STUDIES ON DOPAMINERGIC NEUROPROTECTIVE EFFICACY OF CERTAIN PLANT EXTRACTS IN THE ADULT TRANSITION STAGE *DROSOPHILA* MODEL OF PARKINSON'S DISEASE

by

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Registration No: **PhD/ZOO/00385**

Submitted to

NAGALAND UNIVERSITY

In Partial Fulfilment of the Requirements for Award of the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

DEPARTMENT OF ZOOLOGY

SCHOOL OF SCIENCES

NAGALAND UNIVERSITY

LUMAMI-798627

NAGALAND, INDIA

2024

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DECLARATION

I, Mr. Rahul Chaurasia, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis for the award of any previous degree to me or to the best of my knowledge and to anybody else, and that the thesis has not been submitted by me for any research degree in any other university.

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on Dopaminergic Neuroprotective Efficacy of Certain Plant Extracts in the Adult Transition Stage *Drosophila* Model of Parkinson's Disease"** is a record of original research work done by **Mr. Rahul Chaurasia.** He is a registered research scholar bearing **Regd. No. PhD/ZOO/00385** of the Department and has fulfilled all the requirements of Ph.D. regulations of Nagaland University for the submission of the thesis. The work is original and neither the thesis nor any part of it has been submitted elsewhere for the award of any other degree or distinctions. The thesis is therefore, forwarded for adjudication and consideration for the award of degree of **Doctor of Philosophy in Zoology** under Nagaland University.

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ACKNOWLEDGMENTS

This work was carried out at the Drosophila Neurobiology Laboratory (DNBL), Department of Zoology, Nagaland University (Central), Lumami campus, Nagaland, India.

I was privileged enough to be under the guidance of ***Prof. Sarat Chandra Yenisetti****. His perseverance and eagerness to learn about many facets of neurobiology and PD have enabled him to solve effectively the most difficult problems/trials, which considerably aided in the completion of this thesis. Furthermore, his scientific temperament and approach have inspired and helped me comprehend the power of critical thinking, which I believe will be very beneficial to me in the future. I am appreciative of his instruction since it equipped me with the knowledge and abilities to become a genuine modern biologist and improved my ability to articulate my thoughts. Throughout the journey's ups and downs, he always made time for me and provided me with good advice and encouragement, which enabled me to grow as a person.*

I am eternally grateful to my father, ***Mr. Ramesh Chandra Chaurasia****, and mother,* ***Mrs. Ramsakhi Chaurasia****, who have been my rock and source of unending love and patience over this whole odyssey. They consistently uplifted my spirits, supported me during challenging moments, and empowered me. I owe my heartfelt gratitude to my "Amma",* ***Radhabai Chaurasia****, and Mr. and Mrs.* ***ML Chaurasia*** *for their generous love, support, and motivation.*

I would like to pay my deepest gratitude to ***Dr. Abhik Das*** *(DNBL), who was instrumental in bringing Chapter 4 to life and provided invaluable assistance throughout the HPLC based project implementation. Also, his help with the thesis preparation is highly appreciated.*

I extend my sincere and humble gratitude to ***Dr. Mohamad Ayajuddin*** *(DNBL), who was an invaluable mentor, and working with him on a collaborative fluorescence microscopy based project was very insightful. Further, his contributions in Chapter 2 and Chapter 3 are much valued and highly acknowledged.*

*I would like to express my heartfelt gratitude to **Ms. Abuno Thepa** (DNBL), Ms. **Nukshimenla Jamir** (DNBL), and **Mr. Kelevikho Neikha** (DNBL) for their invaluable assistance in proofreading and formatting the thesis. In addition, the work related discussions with them were consistently productive. The time spent outside of the lab with them was unforgettable after a tight and stressful schedule in the laboratory.*

*I greatly appreciate the contributions of **Dr. Limamanen Phom** (DNBL). The works and ideas for this thesis would not have emerged without the discoveries made through his diligent effort. I greatly value and appreciate his suggestions and guidance.*

I am sincerely grateful for the facilities, resources, and support received from the Department of Zoology throughout this PhD tenure.

I humbly express my deepest respect for my experimental flies, whose sacrifices have made this research fruitful.

I express my sincere gratitude to the funding agencies.

