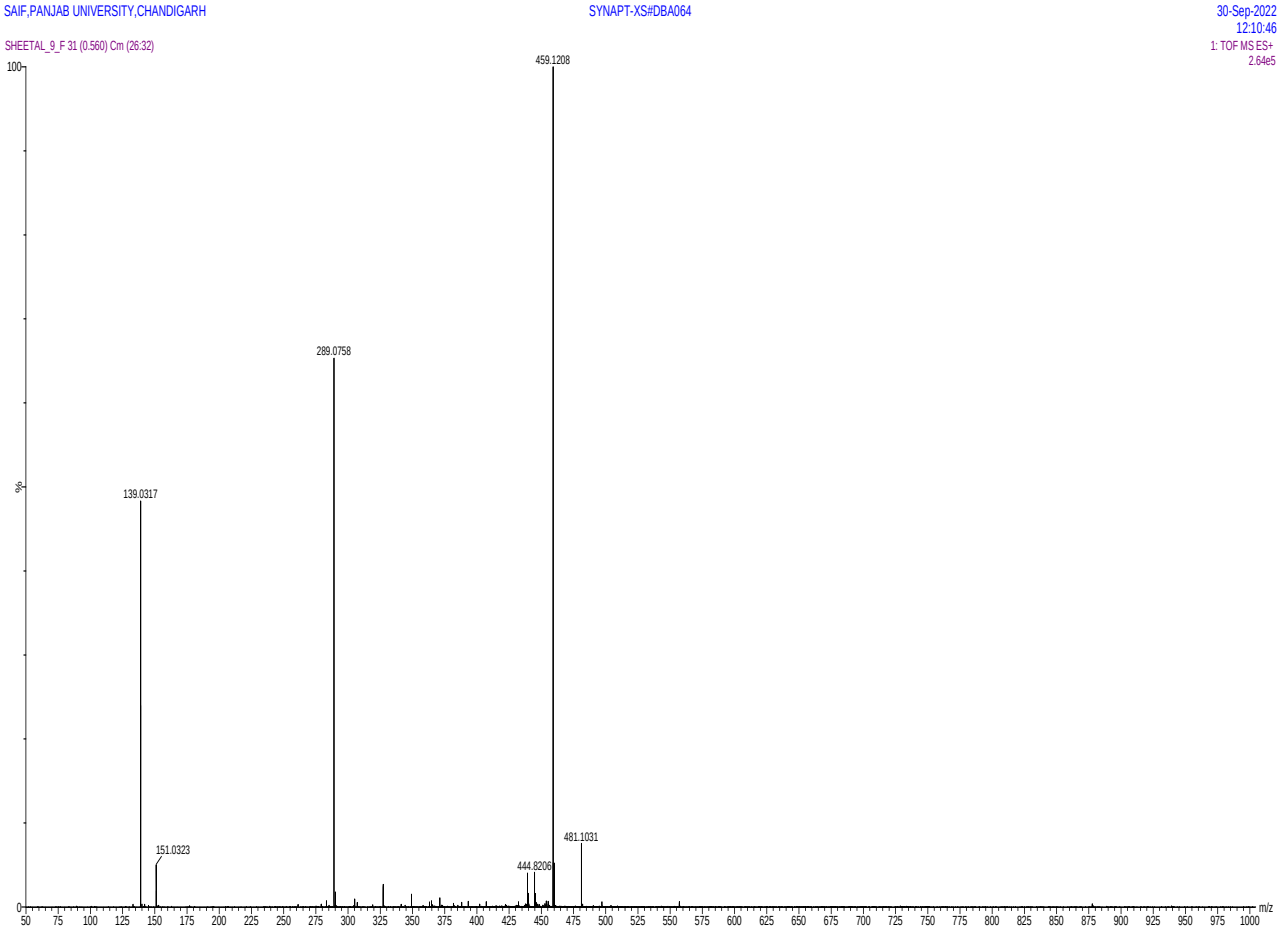

Fig 5.27 ESI-MS spectrum of FC10; FC10 (Fractionated compound)

Table 5.11 Fourier Transform Infrared Spectroscopy FTIR Analysis of FC10 functional groups; FC10 (Fractionated compound)	
Wavenumber (cm^{-1})	**Functional group**
3474	O-H group
3356	O-H stretching
3253	O-H vibration
2168	C=O vibration
1690	C=O
1628	Aromatic C=O/ C=C in aromatic ring
1527 - 1534	Aromatic C=O

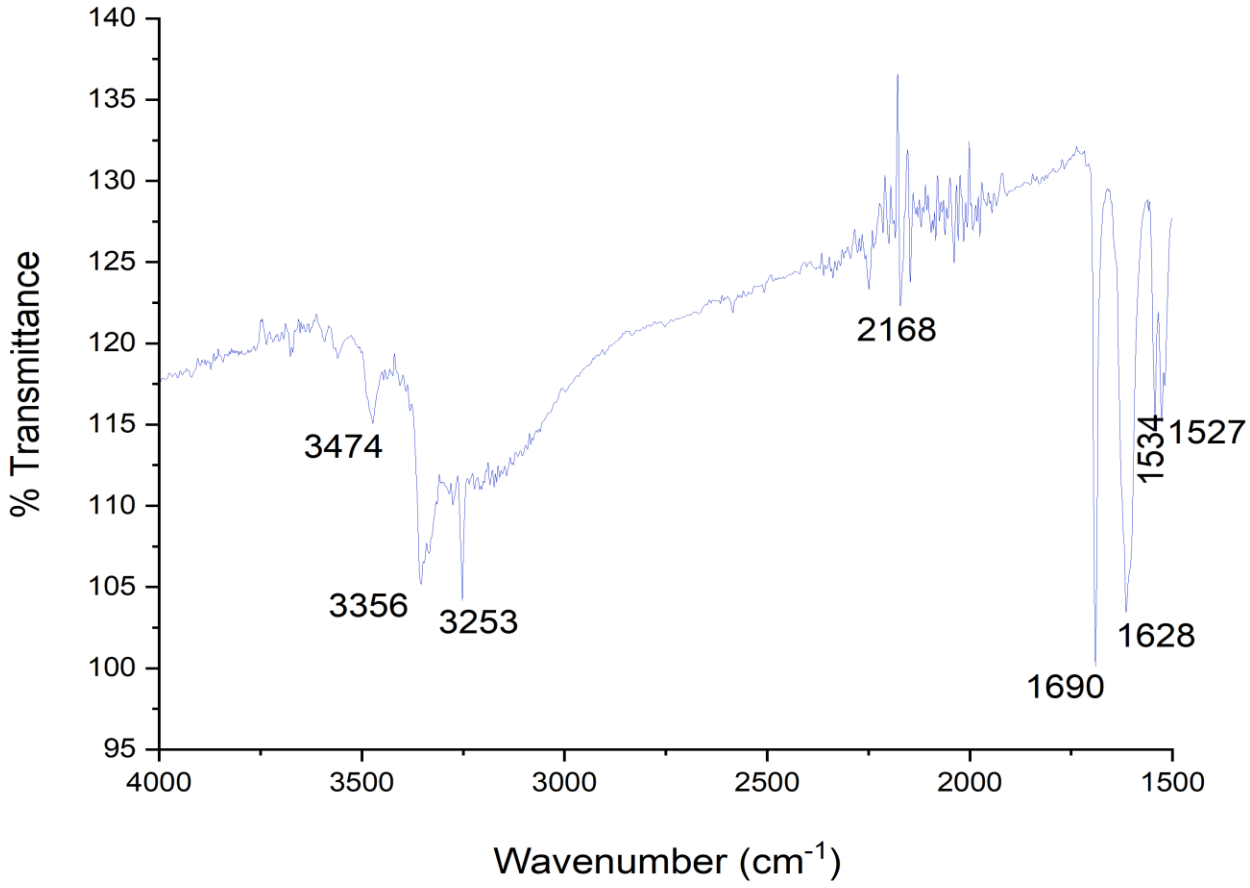

Fig 5.28 FTIR spectrum of FC10; FC10 (Fractionated compound)

NMR spectroscopy was analysed to confirm hydrogen and carbon atoms types and numbers. In proton-NMR (^{1}H-NMR), different peaks of varied NMR represent different position of hydrogen proton. Hence, to elucidate the structure precisely, all the protons type and variation in environment was predicted (Fig 5.29). The singlets at δ_H = 2.46, 2.46 represents presence of two protons with same environment, whereas downfield peaks at δ_H = 4.89, 5.28 represents 2 different positions. A number of low intensity peaks were observed in the region δ_H = 5.86 - 5.80, which might represent the presence of different protons belongs to electronegative region. Presence of peaks at δ_H = 6.36 and 6.75 differentiate the expected compound from other gallate families helps in understanding the possible structure of the compound. ^{13}C NMR revealed the presence of ~ 22 carbon peaks representing different spatial configurations of groups attached with carbon (Fig 5.30). From IR and NMR analysis, it was hypothesized that the structural characteristics of the bioactive fraction were found similar to that of Epigallocatechin Gallate (EGCG).

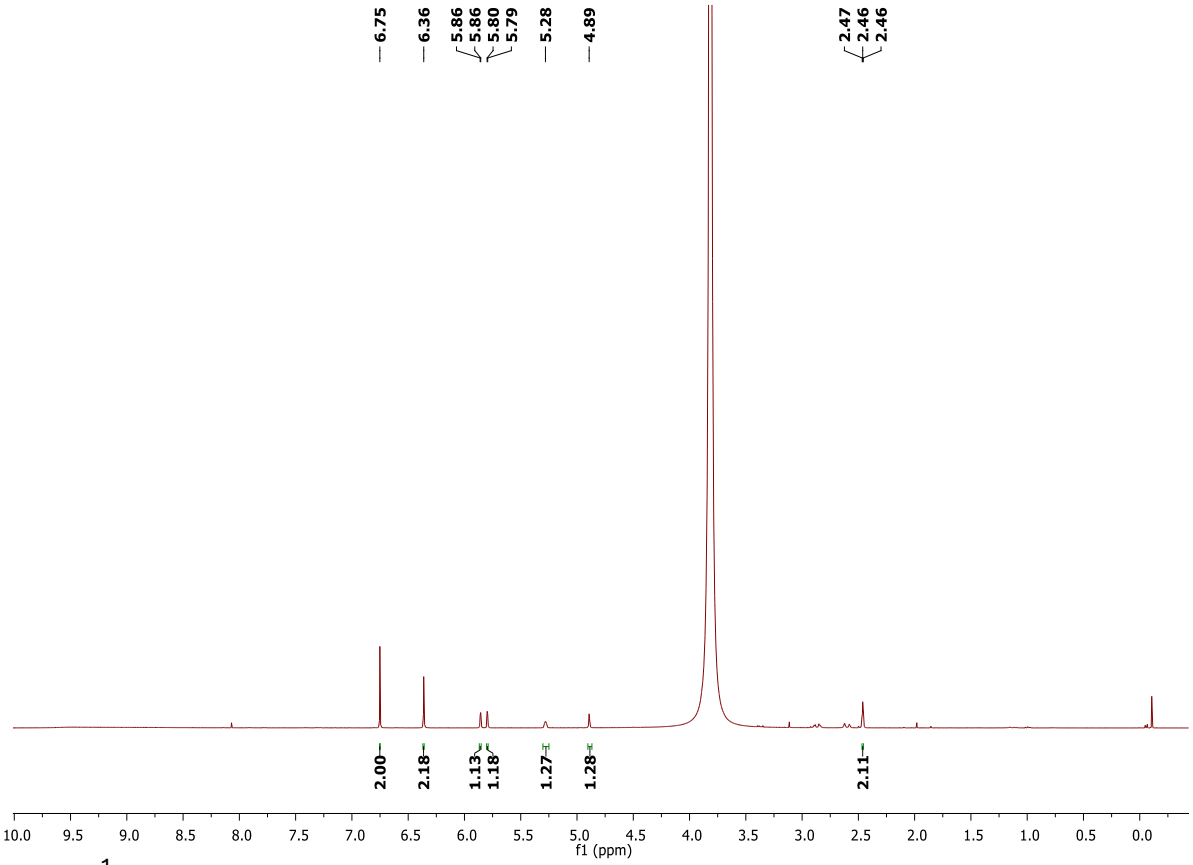

Fig 5.29 ^{1}H NMR image of FC10; FC10 (Fractionated compound)

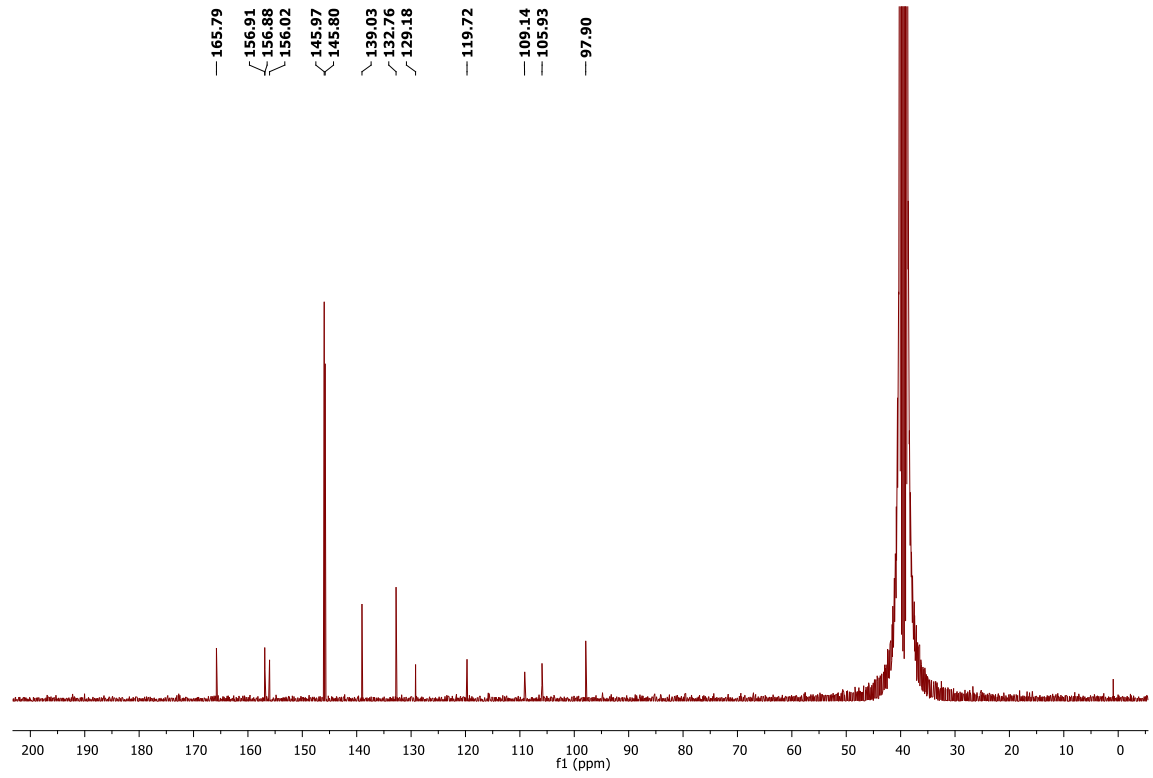

Fig 5.30 ^{13}C NMR image of FC10; FC10 (Fractionated compound)

Hence, chemical name of the fraction 10 was proposed to be [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)choman-3-yl]3,4,5-trihydroxybenzoate. Molecular formula of the compound is studied as $C_{22}H_{18}O_{11}$ (458.4 g/mol) along with proposed structure of the compound (Fig 5.31).

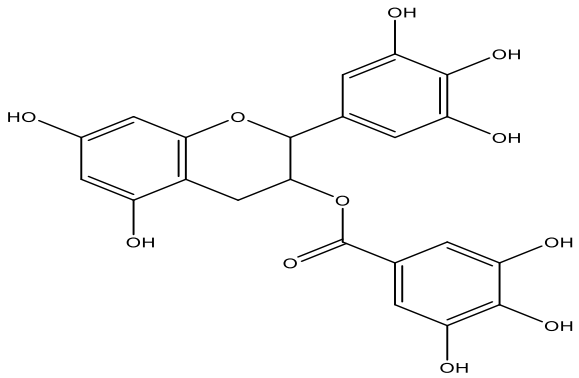

Fig 5.31 Proposed structure of FC 10; FC (Fractionated compound)

5.13 DC_{50} determination

Half maximal degradation concentration (DC_{50}) value of compound EGCG was deduced from dose dependent curve (Fig 5.32). The compound exhibited DC_{50} values of 1.101 ± 0.04 µM. It was quite evident that the compound EGCG caused concentration dependent disaggregation of oligomeric form of protein. Hence, to quantify the changes in β-sheet confirmations, we further proceeded to perform ThT assay.