- *Indian Council of Medical Research (ICMR), Government of India, New Delhi, India, for the Senior Research Fellowship award (**3/1/2/164/Neuro/2021-NCD-I**).*
- *DBT U-EXCEL (Unit of Excellence), Department of Biotechnology, Government of India, New Delhi, India for the Senior research fellowship award.*
- *DBT U-EXCEL (Unit of Excellence), Department of Biotechnology, Government of India, New Delhi, India for the Junior research fellowship award.*
- *University Grant Commission (UGC), Nagaland University, Nagaland, India for the Non-NET fellowship award.*

Rahul Chaurasia

(28th August, 2024, Nagaland, India)

Dedicated to:

My Ma, Papa, and Amma for their constant love, support, and blessings

Omniscient Yogi, "Mahadev"

CONTENTS

LIST OF FIGURES AND TABLES **PAGE NO.**

Chapter 1

Chapter 2

Chapter 3

Chapter 5

ABBREVIATIONS

α-syn: α-synuclein

4-HNE: 4-hydroxy-trans-2-nonenal

6-OHDA: 6-hydroxydopamine

aaNAT: Arylalkyl amine N-acetyltransferase

AChE: Acetyl-cholinesterase

AD: Alzheimer's Disease

ALS: Amyotrophic lateral sclerosis

ALSS: Adult life-stage specific

anti-TH: anti-tyrosine hydroxylase

AP-1: Activator protein 1

ATP: Adenosine triphosphate

Aux: Auxilin

BAX2: Bcl-2 Associated X-protein

BBB: Blood-brain-barrier

Bcl2: B-cell lymphoma 2

BDNF: Brain Derived Neurotrophic Factor

CAT: Catalase

CMG: Curcumin Monoglucoside

CNS: Central nervous system

COMT: Catechol-o-methyltransferase

COX-2: Cyclooxygenase-2

CS: Canton-S

CSF: Cerebrospinal fluid

CU: Curcumin

DA: Dopamine

DAergic: Dopaminergic

DHODH: Dihydroorotate dehydrogenase

DOPAC: 3,4-Dihydroxyphenylacetic acid

dUCH: *Drosophila* ubiquitin carboxyl-terminal hydrolase

ECD: Electro chemical detector

ER: Endoplasmic reticulum

ETC: Electron transport chain

FI: Fluorescence intensity

GFAP: Glial fibrillary acidic protein

GPx: Glutathione peroxidase

GSH: Reduced glutathione

GST: Glutathione s transferase

HP: Health Phase

HPLC: High-performance liquid chromatography

HVA: Homovanillic acid

IL-6: Interleukin-6

iNOS: Inducible nitric oxide synthase

INPP4A: Inositol polyphosphate-4- phosphate type IA

JAK/STAT: Janus kinase/signal transducers and activators of transcription

JNK: c-Jun N-terminal Kinase

Keap-1: Kelch-like ECH-associated protein 1

LDH: Lactase dehydrogenase release: for apoptotic and necrosis cell death

L-DOPA: Levodopa

LRRK2: Leucine-rich repeat kinase 2

MAO: L-Monoamine Oxidase

MAPK: Mitogen-activated protein kinase

MDA: Malondialdehyde

MDTM: Methanol-Dichloromethane-Triethylamine-Methanol

mGDPH: Mitochondrial glyceraldehyde-3-phosphate dehydrogenase

MIP: Maximum intensity projection

MPP^+: 1-methyl-4-phenylpyridinium

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mtDNA: Mitochondrial DNA

mTOR: Mechanistic/mammalian target of rapamycin

MTT: 3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADA: N-acetyl dopamine

NADPH: Nicotinamide adenosine dinucleotide phosphate hydrogen

NBAD: N-β-alanyl dopamine

NDD: Neurodegenerative disorder

NDF: Neuronal dysfunction

NF1: Neurofibromatosis type 1

NF-κB: Nuclear factor-κB

NGS: Normal Goat Serum

NHP: Non-human primates

NIRF: Near-infrared fluorescence imaging

NOS: Nitric Oxide Synthase

NPSH: Non-protein thiol

Nrf2: The nuclear factor erythroid 2–related factor 2

NRU: Neutral Red uptake: Mitochondrial lactate dehydrogenase and lysosomic cytotoxic assessments

OK: Oregon K

OS: Oxidative stress

OXPHOS: Oxidative phosphorylation

PAL: Protocerebral anterior lateral

PAM: Protocerebral anterior medial

PBS: Phosphate-buffered Saline

PBST: PBS containing TX-100

PC: Protein carbonyl

PD: Parkinson's Disease

PFA: Paraformaldehyde

PINK1: PTEN-induced kinase 1

PPD: Protocerebral posterior deutocerebrum

PPL: Protocerebral posterior lateral

PPM: Protocerebral posterior medial

PQ: Paraquat, 1,1'-dimethyl-4,4'-pyridinium

PTEN: Phosphatase and tensin homolog

RB1: Retinoblastoma 1

ROS: Reactive oxygen species

SDH: Succinate dehydrogenase

SGOT: Serum glutamic oxaloacetate transaminase

SGPT: Serum glutamic pyruvic transaminase

SN: Substantia Nigra

SNpc: Substantia nigra pars compacta

SOD*:* Superoxide dismutase

TBI: Traumatic brain injury

TCA: Trichloro Acetic Acid

TH: Tyrosine hydroxylase

TNF: Tumor necrosis factor

TP: Transition Phase

TRITC: Tetramethylrhodamine

TX-100: Triton X-100

UCH-L1: Ubiquitin C-terminal hydrolase L1

VMAT-2: Vesicular monoamine transporter-2

VUM: Ventral unpaired medial

XTT: 2,3-bis (2-methoxy-4-nitro-5- sulfophenyl) -5- [(phenylamino) carbonyl]-2H-tetrazolium hydroxide

Chapter 1

Review of Literature

Chapter 1

1.1 Introduction

Parkinson's disease (PD) is a multifactorial disease characterized by progressive and selective degeneration of dopaminergic (DAergic) neurons with abnormal expression of α-synuclein (α-syn), a presynaptic neuronal protein related to the regulation of the dopamine (DA) and a major fibrillar component of Lewy bodies (LB) in the *substantia nigra* region of the brain ensuing wane at the later stage of human life (Miranda et al., 2022; Meissner et al., 2011). The origin of PD may be linked to gender, growing age, hereditary background, environmental factors, nutritional insufficiency, or brain damage (Stoker et al., 2018). PD affects more than 1% of the population above 65 years of age and is predicted to be doubled by 2030 (WHO, 2023; Aarsland et al., 2021; Hoyert et al., 2012). There is no cure for PD except the pharmacological interventions to alleviate the motor symptoms via reinstating striatal DA tone using dopa mimetic drugs, such as DA precursor and DA agonists, and inhibitors of monoamine oxidase-B (MAO-B), catechol-O-methyl-transferase (COMT), and decarboxylase enzymes. Furthermore, numerous scientific groups worldwide carry out studies on managing PD, focusing on pathogenesis and novel approaches for creating clinical medicine/efficient therapeutic strategies to improve the quality of life for PD patients.

1.2 PD therapeutic strategies and limitations

The current pharmacological treatments for PD are anticipated, but the approaches mainly rely on symptomatic therapies. However, these pharmacological interventions only alleviate the related motor symptoms by restoring striatal DA levels using supplementations, such as DA precursor levodopa (L-DOPA) and DA agonists viz., apomorphine, bromocriptine, pramipexole, cabergoline, rotigotine, ropinirole, pergolide, lisuride, piribedil and inhibitors of MAO-B viz., selegiline, rasagiline, safinamide and