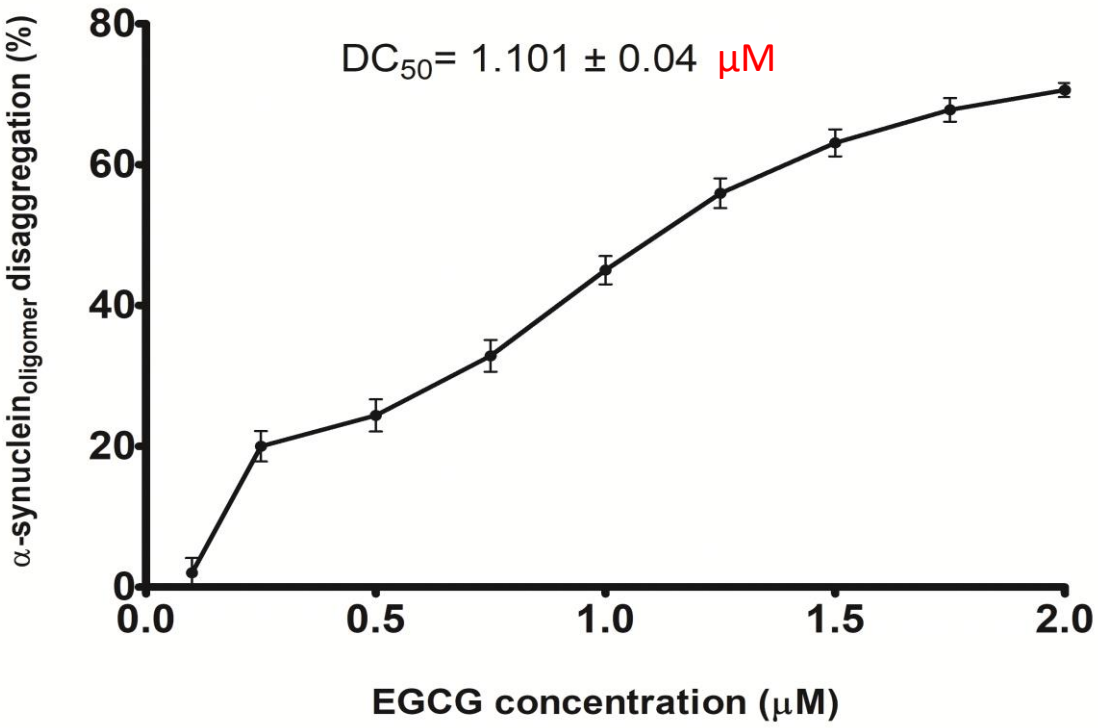

Fig 5.32 Sandwich ELISA representing DC_{50} of oligomeric alpha-synuclein after treatment with a range of 0 – 2 µM dose of Epigallocatechin Gallate (EGCG) compound. The values of DC_{50} are expressed as mean ± standard deviation.

5.14 Disaggregation analysis:

ThT fluorescence assay was performed to quantify the effect of EGCG on disaggregation of oligomeric α-synuclein protein. EGCG caused concentration dependent disaggregation of insoluble form of protein. Maximal disaggregation was achieved at 1.5 μM. A non-significant increase in rate of disaggregation was observed by further increasing concentration of EGCG (Fig 5.33). Hence, it has been concluded that the compound leads to decrease in β-sheet content of oligomeric aggregated protein.

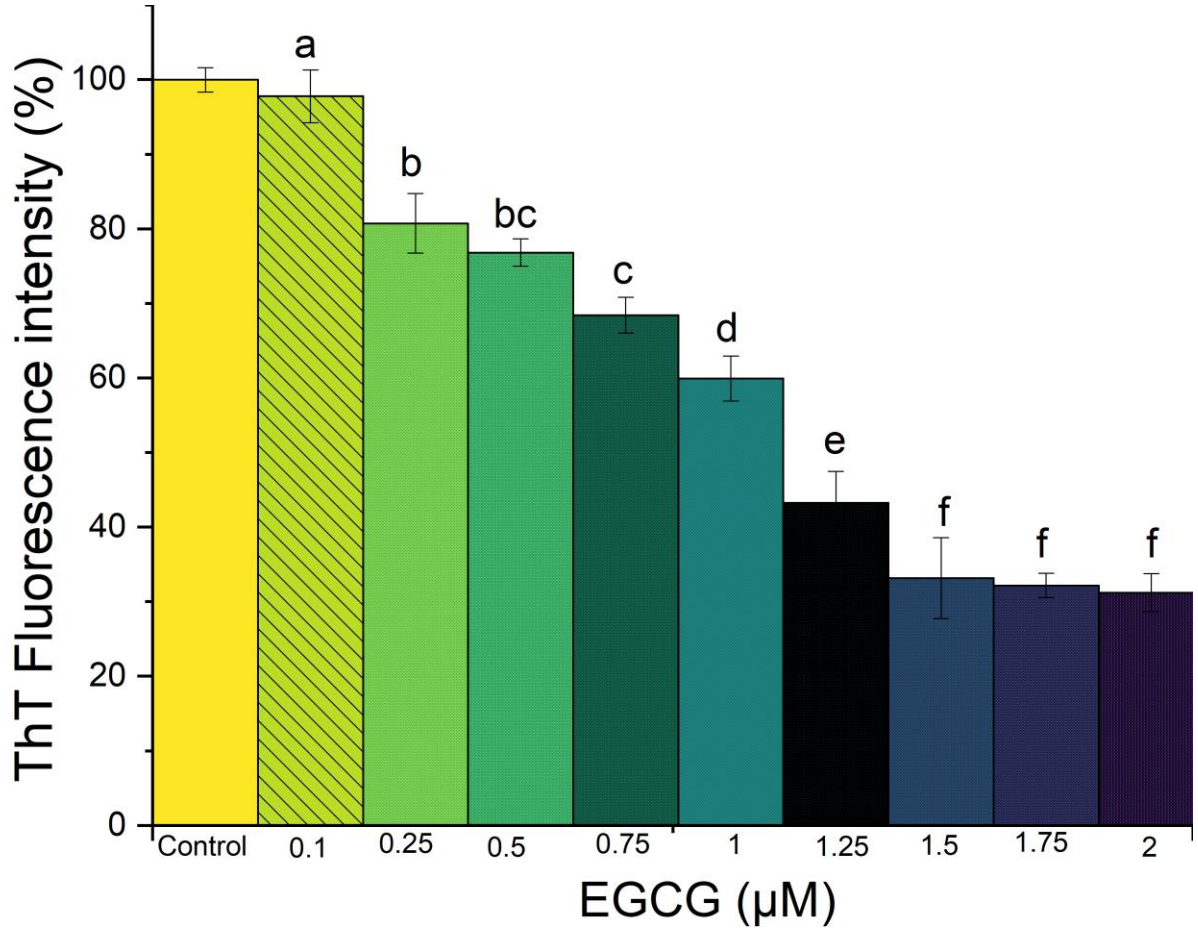

Fig. 5.33 ThT assay representing percentage change in fluorescence intensity at wavelength 487.5 nm of oligomeric alpha-synuclein before and after treated with 0.1 – 2 μM Epigallocatechin Gallate (EGCG) compound; letters (a - f) are analysed as significant letters by Tukey's post hoc test ($p < 0.05$)

CHAPTER-6
DISCUSSION

Discussion

Genetic, biochemicals and animal's studies for over two decades have converged today and strongly establish the crucial role of α-synuclein, a 140 amino acid residue pre-synaptic protein causing shaking palsy i.e., Parkinson's Disease. Parkinson's disease is tightly linked to the propensity of α-synuclein to aggregate into toxic structures which play a role in development and progression of this neurodegenerative disorder (Lundvig et al., 2005).

Monomeric forms of α-synuclein gets self-associated to form soluble α-synuclein oligomers which eventually convert into α-synuclein fibrils. Despite being disordered α-synuclein monomers are non-toxic whereas oligomers and fibrils are more aggregated structures and lead to the formation of β-sheet aggregates. Among these conformers, α-synuclein fibrils and oligomeric α-synuclein forms (Alam et al., 2019). Fibrillar aggregates known as Lewy Bodies (LB) are the markers of the neuronal degradation in the substantia nigra as well as in the locus coeruleus regions of the brain in Parkinson's dementia (Wakabayashi et al., 2007).

A group of neurodegenerative diseases associated with Lewy bodies (misfolded oligomeric aggregates) which exist as intracytoplasmic inclusions as well as glial cytoplasmic inclusions are referred to as "α-Synucleinopathies" (Fink, 2006; Lees et al., 2009; Schmitz et al., 2023). α-Synucleinopathies comprise of Dementia with Lewy bodies (DLB), Parkinsons' disease (PD), MSA (Multiple System Atrophy) and PDD (Parkinson's disease with dementia) (Jellinger, 2010).

Another interesting aspect which was that the monomers of α-synuclein are non-toxic in nature existing in a concentration range of 5-50 μM in the nerve terminals (Perni et al., 2017). This monomer of α-synuclein under physiological conditions is found to play essential role in regulating oxidative stress, dopamine regulation, neuronal differentiation, and promoting SNARE complex formation besides neurotransmitter release (Burre et al., 2010).

As α-synuclein is a multifunctional protein, however its role in synaptic activity is through the regulation of vesicle docking and neurotransmitter release has been clearly documented (Ghiglieri et al., 2018). Toxic forms of α-synuclein disrupts a variety of pathways including the neurotoxic pathway which leads to neurodegeneration. Such pathways comprise of synaptic dysfunction, autophagy/ lysosomal dysregulation, mitochondrial disruption and oxidative stress.

As α-synuclein aggregates in the host cells, it not only affects the cellular pathways but also spreads intercellularly. Therefore, therapeutic interventions of PD management aim to develop a disease modifying therapy by targeting the spread, production, aggregation and degradation of toxic forms of α-synuclein. The presumptive approaches could be developing putative α-

synuclein receptors which be preventing the α-synuclein spread or reducing the α-synuclein so that its propensity to aggregate and form toxic structures is greatly reduced or its pathway of the production is disrupted, the use of small molecules which could prevent aggregation or disrupt the aggregated α-synuclein, increasing the α-synuclein degradation by autophagy / lysosomal flux or immunotherapy (Fields et al., 2019).

Thus, one of the plausible alternatives for preventing the PD disease progression is disaggregation of oligomeric forms of the α-synuclein (Brundin et al., 2017; Fields et al., 2019). Since times immemorial, nature has been a source of chemical scaffolds which have been developed into therapeutic agents beginning from compounds such as acetylsalicylic acid and morphine which have served as pain killers. Today more than 80% of the modern drugs have their origin from plants directly or indirectly (Fabricant and Farnsworth, 2001; Najmi et al., 2022; Newman and Cragg, 2016). Several plant extracts and phytochemicals have been reported to exhibit neuroprotective action through the anti-oxidant and anti-inflammatory activities (Sarrafchi et al., 2015; Morgan and Grundmann, 2017; Amro et al., 2018). Major bioactive ingredients in plants comprise of phytochemicals and plant secondary metabolites which are the integral constituents of the neuroprotective foods and herbs. These natural entities exert pleiotropic effect and moderate a variety of pathways in animals and cellular models of PD thereby "exerting" a neuroprotective effect (Zanforlin et al., 2017).

Vegetables, fruits, red wine, chocolate and tea exhibit a variety of anti-oxidant functions thereby scavenging the reactive oxygen species (ROS), reactive nitrogen species (RNS) and activation of the anti-oxidant enzyme system. The main components present in these foods comprise of different chemical classes such as flavonoids, stilbenes, phenylpropanoids and terpenes (Naoi et al., 2022). In the recent past several phytochemicals have been found to exhibit a protective action against α-synuclein fibrillation which largely belong to the polyphenolic groups (Chau et al., 2021; El Gaamouch et al., 2021). *In vitro* and *in vivo* studies have previously reported the protective action of polyphenolic compounds such as curcuminoids, baicalein, green tea, (-) epigallocatechin gallate (EGCG), grape and blue berry extracts protect against neuronal damage of PD (Chao et al., 2012).

Other phenolic compounds which have demonstrated inhibition of formation of α-synuclein fibrils and de-stabilize pre-formed fibrils are myricetin, curcuminoid, rosmarianic acid, Nordihydroguaiaretic acid and ferulic acid (Ono and Yamada, 2006). *Camellia sinensis*, popularly known as tea, is a native plant species of Southeast Asia and Asia, where it was initially consumed as a beverage because of its refreshing properties primarily due to the presence of

polyphenols, flavonoids, and catechins present in the leaf decoction. Apple (*Malus domestica*) is also rich in phenolics after cranberries. The five different polyphenols present in apple comprise of phenolic acids, anthocyanins and dihydrochalcones (Ceymann et al., 2012). The well-known flavonoids present in apples are quercetins viz., quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-ramnoside apart from other compounds such as cyanidin-3-galactoside, epicatechin, chlorogenic acid, coumaric acid phlorizin and gallic acid (Boyer et al., 2004; Wojdylo et al., 2008).

As evident from animal mode of PD, tea polyphenols exert neuroprotective and neurorestorative effects (Caruana and Vassallo, 2015). Black tea extract has also been evaluated. It possesses strong activity towards antagonizing the aggregation of α-synuclein (Ono et al., 2020). The major polyphenolic constituents found in tea are, catechins, including -epigallocatechin (EGC), (-)-epicatechin gallate (ECG), epigallocatechin-3-gallate (EGCG) (Bavi et al., 2023). As it has been well experimented and documented those polyphenolic compounds originating in plants possess the potential to be used as new therapeutic agents due to their anti-oxidant as well as toxic protein degradative abilities, it is of utmost importance to develop them to be used in clinical settings i.e. as therapeutic agents/ commercial drugs.

However, these polyphenolic compounds are primarily produced in much lower quantities within the plants, and their extraction process makes them expensive, hence they are generally known as “low volume high value” products. Hence their copious supply for commercial use is a matter of concern as mass annihilation of the source plant to extract a particular polyphenolic compound would threaten the ecosystem and eventually lead to biodiversity loss. Owing to this bottleneck in successful utilization of some plant based polyphenolic compounds as therapeutic agents/ nutraceuticals alternative routes of commercial production of the polyphenolic entities need to be explored.

Microorganisms (bacteria, actinomycetes and fungi) have been isolated / recovered from different ecological niches and subjected to phenotypic assays to identify the bioactive / pharmacological properties of the lead cultures (Katz and Baltz, 2016). Endophytes is a term assigned to the association of microorganisms with the plants, in which they reside within them symbiotically, without any overt signatures of their existence for at least a period of their life cycle (Bacon and White, 2000).

Today endophytic fungi existing in different ecological niches have become the prime focus of several research groups across the globe since they serve as metabolic factories not only for novel bioactive compounds of pharmaceutical / agrochemical importance but also as a source

of putative phytochemicals of the plants in which they reside and co-evolve (Hashem et al., 2023). As it has been amply demonstrated that endophytic fungi possess the capacity to biosynthesize a variety of putative phytochemicals of their host plants such as Huperzine, Camptothecin, Hypericin, podophyllotoxin and Camptothecin due to coevolution and horizontal gene transfer (Aly et al., 2010; Devi et al., 2023). A significant array of natural chemical entities is expressed by endophytic fungi as antioxidants, which is generally expressed during their symbiotic relationship within their host to overcome different stress signals in the form of ROS (Hamilton et al., 2012; Kouipou Toghueo and Boyom, 2019; Abo Nahas et al., 2023). However, the anti-oxidants produced by endophytic fungi have not been subjected to evaluation of their potential to prevent aggregation of proteins into misfolded aggregates with toxic attributes.