COMT viz., entacapone, tolcapone and decarboxylase inhibitors, viz., carbidopa, benserazide, and antimuscarinic drugs, viz., trihexyphenidyl, benztropine, orphenadrine, procyclidine, biperiden, and the N-methyl-D-aspartate (NMDA) antagonist, amantadine. L-DOPA brought a revolution in the field of management of PD and established as the gold standard treatment by significantly improving PD symptoms, quality of life, and normalizing life expectancy, even countering late-onset PD symptoms (Nakmode et al., 2023; Tambasco et al., 2018; Jankovic, 2002). Nevertheless, the administration of L-DOPA has limitations due to its half-life in the blood (about 90-120 minutes), which causes fluctuations in blood levels that result in changes in clinical symptoms in the advanced stage, manifesting as the wearing-off phenomenon, which is the re-emergence of PD symptoms as the effect of L-DOPA diminishes near the end of the dose interval, usually 3 to 4 hours after the dose. It has also been proposed by Hickey and Stacy (2011) that L-DOPA-mediated interventions show uncertainty as they go through the "On-Off" phase, where during the "On" phase, PD patients respond to the therapy and symptoms are controlled, whereas during the "Off" phase patients do not respond to the therapy and symptoms including rigidity, tremors, and slow movement reappears. Even long-term treatment may lead to considerable toxicity by inducing motor abnormalities and spontaneous movements, known as L-DOPA-induced dyskinesia (LID), which manifests as DA- resistant motor and non-motor neurotoxicity (Rascol et al., 2003). Moreover, as the disease progresses, patients are less responsive to DAergic medications and require more frequent doses of DA supplementations (Chou et al., 2018). Therefore, current formulations of L-DOPA contain decarboxylase inhibitors, better known as carbidopa or benserazide. Decarboxylase inhibitors prevent the peripheral metabolism of DA and allow for a greater bioavailability of the drug (Muller et al., 2015). However, the simultaneous administration of L-DOPA and other drugs is recommended to avoid the complications

caused by high doses of a single medication (Dhanawat et al., 2020). These drugs include rasagiline, safinamide, selegiline, and MAO-B inhibitors, COMT inhibitors, such as entacapone and tolcapone, which have been shown to increase DA levels (Oertel et al., 2016). Another category of drugs is DA agonists such as ropinirole and pramipexole, which have been described as safe and effective in both monotherapy and in combination with L-DOPA (Binde et al., 2020; Kulisevsky et al., 2010). This class of medications includes two notable exceptions: apomorphine, which is either administered subcutaneously or via injection as a life-saving measure for patients experiencing motor fluctuations, and rotigotine, which is available as transdermal patches that provide a continuous supply of the medication (Carbone et al., 2019). All these therapies have been of great value in managing PD symptoms. However, wearing out the effect of oral medications, limited therapeutical options for patients with advanced disease, and the failure of clinical trials on disease-modifying agents so far have led researchers to reconsider clinical trial design (Cedarbaum, 2018). More recent unique formulations of L-DOPA have been developed to address L-DOPA therapy shortcomings (Armstrong et al., 2020), where continuous duodenal infusion of levodopa/carbidopa intestinal gel and apomorphine subcutaneous pumps directly into the small intestine of the PD patients results in the marked reduction of motor fluctuations by reducing the plasma L-DOPA variability (Armstrong et al., 2020). However, L-DOPA-induced toxicity is hypothesized to be mediated through pulsative activation of the DA receptor. In normal physiological conditions, DA release is facilitated via phasic (fast-acting receptors that stop responding to continuous stimulation) and tonic (slow-adapting, slow-producing action potentials that occur at a continuous frequency as long as the stimulus persists) firing of the receptors. The primary mechanism that preserves the DA levels and the stimulation of DA receptors stimulated continuously, is modulated through the tonic firing of DA neurons (Papa et al.,

1994; Grace et al., 1984). The propagation of appropriate tonic firing, which facilitates the storage and absorption of DA and L-DOPA from plasma, is ensured by an adequate number of DAergic neuronal terminals throughout the early stages of PD. However, an imbalance in internal DA storage/transport and reduced tonic firing result from the degeneration of DAergic neuron terminals that occurs with PD. Therefore, DAergic neurons thus become dependent on plasma L-DOPA, which has a brief half-life, and changes in tonic firing also cause an imbalance in the intake of plasma L-DOPA levels. Thus, fluctuations in plasma L-DOPA lead to variations in L-DOPA and DA levels, exposing receptors to altered DA concentrations that may trigger a recurrence of PD symptoms (Nakmode et al., 2023). Additionally, as PD progresses, a gradual loss of DAergic neurons alters the regulation of neuronal DA and the responsiveness to symptomatic pharmacological treatments. Therefore, symptomatic therapy is insufficient to stop the onset and advancement of PD. So, to reduce or stop the clinical progression and mobility impairment, a disease-modifying approach that can directly target the etiology rather than offering symptomatic alleviation remains a major unmet clinical need in the management of PD. Therefore, it is suggested that disease-modifying therapeutics, including immunotherapies, small molecules/compounds, and nanoformulations, repurposing of the drugs, are the next "cutting edge tools" that might change the etiopathogenesis of PD at its core (Nakmode et al., 2023). Because of their affordability, safety, and multi-target mode of action, plant products/extracts/nutraceuticals have become effective disease-modifying agents and present a promising avenue for alternative therapy for PD (Lama et al., 2020; Yuan et al., 2016; Modi et al., 2016).