Hence based on this premise, we undertook exploration of the endophytes associated with *Camellia sinensis* and *Malus domestica* from India as a plausible source of α-synuclein disaggregating/inhibiting compounds. From *Camellia sinensis,* 53 endophytic isolates were recovered from the stems and leaves while 26 were isolated from *Malus domestica*. The majority of isolates from tea belonged to the phylum Ascomycetes within three dominant classes- *Sordariomycetes*, *Dothideomycetes* and *Eurotiomycetes*. The phylum ascomycetes especially the classes Sordariomycetes and Dothideomycetes have been recognized as common representatives of endophytic communities in tea (Onlamun et al., 2023; Tibpromma et al., 2022; Win, 2018)

In our study, the genus *Fusarium* was the dominant isolate, whereas in another study, *Penicillium* was found to be dominant (Nath et al., 2015; Nath et al., 2023 b). Yet another study from China reported the presence of four common endophytic fungi *Pleosporales* sp., *Colletotrichum gloeosporioides*, *Peyronellaea glomerata* and *Botryosphaeria dothidea*. The isolation frequency of *Botryosphaeria* was very low compared to *Fusarium* species whilst *Colletotrichum* species was not isolated from stem and leaves of *Camellia sinensis* collected from Assam and Himachal Pradesh. *Fusarium* species has also been reported as an endophyte in the stems of *Camellia sinensis*, collected from west Java, Indonesia (Agusta et al., 2006). *Penicillium* sp., *Aspergillus* sp.*, Cladosporium* sp., and *Fusarium* sp. have also been reported as endophytic in Tea isolated from different tea gardens in Assam, India (Nath et al., 2023 a). On the other hand, *Colletotrichum* sp. was found to be most dominant endophytic species isolated from the leaves and stem samples of Apple leaves in our study. *Colletotrichum* sp. has also been reported as one of the prominent species of fungal endophytes existing in apple in Brazil, apart from *Epicoccum* sp. and *Xylaria* sp. which are reported in our study also (Camatti-Sartori et al., 2005). Afandhi et al. (2018) has also reported the endophytic existence of *Aspergillus* sp., *Penicillium* sp., *Curvularia* sp. and

Alternaria sp. from apple leaves in Indonesia. *Botryosphaeria* sp. and *Melanconiella* sp. were found as incidental endophytes in tea. Similarly in case of apples, *Xylaria* sp. and *Epicoccum* sp. have appeared to be incidental as they have not been reported earlier.

Today it is well understood that α-synucleinopathies have been neuropathologically characterized by the presence of Lewy bodies in the cytoplasm of vulnerable neuronal and glial populations. Despite cumulative evidence existing on the pathological role of improper α-synuclein aggregation in the neurodegeneration process (Chartier-Harlin et al., 2004; Winner et al., 2011) , the exact underlying mechanisms of cytotoxicity in PD still needs clarity and therefore hampers the disease modifying therapeutic interventions. α-synuclein can exist as a soluble unfolded monomer or a functional tetramer under normal conditions. Pathological conditions convert the assembly of soluble oligomeric intermediates that mature into insoluble amyloid fibrils found in Lewy Bodies. Current hypothesis suggests that α-synuclein pre-fibrillar forms represent toxic species, making them a subject for intense investigations (Outeiro et al., 2008; Villar-Pique et al., 2016). Further it has also been proved that the α-synuclein aggregates can spread across the Central Nervous System (CNS) by undertaking a cell-to-cell propagation similar to a prion thereby driving disease progression. Hence, eliminating the toxic α-synuclein aggregates may serve as an attractive target for development of therapeutic interventions which may halt the disease progression.

As α-synuclein has a central role in the disease development and progression, therefore several cellular models have been developed which mimic the important aspects of α-synuclein biology such as aggregation and toxicity have been developed over the years to comprehend the process of PD pathogenesis and other synucleinopathies (Lazaro et al., 2016). *Saccharomyces cerevisiae* (also referred as “yeast”) is a well-known industrial eukaryotic single celled microorganisms which has been used from making bread to production of fermented beverages and alcohol. Biology and genetics of *Saccharomyces cerevisiae* has been thoroughly worked out as it has been used for thousands of years of industrial applications and further can be exploited for understanding basic biological processes that are conserved among all eukaryotes.

*Saccharomyces cerevisiae*serves as a dynamic living laboratory, offering a unique platform for delving into the intricacies of neurodegenerative diseases linked to the misfolding and aggregation of proteins based on the depth of understanding the molecular machines responsible for the protein folding, misfolding and degradation as well as identification of yeast proteins exhibiting prion like behaviour (Delenclos et al., 2019). Heterologous expression of α-synuclein in yeast has been instrumental in unravelling key facets of synucleinopathies,

shedding light on both dose-dependent toxicity and the formation of inclusions (Outeiro and Lindquist, 2003). Different isoforms of yeast cells are examined to understand the molecular mechanism responsible for intracellular accumulation of toxic protein inclusions (Gupta et al., 2018). Other research groups have also explored yeast as a toolbox to understand the molecular mechanisms underlying the impairment of intracellular trafficking, transformed lipid metabolism and increased level of oxidative stress (Outeiro and Lindquist, 2003; Zabrocki et al., 2005). Foundational investigations conducted in *Saccharomyces cerevisiae*have yielded profound insights into the biological intricacies and pathophysiology of α-synuclein and other genes associated with Parkinson's disease. Concurrently, these studies have played a pivotal role in the discovery of small molecular entities capable of alleviating α-synuclein induced toxicity (Dhungel et al., 2015; Fleming et al., 2008; Sampaio-Marques et al., 2012; Su et al., 2010; Tardiff et al., 2014). Thus, yeast model has been proven to be useful tool to understand mutations in α-synuclein leading to the development of PD (Lazaro et al., 2016) as well as post-translational modifications such as SUMOylation (Lazaro et al., 2014), glycation (Vicente Miranda et al., 2017) and phosphorylation (Bras et al., 2018; Tenreiro et al., 2017).

As the development of therapeutic interventions for α-synucleinopathies is a tremendous challenge, recombinant *Saccharomyces cerevisiae* may be used as an excellent screening platform for the development of therapeutic compounds. Hence in the present study, we used recombinant *Saccharomyces cerevisiae,* "SY246" (duly gifted by Dr. Deepak Sharma, Principal Scientist, CSIR-Institute of Microbial Technology, Chandigarh, India) in which induction of insoluble α-synuclein takes place due to oxidative stress. As it has been established that oxidative stress is one of the primary triggers in oligomerization of the α-synuclein to form insoluble aggregates and promote ROS activity (Junn and Mouradian, 2002).

To discover novel therapeutic agents as disaggregators, a simple robust and inexpensive assay should be used for screening a large set of natural extracts or chemical libraries. Therefore, Nitroblue Tetrazolium (NBT) assay was used as a preliminary assay in the present study to identify lead culture filtrates of endophytic fungi isolated from tea and apple which significantly reduce the oxidative stress of the recombinant *Saccharomyces cerevisiae*SY246 under the induced state. NBT initially is a pale-yellow colored and its reduced form appears to be a dark blue insoluble precipitate called diformazan which can be measured spectrophotometrically. To establish the baseline of oxidative stress after α-synuclein induction in the recombinant yeast SY246, NBT assay was carried out under non-induced as well as induced conditions. Piceatannol, a polyphenol was used as a positive control to assess the reduction of oxidative

stress i.e. reduction in ROS (reactive oxygen species) as compared to the test isolates. The best isolate which induced the maximum reduction in ROS generation was #59CSLEAS tentatively identified as *Fusarium* species isolated from Tea (Vats and Saxena, 2023).

Previously NBT assay has been used for screening the Xanthine oxidase (XO) inhibitors from endophytic fungal extracts for developing therapeutic interventions for hyperuricemia and gout (Kapoor and Saxena, 2014). NBT microtitre plate assay has also been used to identify new inhibitors of XO which also reduce oxidative stress from an endophytic fungi *Lasiodiplodia theobromae* (#1048AMSTITYEL) (Kumar et al., 2019). As oxidative stress brings in a structural change in the native protein i.e. converts it into a misfolded or damaged state within the cells and acquiring an abnormal function/action. It becomes imperative to confirm the structural changes in the protein post-oxidative stress (Goswami et al., 2006; Griendling et al., 2016; Hu et al., 2020). Hence there was a need to perform the aggregation kinetics of monomeric α-synuclein. Maximum aggregation of the monomeric α-synuclein was observed at a concentration of 70 µM after 96 hours of incubation at 37 °C at 800 rpm based on the emission spectra of Thioflavin T (ThT). ThT is a benzothiazole dye which specifically stains the cross β- architecture of amyloid fibrils (a strong bathochromic shift from 450 nm to 482 nm) (Hashimoto et al., 1998; Naiki et al., 1989). The enhancement in fluorescence emission happens due to loss of rotational freedom between the benzothiazole and aniline rings (Chen et al., 2021; Estaun-Panzano et al., 2023). Cell free filtrate of #59CSLEAS exhibited main structural changes thereby reducing the intensity of emission spectrum by 1.63 folds as compared to the control. Similar reductions have been observed in the tea polyphenols viz. (-) catechin gallate (CG), (-) epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) which prevented the aggregation of β-amyloid. EGCG induced reduction in α-synuclein by 1.53 folds which was quite close to our fungal extract against α-synuclein. The fluorescence emission in case of CG was quenched by 2.15 folds thereby causing more than 50% inhibition by Aβ aggregation. Catechin gallate has neem found to exhibit a cytoprotective action on human neuroblastoma cell line (SH-SY5Y) thereby maintaining the cell viability by 92% while the non-treated cells (controls) the viability was reduced by 40% (Rho et al., 2019).

Apart from catechins, Tan I and Tan II (Tashinone) inhibited the aggregation of α-synuclein by significantly delaying the time for α-synuclein aggregation with an obvious decrease in the fluorescence intensity (Ji et al., 2016). More recently a synthetic derivative of Resveratrol, AM-12 by Thioflavin T assay has been used to inhibit aggregation α-synuclein monomers but also delays the aggregation of α-synuclein oligomers and fibrils (Chau et al., 2021). ThT fluorescence assay

has also been revealed that Vitamin B12 acts against fibrillogenesis, and cytotoxicity of α-synuclein. It was also found to dissemble the pre-existing mature α-synuclein fibrils and attenuate the consequent toxicity (Jia et al., 2019).

Oxidative stress is the hallmark of an array of neurodegenerative disorders (including Alzheimer's and Parkinson's disease) and metabolic syndrome such as diabetes, obesity, hyperuricemia and cancer. Hence it is necessary to evaluate the quantum of oxidative stress to be ascertained prior to development of a therapeutic intervention/ strategy. There is a variety of indirect methods to assess the oxidative stress such as lipid peroxidation, glutathione reduction and DNA adduct formation (Olowe et al., 2020) to directly assess the oxidative stress quantity. Hence, a fluorescent probe assay using 2',7'-dichloroflourescin (DCFH-DA) was carried out to assess the intracellular concentrations of H_2O_2 (Keston and Brandt, 1965; Wang and Joseph, 1999). The DCFH-DA method relies on the biological conversion of non-fluorescein derivatives, which, following their formation, emit fluorescence upon oxidation by hydrogen peroxide. When applied to intact cells, DCFH-DA penetrates the cell membrane and undergoes enzymatic hydrolysis by intracellular esterase, transforming into a non-fluorescent DCFH. Upon interaction with ROS, DCFH is oxidized, resulting in the production of highly fluorescent di-chloro-fluorescein (DCF). This process enables the comprehensive quantification of oxidative stress levels within the cells (LeBel et al., 1992; Li et al., 2013 a). Resveratrol has been documented for its ability to shield SK-N-BE cells from oxidative stress triggered by α-synuclein or amyloid β. This protective effect is primarily linked to the activation of the SIRT1 gene by resveratrol, which helps mitigate the harmful consequences of oxidative stress or α-synuclein aggregation. The assessment of the reduction in oxidative stress facilitated by resveratrol was conducted through the DCFH-DA assay. The results revealed that resveratrol effectively normalized ROS production to basal levels when compared to cells exposed to hydrogen peroxide for 24 hr (Albani et al., 2009). Our results exhibited 64.6% reduction of oxidative stress in the recombinant α-synuclein model under induced state when treated with a fungal extract of 59CSLEAS as compared to the control. Triclabendazole on the other hand decreased the ROS burden in yeast cells expressing α-synuclein when incubated with ROS sensitive DCFH-DA. Triclabendazole treated yeast cells expressing α-synuclein exhibited a lesser count of cells exhibiting oxidative stress (~ 45%) as compared to the untreated (~ 75%) (Lee et al., 2011). Rotenone has also been found to induce oxidative stress as a result of mitochondrial dysfunction eventually leading to cell death in SK-N-SH cells. Geraniol on the other hand not only reduced the α-synuclein expression in SK-N-SH cells but also induced a neuroprotective effect through reduction of the oxidative stress induced by rotenone as assessed by DCFH-DA assay (Rekha and Inmozhi Sivakamasundari, 2018).

Sandwich ELISA kits from various manufacturers have been predominantly employed to verify and quantify α-synuclein oligomers. This method is specifically designed for oligomer detection, exhibiting minimal cross-reactivity with α-synuclein monomers. The Sandwich ELISA technique can identify oligomers within a broad concentration range, ranging from 0.25 to 20 ng/ml, in cellular and transgenic animal models of α-synucleinopathies (Lassen et al., 2018). Additionally, oligomeric α-synuclein has been successfully identified in various biological fluids, such as saliva (Vivacqua et al., 2016). More recently, the application of sandwich ELISA has extended to the detection of α-synuclein oligomeric formation in urine (Nam et al., 2020). Thus, in the present study we also intended to confirm the presence and absence of the α-synuclein oligomers when treated by cell free filtrate of endophytic fungi to understand its disaggregation potential. In our study cell free filtrate of 59CLSEAS was found to disaggregate oligomeric α-synuclein by 46.5% which was the highest reduction among the potential culture filtrates of the endophytic fungi.

As the cell free culture filtrate of 59CSLEAS isolate from tea was exhibiting the highest degradation potential based on NBT, ThT, DCFH-DA, and Sandwich ELISA, it was further taken up for identification using morphological as well as molecular phylogenetic methods. Morphotaxonomy is the most convenient and reliable way of identifying filamentous sporulating fungi. In the present study the isolate 59CSLEAS, morphologically belonged to the genus *Fusarium* based on spore morphology and microscopy using standard mycological keys, however determining species is a contentious issue and precise taxonomy remain a challenge for the fungal taxonomists (Chandra et al., 2011) and many fungal species have still not been identified to date. Hence a multilocus analysis of genomic DNA of 59CSLEAS was carried out using three loci, namely ITS (internal transcribed spacer), TEF (translation elongation factor 1α) and TUB (β-tubulin). The ITS sequence was chosen because the UNITE community recognized it as a designated "Barcode" region, and it subsequently received official barcode status from The Consortium of Barcode of Life, as documented by Koljalg et al. (2005) and Schoch et al. (2012). Earlier, an endophytic fungal isolate, NFX06, obtained from *Nothapodytes foetida* leaves, was classified as *Fusarium oxysporum* by ITS sequence analysis, as reported by (Musavi and Balakrishnan, 2013). Similarly, *Fusarium verticillioides* and *Fusarium proliferatum* have been differentiated based on the ITS region due to their morphological similarities (Visentin et al., 2009). More recently an endophytic *Fusarium oxysporum* GW has been identified by ITS sequencing which possesses weedicidal as well as biostimulant properties (Asim et al., 2022). The translation elongation factor (TEF) 1 alpha gene is crucial in facilitating the translation process within eukaryotic cells. TEF codes for a vital component of the protein translation machinery and is recognized for its phylogenetic usefulness, particularly in a variety of organisms

such as *Fusarium*. Its high informativeness and the absence of detectable non-orthologous gene copies make it a valuable tool for phylogenetic analysis (Geiser et al., 2004). Barik and Tayung, (2012) have also proposed that TEF 1 α region could serve as a phylogenetic biomarker to distinguish *Fusarium* species, existing as endophytes, pathogens and saprophytes. TUB (β-tubulin) gene has also been used additionally to identify and delineate *Fusarium* species complex as discriminatory power and problems vary with each complex (Torbati et al., 2021). Thus, based on all these loci, morphological and microscopic studies the endophytic isolate 59CSLEAS was confirmed to be *Fusarium oxysporum*. The strain was deposited in the NFCCI (National Fungal Culture Collection of India, Pune) with accession number NFCCI-4191.

Of the variety of polyphenolic compounds present in *Camellia sinensis* (Tea), only a selected few have been evaluated for their α-synuclein disaggregation potential by *in vitro* assays. However, this does not provide the complete picture regarding their α-synuclein disaggregation potential. Hence an *in silico* analysis of the major phytochemicals present in tea was also undertaken in the present study. This would also be helpful in providing an insight of the probable potential compounds which could probably be found in the cell free culture filtrate of *Fusarium oxysporum* (59CSLEAS) which exhibited a potent α-synuclein disaggregating potential in the *in vitro* studies. Discovering compounds that can effectively inhibit the fibrillogenesis of α-synuclein remains a significant challenge. With the vast array of existing compounds, conducting experimental screenings across all available databases within a reasonable timeframe is unfeasible. Virtual screening (VS) is recognized as an effective approach for identifying new bioactive compounds and is currently employed in various drug discovery and development strategies. This method involves swiftly screening the chemical entities in extensive libraries to identify structures with improved ligand-receptor interaction patterns (Vittorio et al., 2020). Docking methods are commonly applied in the majority of virtual screening strategies on large databases. Thus, computational chemistry advancements offer a promising avenue to guide experiments through realistic simulations, suggesting how existing compounds can be manipulated to create potent inhibitors for α-synuclein aggregation at minimal cost. The complex interactions of α-synuclein and phytochemicals were predicted from the molecular docking (Sahihi et al., 2021). Molecular dynamics simulations were carried out on hit compounds complexes to understand the interaction stability under dynamic biological condition. The Theaflavin-3,3-digallate (T33DG), Theaflavin(T) and Theaflavin-3'-gallate (T3G) compounds were found to be strong α-synuclein binders. *In silico* study suggested that there is a possibility of conversion of the α-sheet structure of α-synuclein into a loop, which probably would lead to disaggregation of the pre-formed toxic conformers of α-synuclein as well as restrict their further aggregation (Vats et al., 2022).

In order to isolate the lead molecule exhibiting the α-synuclein disaggregating potential, it was imperative to fractionate the cell free culture filtrate of *Fusarium oxysporum* (59CSLEAS) by liquid-liquid extraction using different solvents viz. hexane, dichloromethane, chloroform, ethyl acetate and aqueous fraction. The crude solvent residue so obtained were again tested for their α-synuclein disaggregating potential by sandwich ELISA method. Crude chloroform extract exhibited highest α-synuclein disaggregation among the different solvent residues tested (Vats and Saxena, 2023). A wide range of bioactive compounds comprising of terpenoids, alkaloids, quinone, steroids, phenols, isocoumarins, phenylpropanoids, lactones and lignans (Radic and Strukelj, 2012). Fusartricin, a sesquiterpenoid ether was isolated from the chloroform fraction of culture filtrate of *Fusarium tricinctum* Salicorn 19 and exhibited antimicrobial activity (Zhang et al., 2015). It has also been found that both chloroform and methanol extracts of *Ginkgo biloba* protected the neuronal cell from β-amyloid insult (Kim et al., 2007). Chloroform extract of *Polyscias fruticose* exhibited amelioration of oxidative stress and decrease in the degeneration of dopaminergic neurons thereby opening avenues of isolation of the bioactive compound (Ly et al., 2022). Polyphenols from Tea have been also extracted by various methods however, a combination of hexane, ethyl acetate, methanol and water was found to recover the polyphenols effectively from tea leaves (Kumar and Rajapaksha, 2005). Phytochemical testing of the crude extracts gives an indication of the nature of the compound based upon which purification and visualization techniques could be effectively designed. Hence phytochemical analysis of the crude residue of *Fusarium oxysporum* (59CSLEAS) was also performed to establish the process of extraction of the lead molecule responsible for disaggregation of α-synuclein. Fusagunolics A and B were isolated as two new phenolic entities from the plant endophytic fungus *Fusarium guttiforme* which exhibited anti-inflammatory and Nitric oxide (NO) inhibitory activity (Yue et al., 2024). *Fusarium* is recognized as a valuable reservoir of bioactive substances, encompassing over a hundred compounds distinguished by their distinctive chemical compositions. These compounds belong to a diverse array of classes, including butenolides, alkaloids, terpenoids, cytochalasins, phenalenones, xanthones, sterols, and derivatives of diphenyl ether, as well as anthraquinones. These compounds exhibit a broad spectrum of bioactivities, demonstrating effectiveness in areas such as antimicrobial, antiviral, anticancer, antioxidant, antiparasitic, and immunomodulatory functions (Ahmed et al., 2023).

The process of isolating a bioactive compound from solvent residues entails employing a range of analytical techniques to separate individual compounds and subsequently identify the one exhibiting the desired bioactivity. Liquid-liquid fractionation is utilized to break down complex mixtures into smaller fractions. These fractions are then carefully chosen based on bioactivity

for additional separation and isolation through techniques such as Thin Layer Chromatography (TLC) and column chromatography. Therefore, employing bioassay-guided fractionation represents the most suitable method to streamline the isolation of bioactive molecules. Bioassay guided isolation has been adopted for isolation of a variety of compounds from fungal endophytes exhibiting different bioactivities such as Anticancer agent from *Fusarium solani* (Abutaha et al., 2020), antimicrobial compounds and equisetin from endophytic *Fusarium* sp., isolated from *Opuntia dillenii* (Ratnaweera et al., 2015), Fusartricin from *Fusarium tricinctum* Salicorn 19 (Zhang et al., 2015) ginsenoside isolated from extract of endophyte *Fusarium* sp. PN8 from *P. notoginseng* (Jin et al., 2017), Cajaninstilbene acid, a natural antioxidant was isolated from extracts of endophytic *Fusarium solani* (ERP-07), *Fusarium oxysporum* (ERP-10), and *F. proliferatum* isolated from *Cajanus cajan* (Zhao et al., 2012). In the present study, TLC fractionation of the chloroform extract was performed. The mobile phase solvents were optimized as chloroform: methanol: ethyl acetate: acetic acid in which 11 bands were observed. Hence this mobile phase was further used for column chromatography which yielded 11 fractions based on TLC profile and further checked for their α-synuclein disaggregating activity. Significant α-synuclein disaggregation activity was observed in fraction 10 which was reconfirmed to be pure chromatographically i.e. by TLC and found to be a single entity.

Structure elucidation of the fraction 10 was performed by using various spectroscopic methods, primarily comprising of LC, ESI-MS, FTIR and NMR (^{1}H and ^{13}C). From the ESI-MS, a molecular mass of the bioactive fraction 10 was found to be 459.120 g/mol which was quite close to the molecular mass of EGCG derived from plants i.e. 458.320 g/mol (Huang et al., 2022 b; Li et al., 2022 a; Yassin et al., 2014). Further the fragmentation pattern was also compared with the EGCG to confirm the tentative structure (Spacil et al., 2010). Green tea phytoconstituents, predominantly catechins including EGCG have been analyzed via NMR analysis (Napolitano et al., 2014). The ^{1}H spectra of EGCG in our study justified the proton characteristics of EGCG in agreement with previous studies (Folch-Cano et al., 2014). On the other hand, ^{13}C-NMR spectra indicated a very high propensity of aromatic carbons ranging in ppm 110-175, whereas the spectral region of ppm 100-150 depicts presence of unsaturated alkene. These chemical shifts of protons and carbon were compared with previously analyzed signals of ECGC and related catechins (Minnelli et al., 2020; Zhong and Shahidi, 2011). FTIR is utilized to clarify the structure of unidentified compositions, revealing the intensity of absorption spectra linked to the molecular composition or the content of specific chemical functional groups (Bobby et al., 2012). Thus, based on NMR, FTIR and mass spectra, the structure of the bioactive fraction was proposed to be epigallocatechin gallate (EGCG). Today a variety of analytical techniques or their

combinations have been developed to recover polyphenolic entities from plants as well as fungi for their prospective applications in food, pharmaceutical, nutraceutical and cosmeceutical sectors (Ajila et al., 2010).

Endophytic *Fusarium oxysporum* has been found to be a bioresource of a host of bioactive compounds with different pharmacological activities. It has also been found to produce the putative phytochemicals of the host in which it resides symbiotically for instance, Podophyllotoxin production by *F. oxysporum* residing in *Juniperus recurva* (Kour et al., 2008; Nguyen et al., 2023), ginkgolide B produced by *F. oxysporum* (isolate SYP0056) (Cui et al., 2012), Vincristine and Vinblastine from endophytic *F. oxysporum* isolated from *Catharanthus roseus* (Kumar et al., 2013).

Apart from the potent α-synuclein disaggregating action of EGCG, it possesses strong anti-oxidant activity (Zhu et al., 2017; Harati et al., 2023). From the present study, the anti-oxidant potential of the fungal EGCG exhibited IC_{50} value of 0.120 ± 0.02 mg/ml which is comparable with IC_{50} value 0.09 mg / ml (Zhu et al., 2014). However, Boulmokh et al. (2021) reported lower IC_{50} of EGCG compound i.e., 0.047 mg/ml. Extensive research investigations on EGCG isolated from tea has demonstrated its noteworthy potential as an antioxidant (Zhu et al., 2014), anti-carcinogenic (Almatroodi et al., 2020; H. Li et al., 2014; Zan et al., 2019), antimicrobial (Nikoo et al., 2018) and neuroprotective (Singh et al., 2015).

In this study the fungal ECEG also exhibited the disaggregation of α-synuclein in dose dependent fashion similar to the tea EGCG, the disaggregation constant, DC_{50} was found to be 1.101 ± 0.04 µM. Caruana et al. (2011) reported an IC_{50} of 0.26 µM for preformed α-synuclein oligomers. However, Jiang et al. (2022) reported an IC_{50} of as 1.06 ± 0.04 µM when EGCG was used as a positive control.

Thus, from the present study we have proved again that endophytic fungi can produce the putative phytochemicals of their host. This is the very first report of a tea polyphenol EGCG (epigallocatechin gallate) being produced by the endophytic *Fusarium oxysporum* isolated from the leaves of *Camellia sinensis* while bioprospecting potential α-synuclein disaggregators from endophytic fungi as a therapeutic disease modifying intervention. The present research work opens avenues for fermentative large-scale production of EGCG in bulk for large scale clinical trials in conjugated and nonconjugated state for Parkinson's disease and other related neurodegenerative disorders.

CHAPTER-7
CONCLUSION

Conclusion

The present study establishes that endophytic fungi are novel sources of oligomeric and fibrillar α-synuclein protein disaggregators. The compounds present in endophytic fungi provide immense possibilities for developing disaggregating compounds with anti-oxidant potential.

1. This is the first systematic screening program exploiting misfolded α-synuclein disaggregator from endophytic fungi using *in vitro* and *in silico* approach combination.
2. This is the first report of isolation of EGCG (FC10), a polyphenol from the endophytic fungus *Fusarium oxysporum* residing in the leaves of *Camellia sinensis* in Assam in Northeastern India.
3. The novel findings in the study reported the concurrently targeted purification of the EGCG compound, having DC_{50} values of 1.101 ± 0.04 μM, which is better than many polyphenols.
4. Hence, we concluded that the cost-effective purification of EGCG from endophytic fungus holds immense applications in the pharma industry as it can open possibilities for strain improvement towards commercial (bulk) production via fermentative pathway, in a cost-effective manner similar to antibiotics production.

CHAPTER-8
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CHAPTER-9
APPENDIX

Table 5.1 Endophytic fungi isolated from various tissues of plants					
S. No.	**Culture code**	**Plant name**	**Plant part**	**Sample location**	**Microscopic identification**
1	1CSLEPL	*Camellia* sp.	Leaf	Palampur	Unidentified
2	3CSLEPL				*Fusarium* sp.
3	4CSLEPL				*Penicillium* sp.
4	5CSLEPL				Unidentified
5	6CSLEPL				*Penicillium* sp.
6	7CSLEPL				Non-sporulating
7	9CSLEPL				*Cladosporium* sp.
8	11CSLEPL				Unidentified
9	12CSLEPL				*Penicillium* sp.
10	19CSLEPL				*Fusarium* sp.
11	20CSLEPL				*Alternaria* sp.
12	21CSLEPL				*Fusarium* sp.
13	22CSLEPL				Non-sporulating
14	36CSLEPL				*Cladosporium* sp.
15	38CSLEPL				*Cladosporium* sp.
16	40CSLEPL				*Fusarium* sp.
17	43CSLEPL				Non-sporulating
18	65CSLEPL				Non-sporulating
19	67CSLEPL				Unidentified
20	68CSLEPL				*Alternaria* sp.
21	78CSLEPL				Unidentified
22	92CSLEPL				*Cladosporium* sp.
23	94CSLEPL				*Cladosporium* sp.
24	96CSLEPL				Non-sporulating
25	99CSLEPL				*Alternaria* sp.
26	10CSLEAS			Assam	*Alternaria* sp.
27	41CSLEAS				*Penicillium* sp.
28	44CSLEAS				*Aspergillus* sp.
29	47CSLEAS				*Penicillium* sp.
30	50CSLEAS				Unidentified
31	59CSLEAS				*Fusarium* sp.
32	70CSLEAS				*Fusarium* sp.
33	117CSLEAS				*Aspergillus* sp.
34	1CSSTAS		Stem		Unidentified
35	2CSSTAS				Unidentified
36	3CSSTAS				Unidentified
37	7CSSTAS				*Penicillium* sp.
38	9CSSTAS				Unidentified
39	12CSSTAS				*Melanconiella* sp.
40	14CSSTAS				*Fusarium* sp.
41	18CSSTAS				*Melanconiella* sp.
42	21CSSTAS				*Botryosphaeria* sp.
43	25CSSTAS				*Fusarium* sp.
44	34CSSTAS				*Chaetomium* sp.
45	35CSSTAS				*Penicillium* sp.
46	36CSSTAS				*Aspergillus* sp.
47	40CSSTAS				*Fusarium* sp.

48	59CSSTAS				*Fusarium* sp.
49	65CSSTAS				*Fusarium* sp.
50	78CSSTAS				*Fusarium* sp.
51	100CSSTAS				Unidentified
52	101CSSTAS				*Penicillium* sp.
53	108CSSTAS				*Aspergillus* sp.
54	3MPLSH	*Malus* sp.	Leaf	Shimla	*Penicillium* sp.
55	4MPLSH				*Aspergillus* sp.
56	11MPLSH				*Xylaria* sp.
57	32MPLSH				*Fusarium* sp.
58	50MPLSH				*Nigrospora* sp.
59	53MPLSH				*Alternaria* sp.
60	62MPLSH				Unidentified
61	67MPLSH				*Collectotrichum* sp.
62	73MPLSH				*Alternaria* sp.
63	77MPLSH				*Epicoccum* sp.
64	82MPLSH				*Alternaria* sp.
65	93MPLSH				*Collectotrichum* sp.
66	96MPLSH				*Lasiodiplodia* sp.
67	98MPLSH				*Botryosphaeria* sp.
68	114MPLSH				*Collectotrichum* sp.
69	117MPLSH				*Collectotrichum* sp.
70	122MPLSH				*Fusarium* sp.
71	139MPLSH				*Alternaria* sp.
72	140MPLSH				*Curvularia* sp.
73	159MPLSH				*Collectotrichum* sp.
74	168MPLSH				*Fusarium* sp.
75	179MPLSH				*Collectotrichum* sp.
76	180MPLSH				*Fusarium* sp.
77	188MPLSH				*Lasiodiplodia* sp.
78	201MPLSH				*Collectotrichum* sp.
79	207MPSSH		Stem		*Collectotrichum* sp.