1.3 Neuroprotective efficacy of plant products/nutraceutical/plant extracts/polyphenols in *Drosophila* model of PD

The objective is to conduct a literature search to locate publications that argue the neuroprotective efficacy of "plant extracts (PE) or nutraceuticals on the neurotoxins (Paraquat (PQ), Rotenone (ROT))-mediated *Drosophila* model of PD". Additionally, articles explored the relationship between plant extracts, *Drosophila*, and PD and studies that investigated “plant extracts in animal models of PD”. I executed an extensive search utilizing five data sources, notably "PubMed," "Web of Science," "Science Direct," "Scopus," and "Google Scholar."

Multiple studies demonstrated evidence for the efficacy of plant products, nutraceuticals, and plant extracts in mitigating neurodegenerative diseases (NDD), such as PD (Scalbert et al., 2005). The disease has clinical and pathogenetic features, including neuroinflammation, mitochondrial dysfunction, protein aggregation, and iron toxicity. Furthermore, PD influences oxidative stress (OS) (Cha et al., 2017; Ataie et al., 2016). However, the critical pathogenesis and therapeutics of PD remain mostly unknown. Studies have revealed that plant products, nutraceuticals, and extracts have neuroprotective efficacy, which could be rendered in treatment or medication (**Tables 1.1 and 1.2**). A well-established source for developing therapeutic drugs for PD is traditional herbal components with antioxidant effects (Park et al., 2018). For instance, *Decalepis hamiltonii* (*Dh*) root extract has recently gained recognition as an innovative all-natural antioxidant. In flies overexpressing both missense mutations (*A30P and A53T*) of α-syn, restoring defective motility and circadian rhythm, reducing reactive oxygen species (ROS) and lipid peroxidation (LP), and improving catalase (CAT) and superoxide dismutase (SOD) activity were all accomplished with dietary supplementation with antioxidants from *Dh* root extract. Furthermore, Dh extract reduced neurotoxicity against

paraquat (PQ) (Jahromi et al., 2015), and it can be used in PD therapy to delay the onset of PD. Similar to *Dh*, avocado (*Persea americana*) is also a source of antioxidant for PD treatment (Bost et al., 2013). The methanolic extracts of *P. Americana* include epicatechin (EC) and Colin red peel (CRE), which enhance the longevity and locomotor activity of parkin knockdown flies exposed to PQ. Similarly, its extract exhibited protection in *parkin* knockdown flies against PQ-induced OS, mobility dysfunction, and LP (Ortega-Arellano et al.,2019). Tangeritin, a flavonoid found in the peels of Mandarin oranges, is a polyphenol that exhibits several biological activities. These activities include neuroprotection, improvement of gap junction intercellular communication, activation of apoptosis, and inhibition of metastasis (Seo et al., 2011; Datla et al., 2001; Hirano et al., 1995; Chaumontet et al.,1994). The DA content of PD flies was elevated by tangeritin exposure, which also restored the reduced locomotor activity and various OS markers, including LP, reduced glutathione (GSH), glutathione s-transferase (GST), protein carbonyl (PC) content, and MAO activity (Fatima et al., 2017). Therefore, supplementation of tangeritin reduced PD symptoms, indicating its potential application in dietary therapy. Polyphenols are abundant in grape and grape seed extracts, which are well-known sources of antioxidants in the treatment of NDD, like PD (Cha et al., 2017; Wang et al., 2010; Long et al., 2009). The feeding of grape extract and grape seed extracts to flies expressing α-syn exhibited a significant increase in the lifespan of female flies and a substantial improvement in the climbing ability of male flies. This confirms that the grape extracts have the potential to protect against the detrimental impacts of free radicals and free radical mediated-LP and DNA damage (Long et al., 2009). The administration of capsaicin exhibited a protective effect on PD flies induced by overexpression of α-syn, resulting in a delayed decline in their climbing abilities. Capsaicin supplementation can slow the onset of PD (Siddique et al., 2012). Tea consumption is widespread globally

because it has biological benefits that include antioxidation, anti-inflammation, and the prevention of several diseases. According to Siddique et al. (2017), black tea can mitigate PD-mediated phenotypes in flies. This protection is achieved by reducing the LP and PC content, increasing the levels of GSH and DA, and reducing the activity of GST in a dose-dependent manner. Notably, decaffeinated coffee and nicotine-free tobacco exhibited a neuroprotective effect in the PD transgenic fly that overexpressed the α-syn and loss-of-function *parkin* gene mutant. This exhibited neuroprotection is achieved through activating the transcription factor *Nrf2* (Trinh et al., 2010). Therefore, these compounds are potential therapeutic candidates in models of PD. Polyphenolic extracts, phenolic acids, and flavanols exhibit antioxidant activity and protective efficacy against PQ-mediated PD models. In flies, the impaired climbing ability induced by PQ was reversed by polyphenols, such as gallic acid (GA), caffeic acid (CA), propyl gallate (PG), and epigallocatechin-3-gallate (EGCG). In particular, the PQ-mediated locomotor dysfunction in flies was protected by PG and EGCG polyphenols (Jimenez-Del-Rio et al., 2010). Additionally, genotropic drug curcumin (CU) exposure to PQ-mediated and α-syn overexpressing PD flies significantly restored the climbing ability, enhanced life span, and reduced OS, resulting in reduction of LP and PC content, as well as apoptosis (Siddique et al., 2014). GA substantially preserved the number of DAergic neurons and enhanced life span and locomotor activities in PQ-mediated PD flies (Ortega-Arellano et al., 2013). Consequently, the diverse plant products, nutraceuticals, and polyphenolic compounds discussed and summarized in tables **1.1&1.2** are potential PD drug therapy sources.

S. No.	Fly	Age/Sex	Neurotoxin treatment strategies	Plant extract intervention strategies	PD phenotypes scored	Remark(s)	Reference(s)
1	Oregon K	5, 50 days old, Male	10mM Paraquat (PQ) for 24 and 48 hrs on filter paper	500µM, 1mM, Curcumin for 24 and 48 hrs on filter paper in Co- and Pre-treatment regimens	a) Mobility defects b) Increased mortality c) Depleted brain dopamine d) Elevated OS markers *viz.*, ROS, LP, PC, HP, SOD, CAT, GST levels, Reduced GSH, Total thiol, AchE activity e) Reduced brain TH protein level f) No DAergic neuronal loss	Rescued PD phenotypes during the health Phase, but not during the transition Phase	Phom et al., 2014; Phom, 2018
2	Oregon R	10, 20, 30 days old, Male	2 hrs starvation, 20 mM PQ for 24 hrs on filter paper	Dietary administration of ethanol extract of apple polyphenols (AP): 2 mg/mL and 10 mg/mL for 30 days	a) Mobility defects b) Reduced lifespan	Partially rescued PD phenotypes	Peng et al.,2011
3	Oregon R	10, 20, 30 days old, Male	2 hrs starvation, 20 mM PQ for 24 hrs on filter paper	Dietary administration of blueberry extracts (BBE):5 mg/mL for 30 days	a) Mobility defects b) Reduced lifespan	Rescued PD phenotypes	Peng et al.,2012
4	Canton S	5 days old, Male	6 hrs starvation, 5 mM PQ for 48 hrs on filter paper	100µM Calycosin for 48 hrs on filter paper	a) Mobility defects, b) Increased mortality c) Depleted brain dopamine d) Elevated OS markers *viz.*, Elevated ROS, Reduced SOD, CAT, GSH activity, Elevated LPO, PC levels e) Reduced brain TH activity f) DAergic neuronal loss (PPL1 and PPM subset) g) Mitochondrial impairments *viz.*, Reduced Complex I, III activity, Reduced MMP intensity, ATP levels, h) Reduced L-DOPA production	Rescued PD phenotypes	Chaouhan et al.,2022
5	Oregon K	8-10 days old, Male	20-40 mM PQ for 24 and, 48 hrs on filter paper	*Bacopa monnieri* extract (BME): 0.05, 0.1% prophylactic treatment of BME for 7 days in feed	a) Increased mortality b) Elevated OS markers *viz.*, Enhanced MDA, HP and, PC levels, Elevated CAT and SOD c) Mitochondrial dysfunction *viz.*, Reduced succinate dehydrogenase, Inhibited Complex-I-III, II-III activity	Rescued PD phenotypes	Hosamani and muralidhara, (2010)
6	Oregon K	2 days old	15-20 mM PQ for 24 and 48 hrs on filter paper	Dietary feeding of *Decalepis hamiltonii (Dh)* aqueous extract:	a) Mobility defects b) Increased mortality	Rescued PD phenotypes	Jahromi et al., 2013