Table 5.6 The possible hydrogen bonding interactions between alpha-synuclein and hit compounds

Molecule name	Binding energy (kcal/mol)	Residues involved in H-bonding		H-bonding distance in Å
		Residue name	Ligand atoms	
T33DG	-7.1	Ala 89	H_{25}-O_{25}	1.9
		Ala 91	O_{23}	1.89
		Thr 75	H_{46}-O_{45}	1.72
		Thr 72	H_{57}-O_{56}	1.88
		Thr 72	H_{61}-O_{60}	1.84
		Gly 93	O_{1}	1.96
		Lys 80	H_{24}-O_{23}	2.06
		Ala 91	O_{23}	2.15
		Gly 93	O_{1}	2.64
		Phe 94	O_{71}	2.28
		Glu 57	H_{59}-O_{58}	1.63
		Glu 57	H_{61}-O_{60}	1.54
		Glu 57	H_{57}-O_{56}	2.5
Theaflavin	-6.7	His 50	O_{41}	2.2
		Val 48	O_{39}-H_{40}	2.117
		Val 77	O_{43}-O_{44}	1.83
		Gln 79	O_{25}-H_{26}	2.05
		Val 77	0_{49}-H_{50}	1.66
		Glu 46	O_{25}-H_{26}	1.66
		Val 48	O_{23}	2.07
		Lys 45	O_{23}-H_{24}	1.92
		Thr 44	O_{27}-H_{28}	2.09
T3G	-6.4	Val 74	O_{60}	1.99
		Val 70	O_{52}-H_{53}	1.75
		Phe 94	O_{56}	2.12
		Ala 91	O_{25}-H_{26}	1.77
		Ile 88	O_{23}-H_{24}	2.11
		Val 74	O_{60}-H_{61}	1.84
		Ala 76	O_{38}	2.09
		Ala 76	O_{38}-H_{39}	1.93

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ORIGINAL PAPER

Endophytic *Fusarium* species, a unique bioresource for disaggregator of misfolded alpha-synuclein

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Abstract

Aggregation of α-synuclein into toxic oligomeric structures has been implicated in the pathogenesis of Parkinson's disease via several key stages of fibrillation, oligomerization, and aggregation. Disaggregation or prevention of aggregation has garnered a lot of attention as a therapeutic strategy to prevent or delay the progression of Parkinson's disease. It has been recently established that certain polyphenolic compounds and catechins present in plants and tea extracts exhibit the potential to inhibit the α-synuclein aggregation. However, their copious supply for therapeutic development is still unsolved. Herein, we report for the first time the disaggregation potential of α-synuclein by an endophytic fungus residing in tea leaves (*Camellia sinensis*). Briefly, a recombinant yeast expressing α-synuclein was used for pre-screening of 53 endophytic fungi isolated from tea using anti-oxidant activity as a marker for the disaggregation of the protein. One isolate #59CSLEAS exhibited 92.4% reduction in production of the superoxide ions, which were similar to the already established α-synuclein disaggregator, Piceatannol exhibiting 92.8% reduction. Thioflavin T assay further established that #59CSLEAS decreased the oligomerization of α-synuclein by 1.63-fold. Subsequently Dichloro-dihydro-fluorescein diacetate-based fluorescence assay exhibited a reduction in total oxidative stress in the recombinant yeast in the presence of fungal extract, thereby indicating the prevention of oligomerization. Oligomer disaggregation potential of the selected fungal extract was found to be 56.5% as assessed by sandwich ELISA assay. Using morphological as well as molecular methods, the endophytic isolate #59CSLEAS was identified as *Fusarium* sp. The sequence was submitted in the Genbank with accession number ON226971.1.

Keywords Oxidative stress · Endophytic fungi · *Camellia sinensis* · Antioxidants · Parkinson's disease

Introduction

Synucleinopathies refer to a group of disorders associated with pathological aggregate formation of protein α-synuclein which are expressed in neuronal or non-neuronal cells of the brain. Aggregation of α-synuclein (α-S) as Lewy bodies is the hallmark of Parkinson's disease (PD), and dementia with Lewy bodies (DLB) (Goedert et al. 2017; Magalhães and Lashuel 2022). The aggregation of α-S is widely linked to neurotoxic pathways which ultimately lead to neurodegeneration. The pathways which primarily get affected in the Parkinson's disease comprise autophagy (lysosomal), deregulation, synaptic dysfunction, mitochondrial disruption, and oxidative stress (Langston et al. 2015; Bernal-Conde et al. 2020). The therapeutic interventions currently for PD primarily comprise restoration of the dopaminergic tone in the striatum. Levodopa has been the cornerstone of PD therapy since 1960s and continues as a therapeutic intervention in 2022. However, long-term use of Levodopa results in dyskinesis (Pezzoli and Zini 2010; Hansen et al. 2022). Other drugs, which are primarily dopamine receptor agonists and inhibitors of dopamine metabolism, have been reported to be less effective. Hence, there is an urgent need to develop better therapeutic interventions including disease-modifying treatments so as to avert the possible Parkinson's pandemic expected to happen in 2040 when the number of patients suffering from Parkinson's disease would touch approximately 14 million (Dorsey et al. 2018). One of the

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possible therapeutic strategies comprises disaggregation/defibrillation of pathologic forms of α-S (Fields et al. 2019).

Majority of the modern drugs used today have their origin from plants. It is well documented that extracts of plants exhibiting neuroprotective properties and phytochemicals possess the ability to inhibit the aggregation or fibril formation of oligomeric form of α-S (Mazo et al. 2017). They also possess the properties to convert the oligomeric structures of alpha-synuclein into unstructured forms and therefore could provide insight into the development of novel drug candidate for the treatment of PD and related synucleinopathies (Javed et al. 2019). Phytochemicals have also been extensively used as structural templates for development into drugs, and the pharmaceutical industry still relies heavily on them for the drug discovery and development process (Heinrich and Beutler 2013). Different classes of phytochemicals, which possess beneficial activities in ameliorating PD, generally comprise terpenoids, flavonoids, saponins, lignans, and glycosides (Jadiya et al. 2011; Shaltiel-Karyo et al. 2012; Siddique et al. 2013; Berrocal et al. 2014; Inoue et al. 2018). Terpenoids, such as celastrol, geraniol, reynosin, thymoquinone, and ginkgolide A, B, and C, have been found to inhibit α-S aggregation or fibril formation, thereby attenuating toxicity in experimental models. Similarly, polyphenolic compounds have also been found to provide neuroprotection as evidenced in experimental and epidemiological studies (Caruana et al. 2011). The prominent food-based beneficial polyphenols comprise oleuropein (present in olive oil), curcumin, resveratrol (present in wine and grapes), catechins (from black and green tea), astaxanthin, and lycopene. They have been found to inhibit α-S aggregation and fibrillation (Caruna et al. 2012; Takahashi et al. 2015). One of the tea polyphenols, EGCG ((-)-Epigallocatechin-3-Gallate) has been reported to inhibit α-S aggregation and fibrillation in a dose-dependent manner. It has also been reported to disaggregate mature and large α-S fibrils into smaller, non-toxic aggregates (Zhao et al. 2017). Catechins, polyphenols, and theaflavins present in tea possess strong antioxidant activity also. In our previous study, we found the potential sites of interaction between the antioxidant compounds isolated from *Camellia sinensis* (tea) and α-S using computational approach. Molecular docking and simulation studies suggest that these compounds possess the potential of decreasing the binding energy by reducing the alpha-sheet structure of α-S (Vats et al. 2022).

Commercial extraction and isolation of phytochemicals exhibiting promising potential to inhibit α-S aggregation and fibrillation or disaggregate the pre-existing mature pathogenic aggregates into non-toxic is an expensive proposition with issues related to continuous supply. Hence, newer alternatives of mass production of these are being explored. Plant–microbe interactions could possibly play a significant role in mass production of the phytochemicals via fermentation route. Every plant on the earth holds a microbiome within itself. These microbes which resides within the plants without any overt signals of their existence have been referred to as Endophytes (Wilson 1995). These endophytes have evolved with their host plants during the course of evolution and have inherited certain genes which render them to produce the plant phytochemicals in a culture medium independent of their host (Strobel 2003). It is also a well-known fact that nature is the ultimate chemist hence, these endophytic fungi may also possess the ability to produce congeners of known phytochemicals which exhibit better disaggregation properties than their parent compounds.

Till date, production of α-S aggregation inhibitors or disaggregators has not been reported from endophytic fungi, and the research is quite nascent in this area. Hence, in the present study, we for the first time report the screening of fungal endophytes of *Camellia sinensis* for their oligomeric α-S disaggregation potential using a recombinant α-S expressing yeast strain. Further, the potential endophytic fungus was identified and the lead fraction was isolated from the culture broth for further characterization of the entity responsible for α-synuclein disaggregation.

Materials and methods

Isolation of endophytic fungi

Asymptomatic samples of *Camellia sinensis* were collected from Assam (27°31′10″N 95°53′18″E) and Palampur (32.109722°N 76.536641°E). The collected samples were placed in sterile zip bags and stored at 4 °C until further use. For endophytic fungal culture isolation, the fresh and disease-free samples were washed under running tap water, air-dried, and surface sterilization was done with minimal modifications (Kapoor and Saxena 2014). Imprints of disinfected plants samples were taken on Potato Dextrose Agar (PDA) plates to validate the sterilization efficiency. Subsequently, 3 mm pieces sterilized plant samples were inoculated onto half-strength PDA plates followed by incubation at 26 ± 2 °C for 8–10 days with 12-h light-and-dark period. The plates were frequently monitored for mycelial growth which were then picked with a sterile fine needle and transferred onto fresh full-strength PDA plate.

After formation of a 7-day-old colony as a pure culture, it was transferred to PDA slants. Each isolate so obtained was encoded based on the plant name, its part, and the place of collection.

Production of culture filtrates

Briefly, 30 ml pre-sterilized Potato Dextrose Broth (PDB) was inoculated with a 5 mm mycelial plug of 7-day-old culture

followed by incubation at 120 rpm, 26 ± 2 °C for 8–10 days with 12-h light-and-dark period. After culmination of the incubation period, the mycelial mass was separated using "Whatman filter paper no. 4" (Sigma Aldrich, USA; Z240567) followed by centrifugation at 10,000 rpm for 10 min at room temperature. The cell-free supernatant was stored at − 20 °C until further use (Kapoor and Saxena 2016).

α-S induction in yeast strain

Recombinant yeast strain of *Saccharomyces cerevisiae* having genotype Mat a, D5A2, lys-, ssa1::Kan, ssa2::HIS3, ssa3::syn-GFP-TRP1, ssa4::syn-GFP-URA3/pRS317-P¬SSA1-SSA1 expressing α-S is a generous gift by Dr. Deepak Sharma, Principal Scientist, CSIR-IMTECH, Chandigarh, India. Yeast cells expressing α-S variants were grown in the Yeast Extract Peptone Dextrose (YEPD) medium until mid- logarithmic phase at 30 °C. The cells were pelleted down, washed thrice using deionized water, and replaced with YPGR medium composed of Yeast extract, Peptone, 2% Galactose, and 2% Raffinose. Further, the inoculated YPGR broth was incubated at 30 °C for 8 h for protein induction. The protein expression was confirmed by GFP quenching under Fluorescence spectrophotometer (Perkin Elmer).

Preliminary screening for α-S disaggregation

Nitroblue tetrazolium (NBT) assay

To assess the α-S disintegration potential of the culture filtrates, NBT method was used as a preliminary screen. The method was based upon the reduction of NBT by ROS, which are produced by yeast during formation of α-S (Javvaji et al. 2020). The NBT is reduced to blue-colored formazan produced due to interaction with superoxide radicals generated during formation of protein. The reduction in the intensity of the blue-colored product indicated lower oxidative stress. Briefly, 25 µl of culture filtrate was incubated with 150 µl of protein expressing media for 18 h at 30 °C, 600 rpm. After completion of the incubation period, 20 µl of NBT (0.5 mg/ml) was added, and the microtiter plates were incubated at 30 °C for 2 h. Further, the amount of formazan produced in the reaction was estimated by measuring the absorbance at 575 nm. The negative wells comprised all reagents except culture filtrates. Piceatannol was used as positive control. Each test was carried out in triplicate, and the data are represented as mean ± SD values. The percentage reduction in superoxide anions is calculated using the formula:

$$[(A - B) - (C - D)]/(A - B) \times 100$$

where A is OD at 575 nm with protein but without sample, B is OD at 575 nm without protein and sample, C is OD AT 575 nm with protein and sample, and D is OD at 575 nm with sample but without protein.

Thioflavin T (ThT) analysis

ThT is a fluorescence dye that has been used for staining cross-β-architecture of amyloid fibrils which leads to change in the spectral characteristics after binding and exhibits strong fluorescence emission. Briefly, 70 µM monomeric α-S (Sigma-Aldrich) and 1 mM ThT dissolved in PBS were used to understand the protein aggregation kinetics. The oligomerization of the monomeric protein was achieved under shaking conditions at 800 rpm at 37 °C by incubating it for 96 h in the absence (control) and the presence of 100 µg/ml samples (disaggregators). Absorbance of the cross-β-architecture was recorded at an intensity 487.5 nm (Wordehoff and Hoyer 2018). Each test was carried out in triplicate and the data are represented as mean ± SD values.

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay

The DCFH-DA assay can provide reliable measurements of ROS levels in the cells. In the presence of ROS, DCFH-DA is rapidly oxidized to 2′,7′-di-chloro-fluorescein, which is highly fluorescent with excitation and emission wavelength at 498 and 519 nm respectively. To perform the DCFH-DA assay, induction of α-S in yeast was done. Briefly, 25 µM of DCFH-DA was used to determine the ROS levels in the strain for quantitative estimation. The dye was incubated with the control, hydrogen peroxide (H_2O_2), and sample for 30 min at 37 °C. The fluorescence was recorded using fluorescent spectrophotometer (Esteves et al. 2009).

Isolation of bioactive fraction

To isolate the bioactive fraction, liquid–liquid extraction was performed using different solvents in order of their increasing polarity. Briefly, hexane, dichloromethane, chloroform, and ethyl acetate were sequentially used to extract the cell-free filtrate in a ratio of 1:2. After three rounds of extraction, the organic layer was pooled and dehydrated using anhydrous ammonium sulfate. Further, the solvent was removed using rotavaporator. The solvent residue so obtained was weighed and reconstituted in methanol and stored at 4 °C till further use.

Sandwich ELISA

Human oligomeric α-S sandwich ELISA kit was purchased from MyBioSource, USA (Cat. No. MBS730762) for ascertaining the oligomeric α-S. Reagents, standard, and sample were prepared as per manufacturer's instructions. Briefly,

the oligomerization of the monomeric protein was achieved under shaking conditions at 800 rpm, 37 °C by incubating it for 96 h in the absence (control) and the presence of 50 µg/ml samples (fungal extracts). The control and the treated oligomeric protein samples were added in the primary antibody coated wells followed by incubation with conjugate solution and washed using 1X wash buffer. The plate was blot dried and substrates A and B were added followed by incubation. The product of the reaction results into a colored complex after incubation period of 30 min. After the incubation period, stop solution was added. The blue-colored complex turns into yellow and absorption recorded at 450 nm (Pagan et al. 2019).

Identification of selected endophytic fungus

The selected endophytic fungus was grown over 6 different growth media viz. Potato dextrose agar (PDA), Corn leaf agar (CLA), Pine leaf agar (PLA), Saboraud dextrose agar (SDA), Water agar (WA), and Cooke Rose Bengal agar (CRBA) to assess their sporulation potential. The plates were incubated at 26 ± 2 °C for 5–7 days under 12 h light/dark cycles. The morphological properties, such as growth rate, colony color, colony diameter, texture pigmentation, and any odor, were observed to identify the fungus.

Microscopic identification

Mycelial mass was picked with a fine tip needle and placed over a clean glass slide containing a drop of water for examination of microscopic characteristics. The mycelial mass was properly teased with the help of a pair of sharp sterile needles. The mounting of the culture was done in lactophenol cotton blue and observed under microscope (Nikon Eclipse ε100). Micrometry of the structures present was done using Image J software, with at least 50 observations per structure.

Molecular identification

For the genomic DNA isolation, 0.2 g fresh mycelia of 3–4 day-old fungal culture were scrapped and subsequently crushed using liquid nitrogen in pestle and mortar. The powdered mycelium was then transferred into the Eppendorf. DNA isolation was done as per manufacturer's instruction with the help of Quick-DNA™ Fungal/Bacterial Miniprep Kit (Cat. No.: D6005) and stored at − 20 °C until further use. Briefly, 25 µl of reaction mixture contains 20 ng extracted DNA, 10 µM ITS1 and ITS4 primers, 2.5 mM dNTP, and 1.5 U of Taq DNA polymerase in 10 X Taq buffer. For PCR amplification, thermal cycling parameters were optimized as initial denaturation at 96 °C for 5 min followed by 39 cycles of 95 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s followed by final extension at 72 °C for 5 min. The amplification of DNA was confirmed via 1.5% agarose gel electrophoresis (White et al. 1990). The amplified PCR product was sent to SolResLab, Haryana for PCR product purification and sequencing. The chromatograms obtained after sequencing were checked for their purity (≥ 95%) and assembled using Sequencher version 5.4.6. The final consensus sequence submitted to GenBank BLAST against the NCBI maintained database was performed to establish the homology with closely related species. The ITS sequences were aligned with the selected species showing maximum similarity using ClustalW option in MEGA X (Kumar et al. 2018). The alignment file involved 23 sequences including 1 sequence to be identified and *Aspergillus niger* sequence was used as an outgroup. Distance-based analysis of the ITS region alignment was done by Maximum Likelihood method using Kimura-2-parameter model (Kimura 1980). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. To infer the consensus tree, 1000 bootstrap replicates were taken into consideration for the representation of evolutionary relationship.

Statistical analysis

For statistical analysis, GraphPad Prism was used. Unpaired student's t test was used for measuring significance within groups. Three or more groups were compared by One-way ANOVA analysis, followed by Tukey's post hoc test. All tests were tailed with 95% confidence interval. Values of $p < 0.05$ were considered significant.

Results

A total of 53 endophytic fungi has been isolated from stem and leaves of *Camellia sinensis*. Based on preliminary morpho-taxonomic attributes, the most prevalent endophytic genus was *Fusarium* (22.6%), followed by *Penicillium* (15.1%), *Alternaria* and *Cladosporium* (9.4%), *Aspergillus* (7.6%), *Melanconiella* (3.8%), and *Botryosphaeria* and *Chaetomium* (1.9%) (Supplementary data 1).

Preliminary screening for the α-S disaggregation of the endophytic isolates was based on the NBT assay. Out of the 53 endophytic isolates, the culture filtrates of fungi exhibiting an inhibition of 70% or higher were considered for further studies. Oxidative stress was considered as an indicator of expression of α-S by the recombinant strain, whereas a reduction in oxidative stress was suggestive of either inhibition of the protein expression or its disintegration. The isolate #59CSLEAS exhibited reduction in oxidative stress by 92.4% which was quite near the positive control Piceatannol

exhibiting 92.8% reduction of superoxide ion formation by NBT assay. The other potential isolates identified in this assay after #59CSLEAS were #9CSSTAS and #108CSSTAS which reduced the oxidative stress by 90.63% and 90.12% respectively (Table 1).

ThT analysis

This assay was used to assess the potential of the fungal culture filtrate to convert the 96-h-old oligomeric protein into α-sheets which was assessed spectrophotometrically. The maximum emission given by the oligomerized protein was 978.37 AU at a wavelength of 487.5 nm. However, a decrease in the emission from 978.37 AU at a wavelength of 487.5 nm was considered as due to disaggregation of the oligomerized protein as explained previously. The culture filtrate of #59CSLEAS, #9CSSTAS and #108CSSTAS induced a decrease in the emission spectrum by 1.63-, 1.59-, and 1.53-fold respectively (Fig. 1). Thus, #59CSLEAS appears to be a promising candidate as a producer of entities which carry out disaggregation or de-fibrillation of pathological forms of α-S.

DCFH-DA assay

The generation of ROS is related to oxidative effect which may be opined to be enhanced during the production/misfolding of α-synuclein. Hence, by DCFH-DA assay, it could be ascertained that decrease in the oxidative stress in the recombinant strain may be due to disaggregation of α-S. Based on the ThT analysis, culture filtrates of all 3 samples were further subjected to DCFH-DA assay and #59CSLEAS exhibited the best disaggregation potential. It was found that culture filtrate of #59CSLEAS was exhibiting a reduction in ROS generation i.e., oxidative stress by 64.6%, whereas 9CSSTAS and 108CSSTAS reduced the stress by 40.22 and 35.75% respectively. The activity of #59CSLEAS is presumably due to disaggregation of the α-S as compared to the control where no disaggregator was used (Fig. 2). Hence, the sample was selected for further evaluation.

Table 1 Fungal isolates exhibiting higher than 70% reduction in oxidative stress

S.No	Treatment	% Reduction in oxidative stress*
2	Piceatannol	92.9 ± 0^{a}
3	#59CSLEAS	92.4 ± 0^{a}
4	#9CSSTAS	90.6 ± 0.01^{a}
5	#108 CSSTAS	90.1 ± 0.01^{a}
6	#10 CSLEAS	86.9 ± 0.02^{ab}
7	#50 CSLEAS	84.2 ± 0^{ab}
8	#101 CSSTAS	75.1 ± 0.19^{b}

*The data presented are mean ± standard deviation of three replicates. Means with different superscript letters are calculated by Tukey's post host test (p˂0.05)

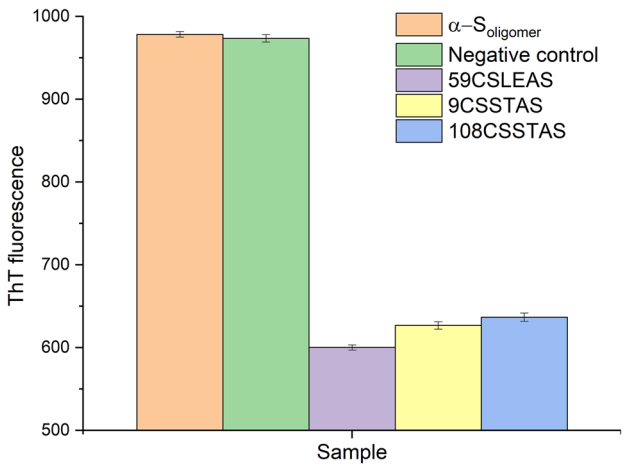

Fig. 1 ThT assay representing shift in fluorescence intensity at wavelength 487.5 nm of oligomeric alpha-synuclein before and after treated with Negative control, endophytic fungal filtrates of #59CSLEAS, #9CSSTAS, #108CSSTAS

Sandwich ELISA

In the Sandwich ELISA assay, each sample was treated in different wells and the results were compared with the standard to determine concentration of α-$S_{oligomers}$. It was found that out of the four solvent fractions, chloroform fraction exhibited highest disaggregation potential i.e., 56.5% followed by ethyl acetate with 46%. On the contrary, dichloromethane and hexane fractions showed very little to no disaggregation capabilities i.e., 20.54% and

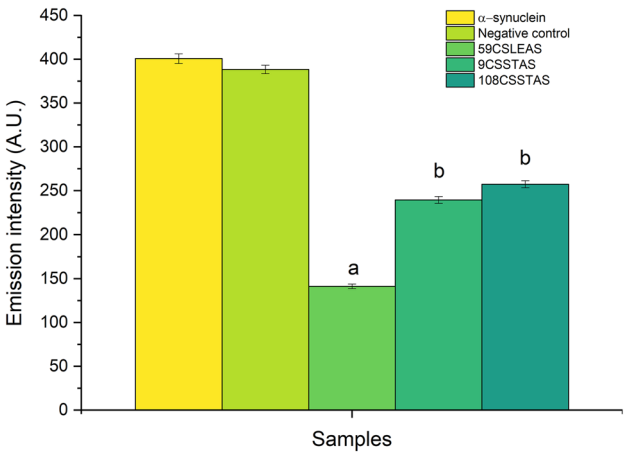

Fig. 2 Dcfh-da analysis of alpha-synuclein before and after treating with disaggregator; a & b are analyzed as significant letters by Tukey's post-hoc test ($p < 0.05$)

8.32% respectively (Fig. 3). Methanol was used as negative control to analyze the study more precisely, where no decrement in the oligomeric protein concentration was observed.

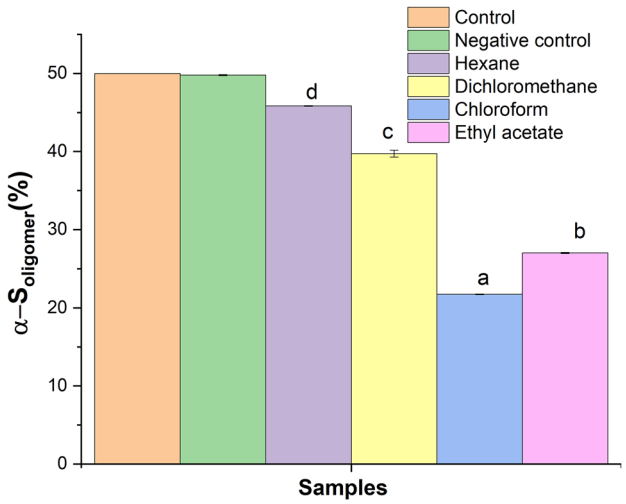

Fig. 3 Effect of different solvent fraction of #59CSLEAS on disaggregation effect of oligomeric alpha-synuclein using Sandwich ELISA kit; a, b, c and b are analyzed as significant letters by Tukey's post hoc test ($p < 0.05$)

Identification of the endophytic fungi

The selected isolate #59CSLEAS was identified based on both classical morpho-taxonomy and molecular taxonomy tools.

Morphological characteristics

Identification of the α-S disaggregator producing endophytic fungus #59CSLEAS was carried out on the basis of specific reproductive and morphological features. A whitish colony was observed when the fungus was grown on SNA and CMA, while a white colony was initially observed on PDA which turned into peach color as the colony grew. On CRBA, the colony color was violet. PLA and WA did not promote the fungal growth (Fig. 4). The colony diameter of #59CSLEAS on PDA was 5.5 ± 0.5 cm on the fifth day. Macroconidia are curved and thick having an average length of 38.1 ± 4.2 µm and average width 38.1 ± 4.2 µm and 7.2 ± 0.3 µm average width. Macroconidia were septate exhibiting 2–3 septa.

Molecular phylogenetic identification

The ITS sequence of #59CSLEAS aligned with the type and non-type sequences to establish the phylogenetic placement. The isolate #59CSLEAS clearly aligned with *Fusarium*

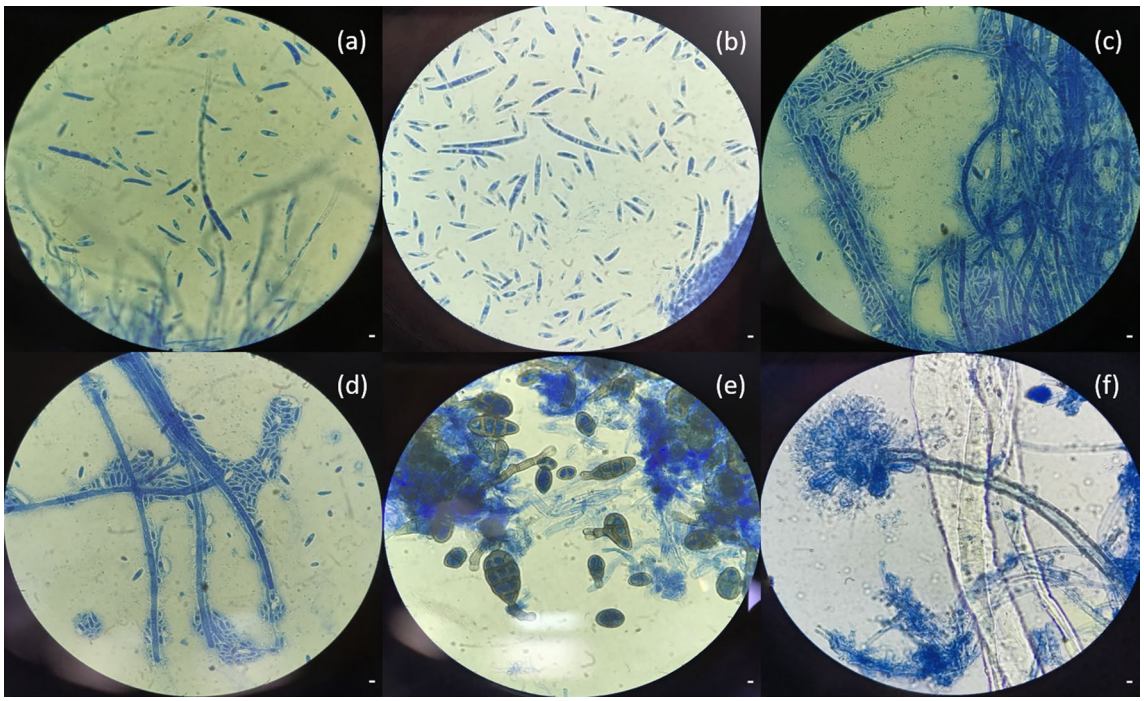

Fig. 4 Endophytic fungal macroconidia **a** #59CSLEAS grown on SNA, **b** #59CSLEAS grown on CMA, **c** #59CSLEAS grown on PDA, **d** #59CSLEAS grown on CRBA, **e** 9CSSTAS and **f** 108CSSTAS on PDA media. (Bar: 10 mm)

oxysporum strain (MZ156849.1) and was clearly demarcated from other species of *Fusarium* (Fig. 5) (Table 3, Supplementary File). Thus, #59CSLEAS is designated as *Fusarium* sp. This strain has been deposited at NFCCI (National Fungal Culture Collection of India, Pune) with accession number NFCCI-4191 and its rDNA in GenBank with accession number ON226971.1.

Discussion and conclusion

Over the past two decades several genetic, biochemical and animal studies have converged and strongly suggest the fundamental role of a 140 residue pre-synaptic α-S protein in the role of pathogenesis of Parkinson's disease (PD) and related disorders. These disorders collectively have been referred as "α- Synucleinopathies" (Fink 2006; Lees et al. 2009).

Parkinson's disease is tightly linked to the propensity of α-S to aggregate and form fibrils (Lundvig et al. 2005). Generally, monomeric α-S gets self-associated to form soluble α-S oligomers which eventually get converted into α-S fibrils. α-S Monomers are disordered structures, whereas oligomers and the fibrils are more aggregated structures which comprise α-sheet aggregates (Alam et al. 2019). Among these conformers, the major toxic agent in PD is oligomeric α-S and α-S fibrils, which serve as a reservoir of toxic oligomeric forms (Winner et al. 2011; Celej et al. 2012). However, α-S monomers are non-toxic in nature. Thus, α-S aggregation appears to be a potential and attractive target for developing therapeutic interventions (Brundin et al. 2017; Fields et al. 2019). Off late several compounds have been found to be protective against α-S fibrillation which primarily belong to the polyphenolic group (El Gaamouch et al. 2021; Chau et al. 2021). Polyphenols are important constituents of human diet and medicinal plants (Caruana et al. 2016; da Costa et al. 2017).

Since times immemorial, nature has provided novel chemical scaffolds which have been developed into therapeutic agents beginning from compounds like morphine and acetylsalicylic acid which served as pain killers (Najimi et al. 2022). Currently, more than 80% of modern drugs have their origin from plants directly or indirectly. *Camellia sinensis*, popularly known as tea, is a native of Southeast Asia and Asia, which is consumed as a beverage due to its refreshing properties primarily attributed to the presence of polyphenols, flavonoids, and catechins present in the leaf decoction. Tea polyphenols have also been found to possess neuroprotective and neurorestorative effects as evident from animal models of PD (Caruana and Vassallo 2015). Further, black tea extract has also been evaluated to possess strong activity toward antagonizing the aggregation of α-S into toxic oligomeric conformers (Ono et al. 2020).

In our previous in silico studies based on different antioxidant/polyphenolic compounds present in *Camellia sinensis*, we proved that there was a possibility of conversion of the α-sheet structure of α-S into a loop, which probably would lead to disaggregation of the pre-formed toxic conformers of α- S as well as restrict their further aggregation (Vats et al. 2022). Thus, the present study was oriented to explore the endophytic fungi present in *Camellia sinensis* for screening their α-S disaggregation potential. Endophytic fungi

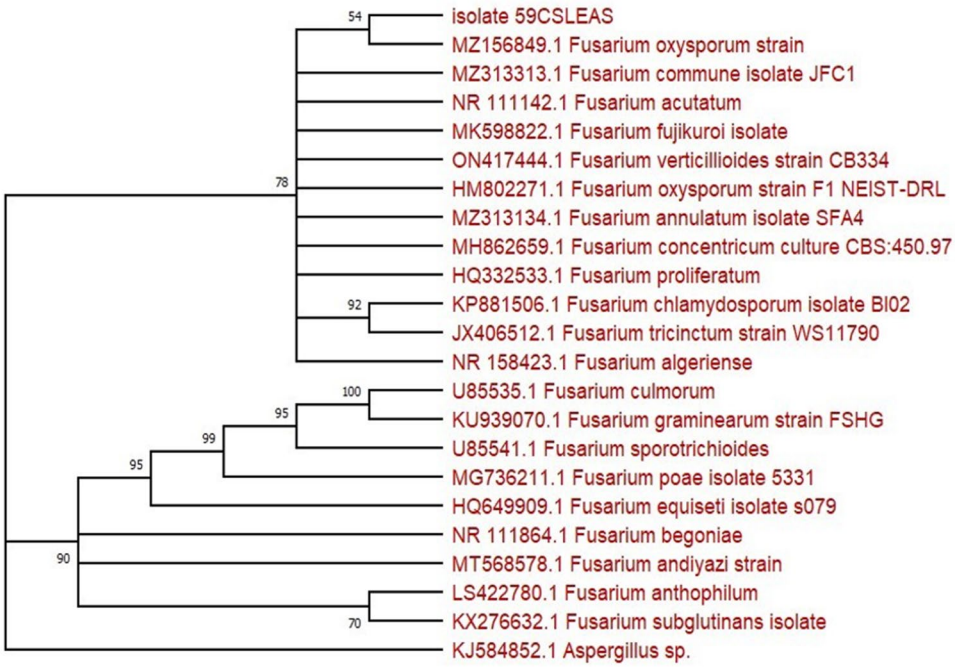

Fig. 5 Maximum likelihood tree based on ITS1-5.8 s-ITS4 representing molecular taxonomy and phylogenetic analysis of culture "#59CSLEAS" (bootstrap test: 1000 replicates)

constitute those microorganisms which inhabit with the core of the plants without exhibiting any overt symptoms. These exist symbiotically with their host plant at least for a part of their life cycle, co-evolving with them and therefore exhibit a continuum in physiological states, colonization pattern and secondary metabolism (Schulz and Boyle 2005). These symbionts reportedly acquire the ability to produce putative phytochemicals of their host plant possibly during the process of co-evolution with the host (Venugopalan and Srivastava 2015; Bielecka et al. 2022).

Despite plants being extensive sources of phytochemicals exhibiting α-S disaggregating properties, their copious supply is a matter of concern. Hence, endophytic fungi offer opportunities to produce these phytochemicals via fermentation route. Therefore, we undertook the very first study of exploring the endophytic fungi from *Camellia sinensis* as a source of α-S disaggregating compounds. In the present study, ~ 53 endophytic fungal isolates reside in the healthy tissues of *C. sinensis* that were isolated. The dominant fungi present in these isolates were found to be *Fusarium* sp. Previously, 143 endophytic fungi have been reported from *Camellia sinensis* with *Fusarium* sp. exhibiting the highest frequency of occurrence (Xie et al. 2020).

Screening platform used for α-S disaggregator was a recombinant *Saccharomyces cerevisiae*, expressing α-S under galactose promoter (gifted by Dr. Deepak Sharma, IMTech Chandigarh). Oxidative stress is one of the factors responsible for protein aggregation in mammalian systems. Hence, oxidative stress of *S. cerevisiae* under induced and non-induced condition could be considered as an initial marker for screening of α-S disaggregation potential of the fungal extracts (Witt and Flower 2006). NBT assay was used to determine the production of superoxide radicals generated in the presence and absence of the fungal extract under α-S induced state. The standard used for the preliminary assay was the grape polyphenol Piceatannol. A reduction of superoxide radical production by 92.8% was observed when Piceatannol, which was used. In the pre-screen, it was found that some of endophytic isolates, viz. #59CSLEAS, #9CSSTAS, and #108CSSTAS exhibited nearly the same potential as Piceatannol. NBT assay is a preferred method of estimating the superoxide ion to reduce p-Nitroblue tetrazolium (NBT) (1,1′, − 3,3′-dimethoxy(1,1′-biphenyl)-bis-[2-(4-nitrophenyl)-methyl-2H-tetrazolium dichloride) to formazan at pH 7.4.

Generally oxidative stress can result in the accumulation of misfolded and damaged proteins in the cells (Goswami et al. 2006; Griendling et al. 2016; Hu et al. 2020). ThT assay is carried out to confirm the structural change in the protein after reduction with superoxide radicals. Thioflavin T is a fluorescent dye which interacts with fibrils having α-sheet structure and exhibits enhanced fluorescence. This enhanced fluorescence is thought to be due to the loss of rotational freedom between the benzothiazole and aniline rings (Chen et al. 2015; Estaun-Panzao et al. 2023).

Culture #59CSLEAS induced maximum structural changes as evident by the reduction of the emission spectrum by -1.63-fold as compared to the control in the formation of α-sheets. Similar reduction has been observed for some tea polyphenols viz. (−)-catechin gallate (CG), (−) epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG) which prevented the aggregation of β-amyloid. (−) Epigallocatechin gallate induced reduction in α-aggregation by − 1.59-fold which was quite close to our fungal extract against α-S. The best activity was exhibited by CG which reduced the fluorescence emission by − 2.15-fold suggesting that it is causing more than 50% inhibition of Aβ-aggregation. It has also been reported that CG exhibited a cytoprotective action on SH-SY5Y human neuroblastoma cells, thereby maintaining a cell viability of 92% while the non-treated cells i.e., controls exhibited Aβ toxicity with a viability of 40% (Rho et al. 2019). Thus, one can infer from our results that #59CSLEAS holds a promising compound as compared to the three pure polyphenolic entities.

Oxidative stress is the hallmark of various degenerative diseases including Alzheimer's, atherosclerosis, cancer, and PD. Hence, it becomes pertinent to estimate the oxidative stress, the mechanisms involved to generate the oxidative stress for developing a therapeutic strategy. There are several indirect methods to assess the oxidative stress, such as lipid peroxidation, glutathione reduction, and DNA adduct formation. However, none of them provides a direct estimation. 2′,7′-dichloroflourescin (DCFH-DA) is a dye used as a probe in fluorometric assay to assess the intracellular oxidative stress by evaluating the formation of intracellular H_2O_2 (Keston and Brandt 1965; Wang and Joseph 1999). The principle of using DCFH-DA is that non-fluorescein derivatives so formed by its biological conversion will emit fluorescence subsequent to oxidation by hydrogen peroxide. On intact cells, DCFH-DA permeates inside where it is enzymatically hydrolyzed by intracellular esterase to a non-fluorescent DCFH which on interaction with ROS is oxidized into highly fluorescent di-chloro-fluorescein (DCF). This helps in overall quantification of oxidative stress in the cells (LeBel et al. 1992).

Resveratrol has been reported to protect SK-N-BE cells from oxidative stress induced by α-S or amyloid β. This is largely attributed to the activation of SIRT1 gene by resveratrol to prevent deleterious effects due to oxidative stress or α-S aggregation. The estimation of reduction in oxidative stress by resveratrol was estimated by DCFH-DA assay. It was found that resveratrol could revert the ROS production to basal level as compared to cells exposed to hydrogen peroxide for 24 h (Albani et al. 2009). Our results also depict a similar trend with respect to oxidative stress on the recombinant α-S yeast model in the present

of fungal extract of #59CSLEAS. A significant reduction of oxidative stress was observed in fungal extract treated α-S yeast model as compared to the untreated. Similarly, another molecule—triclabendazole was found to decrease the ROS burden in the yeast cells expressing α-S when incubated with ROS-sensitive DCFH-DA. It was found that yeast cells expressing α-S when treated with triclabendazole, only ~ 45% of the cells exhibited oxidative stress while those untreated exhibited a higher oxidative stress to the tune of ~ 75% (Lee et al. 2011).

Sandwich ELISA kits from different manufacturers have largely been used for confirmation and quantification of $\alpha\text{-S}_{oligomers}$. This happens to be a specific method for the detection of oligomers since it has a low cross-reactivity for $\alpha\text{-S}_{monomers}$. Sandwich ELISA can detect oligomers in a wide range i.e., 0.25 − 20 ng/ml in cellular and transgenic animal models of α-synucleinopathies (Lassen et al. 2018). Oligomeric α-S has also been detected by sandwich ELISA in different biological fluids including saliva (Vivacqua et al. 2016). More recently, sandwich ELISA has also been used for the detection of oligomeric formation of α-S in urine (Nam et al. 2020). Hence, all the assays viz. NBT, ThT, DCFH-DA, and Sandwich ELISA confirm that the selected endophytic fungus #59CSLEAS produces bioactive compounds which possess the ability to disaggregate oligomeric α-S.

The morphological (culture plate appearance, colony size, coloration, and colony appearance) as well as microscopic attributes (spore structures as their measurements) correlate with *Fusarium* species using standard mycological keys. However, ITS (internal transcribed spacer) region was further amplified to ascertain or confirm its identity through morphological/microbiological methods. ITS sequence was selected since it has been designated as a "Barcode" region by the UNITE community followed by the acceptance from The Consortium of Barcode of Life as the official barcode (Köljalg et al. 2005; Schoch et al. 2012). Previously, an endophytic fungal isolate NFX06 from the leaves of *Nothapodytes foetida* leaf was identified as *Fusarium oxysporum* by ITS sequence analysis (Musavi and Balakrishnan 2013). *Fusarium verticillioides* and *Fusarium proliferatum* have also been distinguished on the basis of ITS region since they exhibit a close similarity morphologically (Visentin et al. 2009).

Based on all above studies, it can be inferred that *Fusarium* species #59CSLEAS appears to be lead endophyte which would provide a molecule that could be developed into a therapeutic entity for PD subsequent to purification of the compound, determination of IC_{50}, and disaggregation kinetics of α-S.

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Author contributions SS conceived the research. SV performed the research and acquired the data. Both authors analyzed and interpreted the data and were involved in drafting the manuscript.

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Declarations

Conflict of interest The authors have no financial or non-financial interest to disclose.

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Consent to participate All authors agree on what is described about their participation in this work.

Consent to publication All authors agree on the submission of the manuscript.

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Identification of alpha-Synuclein Disaggregator from *Camellia sp.* Insight of Molecular Docking and Molecular Dynamics Simulations

Sheetal Vats,[b] Rajesh Kondabala,[c] and Sanjai Saxena*[a]

Misfolded protein formation and aggregation are the central hallmarks for various neurodegenerative disorders. When it comes to Parkinson's disease (PD), alpha-synuclein (α-syn) is the culprit protein. The presence of α-syn protein in lewy bodies and lewy neurites confirmed its presence in the occurrence of PD. The protein is natively present in the soluble monomeric forms, but certain factors such as oxidative stress convert the structure into insoluble oligomeric formats. This study focuses on the inhibitory effects of various antioxidant compounds on α-syn oligomerization. We had collected the list of compounds present in the *Camellia sp.* plant. Using a computational approach, we found the potential interaction sites between the antioxidant compounds and α-syn using a computational approach. Molecular docking and simulation studies suggest that the compound Theaflavin-3-3-digallate shows best interactions with −7.1 kcal/mol and can reduce the alpha-sheet structure of α-syn structure to loop region.

Introduction

Parkinson's disease (PD) is considered the 2nd most common neurodegenerative disorder after Alzheimer's disease. More than 10 million people are living with PD worldwide. The occurrence of the disease increases with age, but an estimated 4% of PD patients are under 50. The loss of dopaminergic neurons in the substantia nigra pars compacta region of the brain has been determined as the pathological characteristics of the disease.[1] PD results in a series of motor and non-motor symptoms, including rigidity, bradykinesia, resting tremor and sleep disturbance, dementia, constipation, and many others.[2] Lewy bodies' intracytoplasmic inclusion of alpha-synuclein (α-syn) protein is considered the pathological hallmark of PD.[3] α-syn is a pre-synaptic intracellular protein having 14 KDa molecular weight consisting of 143 amino acids.[4] It is a small acidic protein consisting of three main domains; 1) N-terminal domain (1–87 AA) consist of seven series of 11 AA which helps in maintaining protein helical structure due to protein-lipid interaction, 2) NAC region (61–95 AA) mainly studied its role in fibril formation and aggregation of the protein, 3) C-terminal domain (96–140 AA) responsible for maintaining the helical structure and inhibiting protein aggregation.[5] The role of α-syn protein in normal cell functioning is dopamine transmission,[6] maintenance of synaptic plasticity for effective neurons communication,[7] ER-Golgi trafficking, and protection of dopaminergic cells against apoptosis.[8] α-syn protein usually is a natively unfolded protein and does not possess any defined structure in the aqueous solution. However, specific aberrations have been observed in the protein structure due to certain pathological conditions that may convert natively unfolded monomers into post-translationally modified oligomeric forms. Certain factors are responsible for causing these pathological structural changes in the protein, such as post-translational modifications,[9] mutations in the SNCA gene,[10] and oxidative stress. In PD patients, abnormal complex I activity of electron transport chain has been observed,[11] which may subject cells to oxidative stress and energy failure due to ROS production and formation of hydroxyl radicals.[12] On the other hand, Dopaminergic neurons (DA) metabolism leads to hydrogen peroxide and superoxide radical formation, which ultimately results in ROS generation.[13] With age, various chaperones cannot induce polyubiquitination, causing the accumulation of such misfolded proteins and eventually forming lewy bodies. Abnormally oxidized tissue content of misfolded proteins increased with age.[14]

The available drugs for PD are chemically active converted into dopamine or dopamine agonists in the brain that may inhibit dopamine metabolism. Patients using these medications have several side effects, such as nausea and headache. Hence, there is a great demand for natural novel compounds to inhibit and disaggregate α-syn oligomers. Antioxidants, acting as free radical scavengers, have been suggested to prevent or may reduce the progression rate of PD by decreasing the rate of oligomerization of α-syn protein.[15,16] To date, abundant studies

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have reported antioxidants and their mechanism for destabilizing formation of α-syn oligomers. A study suggested the role of antioxidants in α-syn protein disaggregation by acting on the protein's NAC region and preventing the formation of amyloid-like fibrils.[17] The effect of other flavonoids, rifampicin, and baicalin, is studied, which after binding with quinone, covalently modify the protein molecules and disaggregate existing fibrils by stabilizing a soluble oligomer.[18,19] The impact of 14 naturally occurring polyphenolic compounds is reviewed and referred to compounds as anti-destabilizer and anti-fibrillogenic against the α-syn protein.[20] Our study mainly focuses on the *in-silico* analysis of α-syn protein inhibitors. We studied the structure-based virtual screening approach as an effective tool to discover potential hit molecules from the selected antioxidant compounds present in the *Camellia sinensis* plant. *Camellia sinensis* is a well-known plant with various bioactive compounds having antioxidant properties and is the studied plant species rich in catechins, flavonoids, and polyphenols.[21,22,23,24,25] The plant is commonly known as a tea shrub and is native to southeast Asia and China. The polyphenols present in the plant are studied to exert powerful neuroprotective effects in animal-based parkinsonism models.[26] The polyphenols can counteract oxidative stress and neuroinflammation, promote autophagy, and decrease the rate of the protein aggregation process.[27]

Table 1. Showing the results of the virtual screening approach.

Name of the ligand	Binding Free Energy (kcal/mol)	Ligand Efficiency (kcal/mol)	Torsional Energy (kcal/mol)
Theaflavin-3-3-digallate	−7.1	0.1	4.6695
Theaflavin	−6.7	0.0753	6.5373
Theaflavin-3'gallate	−6.4	0.1123	3.4243
Theaflavin-3-gallate	−6.2	0.0849	4.9808
Gallocatechin gallate	−6.1	0.122	3.7356
Catechin gallate	−5.4	0.1317	1.2452
Luteolin	−5.3	0.171	1.5565
Quercetin	−5.2	0.1576	1.8678
Gallocatechin	−5.2	0.1529	2.1791
Epigallocatechin	−5.1	0.15	2.1791
Myricetin	−5.1	0.1457	2.1791
Apigenin	−5	0.1724	1.2452
Kaempferol	−4.9	0.1581	1.5565
Epigallocatechin gallate	−4.9	0.14	2.1791
Epicatechin	−4.8	0.15	1.8678
Caffeic acid	−3.9	0.1696	1.5565
Caffeine	−3.8	0.2	0
Theophylline	−3.8	0.2533	0
Gallic acid	−3.6	0.2	0.3113
Adenine	−3.4	0.2267	0

Results and Discussion

Structure-based Virtual Screening

All 20 compounds are subjected to docking analysis and presented a docking score in the form of binding energy within the range of −3.4 to −7.1 kcal/mol towards the structure of α-syn with PDB.ID: 2N0A. Table 1 shows the binding affinities (docking score) and other docking parameters of each compound used in this study. Interaction analysis of all possible docked conformers of all 20 compounds is carried out to investigate their binding pattern and possible interactions towards the 2 N0 A binding pocket. Among 20 compounds, the highest binding energy is observed in the case of Theaflavin-3-3-digallate (T33DG) with −7.1 kcal/mol followed by Theaflavin (T) and Theaflavin-3'-gallate (T3G) with −6.7 and −6.4 kcal/mol, respectively. The top three hit compounds are selected for further investigation using molecular dynamics simulations.

The intermolecular interactions between the α-syn protein and hit compounds are shown in Figure 1. From molecular docking analysis, the ligand Theaflavin-3-3-digallate interacts with Lys80, Ala91, Gly93, Glu57, and Phe3 through approximately 7 hydrogen bonds. Theaflavin binds with Val77, Val48, Glu46, Lys45, and Thr44 through 5 hydrogen bonds, and Theaflavin-3'-gallate binds with 4 hydrogen bonds, i.e., Ile88, Val74, Ala76.

Molecular dynamics simulation

Seventy amino acids from α-syn in structure PDB.ID: 2N0A is involved in amyloid formation. Therefore, we have considered that only 70 amino acids possess domain for molecular dynamics (MD) simulation studies. The other structure part is the loop region that shows higher conformational changes. The stability, conformational changes, and intermolecular interactions of the hit compounds with α-syn are found through MD simulations.

The α-syn has a +3 charge. To neutralize the simulation system, we have added a −3 Cl charge. The rmsd of a single α-syn at its unbound state with another α-syn or with a compound is shifted from 2.5 to 19.5 Å, which is unusual in the globular proteins. The globular proteins preferred rmsd change in a range of 1–3 Å. The langer changes in the rmsd of α-syn indicate the protein is undergoing enormous conformational change during the simulation. In the case of the RMSF analysis, the protein has more fluctuations in the loop region, where the β-strand region remains rigid. In the rmsf plot, it is found that the region with amino acid index 22 to 24 and 47 to 49 represents the β-stands. The secondary structure of the α-syn protein is monitored throughout the simulation. The SSE (secondary structure elements) analysis summarizes that the protein consists of a 10.33% beta-strand region, 0.21% helix region, and 89.46% volatile loop region. The charge of the α-syn and Theaflavin-3-gallate complex simulation system is 21 Na and 24 Cl by adding 0.15 M NaCl to the system. The total charge of the simulation system by neutralizing protein with −3 Cl and adding 0.05 M NaCl is 24 Na and 24 Cl. The average RMSD of the α-syn bound with Theaflavin-3-gallate is 4.563 Å. Initially, the rmsd is 16, Å which is later stabilized at 11 Å. The secondary structure of the α-syn protein is monitored throughout the simulation. The SSE (secondary structure elements) of

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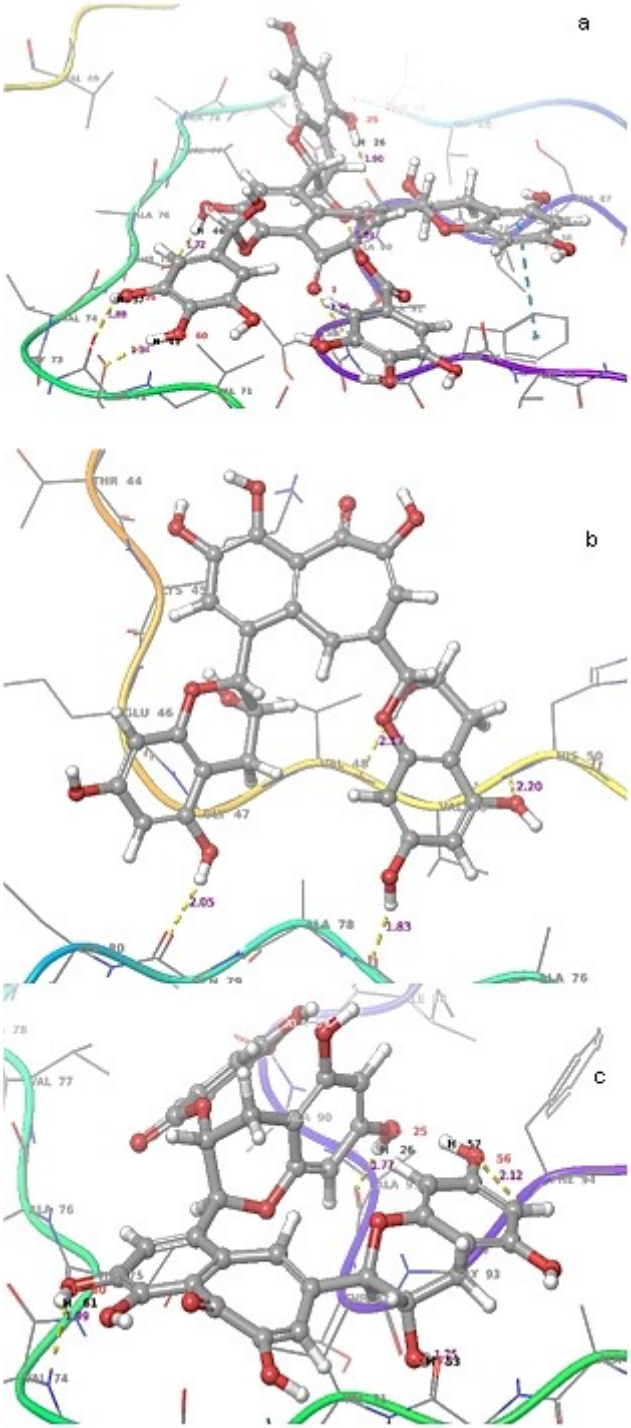

Figure 1. Intermolecular interactions between (a) T33DG (b) T and (c) T3G with α-syn protein.

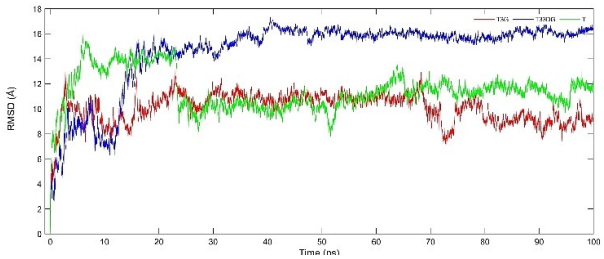

Figure 2. RMSD plot of α-syn and α-syn bound with T3G, T33DG and T respective.

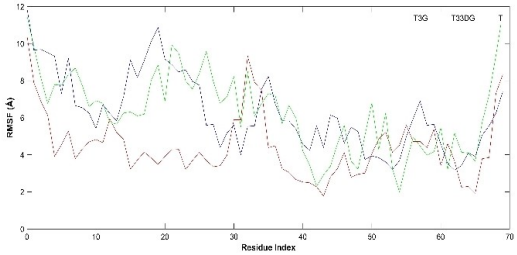

Figure 3. RMSF plot of α-syn and α-syn bound with T3G, T33DG and T respective.

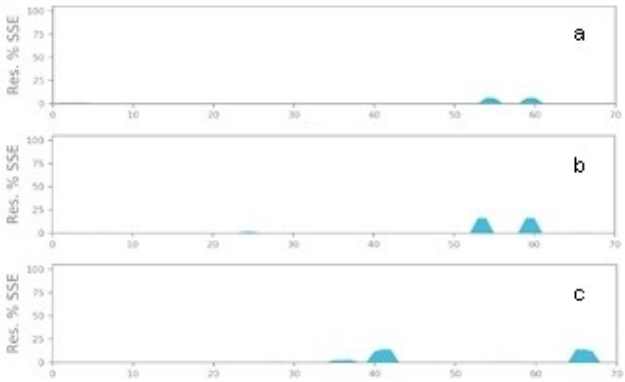

Figure 4. The secondary structure α-syn complexed with (a) T33DG, (b) T and (c) T3G compounds.

Theaflavin-3-gallate bound α-syn analysis summarizes the protein consists of 0.99% beta-strand region, 0.01% helix region, and 99% region is volatile loop region. The charge of the α-syn and Theaflavin-3-3-digallate complex simulation system is 19 Na and 22 Cl by adding 0.15 M NaCl to the system. The total charge of the simulation system by neutralizing protein with −3 Cl and adding 0.05 M NaCl is 22 Na and 22 Cl.

The average rmsd of the α-syn bound with Theaflavin-3-3-digallate is 4.563 Å. Initially, the rmsd is 9 Å, stabilizing at a 10 ns time scale with 16 Å. The secondary structure of the α-syn protein is monitored throughout the simulation. The SSE (secondary structure elements) of Theaflavin-3-3-digallate bound α-syn analysis summarizes that the protein has a 0.38% beta-strand region, 0.04% helix region, and 99.58% region is volatile loop region. The charge of the α-syn and Theaflavin complex simulation system is 21 Na and 24 Cl after adding 0.15 M NaCl. The total charges of the simulation system after neutralizing protein with −3 Cl and by adding 0.05 M NaCl are 24 Na and 24 Cl. The average rmsd of the α-syn bound with Theaflavin is 5.942 Å. Initially, the rmsd is 15, Å which is

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Table 2. The possible hydrogen bonding interactions between α-syn and hit compounds.

Molecule name	Binding energy (kcal/mol)	Residues involved in H-bonding Residue name	Ligand atoms	H-bonding distance in Å
T33DG	−7.1	Ala 89	H_{25}-O_{25}	1.9
		Ala 91	O_{23}	1.89
		Thr 75	H_{46}-O_{45}	1.72
		Thr 72	H_{57}-O_{56}	1.88
		Thr 72	H_{61}-O_{60}	1.84
		Gly 93	O_{1}	1.96
		Lys 80	H_{24}-O_{23}	2.06
		Ala 91	O_{23}	2.15
		Gly 93	O_{1}	2.64
		Phe 94	O_{71}	2.28
		Glu 57	H_{59}-O_{58}	1.63
		Glu 57	H_{61}-O_{60}	1.54
		Glu 57	H_{57}-O_{56}	2.5
Theaflavin	−6.7	His 50	O_{41}	2.2
		Val 48	O_{39}-H_{40}	2.117
		Val 77	O_{43}-O_{44}	1.83
		Gln 79	O_{25}-H_{26}	2.05
		Val 77	0_{49}-H_{50}	1.66
		Glu 46	O_{25}-H_{26}	1.66
		Val 48	O_{23}	2.07
		Lys 45	O_{23}-H_{24}	1.92
		Thr 44	O_{27}-H_{28}	2.09
T3G	−6.4	Val 74	O_{60}	1.99
		Val 70	O_{52}-H_{53}	1.75
		Phe 94	O_{56}	2.12
		Ala 91	O_{25}-H_{26}	1.77
		Ile 88	O_{23}-H_{24}	2.11
		Val 74	O_{60}-H_{61}	1.84
		Ala 76	O_{38}	2.09
		Ala 76	O_{38}-H_{39}	1.93

reduced to 10 Å after 20 ns time scale. The secondary structure of the α-syn protein is monitored throughout the simulation. The SSE (secondary structure elements) of Theaflavin bound α-syn analysis summarizes the protein consists of 0.99% beta-strand region, 0.01% helix region, and 99% region is volatile loop region. The RMSD analysis confirmed that the Theaflavin-3-3-digallate (T33DG) showed structural stability during the MD simulations compared with Theaflavin (T) and Theaflavin-3'-gallate (T3G) (Figure 2).

The fluctuation of protein residue side chains upon binding with ligands is investigated using RMSF in the MD simulation. The protein's C terminal (6 Å) has low fluctuation compared to the N terminal region (10.5 Å).

It is observed that the ligand-binding sites (40–70 residues) show lower fluctuations in protein-ligand complexes due to the intermolecular interactions. The comparative RMSF of non-bounded protein and complexes is shown in Figure 3. From secondary structure analysis of the protein upon binding with T33DG, T, and T3G compounds, it is found that the beta-sheet region is converted into the loop. In the T33DG complexed structure, the beta-sheet region almost completely disappeared compared to the other two.

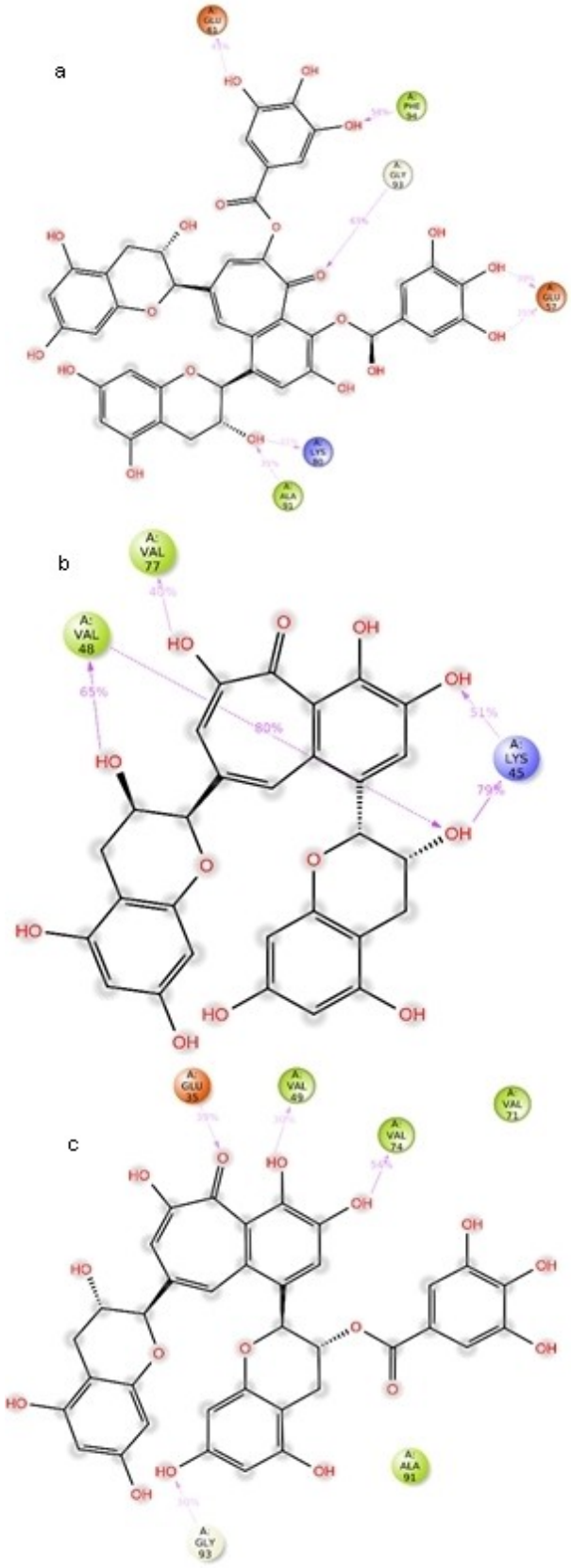

Figure 5. The interaction diagram of (a) T33DG, (b) T, and (c) T3G with α-syn from MD simulation.

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This analysis supports that the compound T33DG can disaggregate the α-syn amyloids. The secondary structure comparison of the complex structure is shown in Figure 4. The molecular dynamics simulations showed that the intermolecular interactions such as hydrogen bond, hydrophobic contact, ionic contact, and salt bridge are analyzed over 100 ns. The analysis states that the regions crucial for aggregation have strongly interacted with the ligands. Table 2 shows the possible hydrogen bonds formed by the hit compounds T33DG, T, and T3G with α-syn protein. It is observed that the π-π bond is found only between T33DG and Phe94 residue of α-syn. Figure 5 shows the hydrogen bonds and their strengths in the simulation procedure. It is found that the compound T33DG shows more number of interactions crossing the threshold strength of the hydrogen bonding, which is over 30%.

Conclusion

To date, no promising cure is reported for PD. The figure for PD patients is increasing abruptly, and by 2040 the number of people with PD is projected worldwide to exceed 12 million. The disease occurrence increases with age, but 10% of patients are under 50. The inhibition of α-syn aggregation has emerged as a promising strategy for treating PD. This study worked with various antioxidant class compounds present in *Camellia sp.* From the *In-silico* analysis, it is found that the hit compounds, Theaflavin-3-3-digallate (T33DG), Theaflavin (T), and Theaflavin-3'-gallate (T3G), show better interactions. Bioassay of the hit compounds with monomeric and aggregated α-syn will help in better understanding the disaggregation of the lewy bodies.

Supporting Information Summary

The *in silico* experimental section of the current article is provided in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: α-syn · *Camellia sp.* · Molecular dynamics · Parkinson's disease · Virtual screening

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