				0.1, 0.5 % for 14 days in food media	c) Elevated OS markers *viz.*, Elevated MDA, AchE, CAT, SOD levels, GSH depletion		
7	Canton S	3 days old, Female	3 hrs starvation, 20mM PQ for 24 and 48 hrs on filter paper	Gallic acid (GA), Ferulic acid (FA), Caffeic acid (CA), Coumaric acid (Cou A), Propyl gallate (PG), Epicatechin (EC), Epigallocatechin (EGC), Epigallocatechin gallate (EGCG): Except for GA and Cou A, PG (0.1 mM), FA (0.5 mM), CA (0.5 mM) and catechins (EC, 0.5 mM; EGC, 0.1 mM; EGCG, 0.1 mM) pre-feeding for 72 hrs on filter paper	a) Mobility defects b) Increased mortality	Rescued PD phenotypes	Jimenez-Del-Rio et al., 2010
8	*D. melanogaster*	3 days old, Male	4 hrs starvation, 5 mM PQ for 12 and 24 hrs on filter paper	Mulberry fruits (MF): pre-treatment with 1% MF extracts	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Increased MDA, ROS, CAT, SOD and AchE levels, GSH depletion	Rescued PD phenotypes	Mahesh et al., 2022
9	Canton S	3-5 days old, Male	5 mM PQ for 18, 24, and 48 hrs on filter paper	GardeninA (GA) (*Gardenia resinifera*): 10 µM GA pre-feeding for 4 days on filter paper	a) Mobility defects b) Increased mortality c) Elevated OS marker *viz.*, Elevated LP level d) DAergic neuronal loss (PPM and PPL1 subset)	Rescued PD phenotypes	Maitra et al., 2021
10	Oregon K	6 days old, Male and Female	5.375- and 20-mM PQ for 24 hrs, alternatively for 8 days	4-Hydroxyisophthalic acid (DHA-I) form roots of *Decalepis hamiltoni*- DHA-I, 0.01% (0.55 mM), 0.02% (1.1 mM) and 0.05% (w/v) (2.75 mM) supplemented in diet for five days; 0.02% of DHA-I for neuroprotective action/assays	a) Mobility defects b) Decreased mean lifespan c) Elevated OS markers *viz.*, Enhanced MDA, ROS, CAT, SOD: Cu/Zn-SOD, Mn-SOD, and AchE, GSH depletion d) DAergic neuronal loss (Vacuolar lesions)	Rescued PD phenotypes	Niveditha and Shivanandappa, 2018
11	*D. melanogaster* (B Line)	Male and Female	12.5 mM PQ on filter paper	*Rhodiola rosea* (SHR-5) extract: 125 or 25 mg/mL for 7 and 14 days in the diet	a) Increased mortality b) Elevated OS markers *viz.*, Enhanced CAT, SOD: Mn-SOD levels	Partially rescued PD phenotypes	Schriner et al., 2009a
12	Canton S	1 day old, Female	200 µg/mL (0.8 mM) PQ for 3 days in diet	Dietary supplementation of eicosapentaenoic (EPA) and Docosahexaenoic (DHA) acids (ω-3): 1 mg/mL (2 mM) for 3 days	a) Loss of citrate synthase, b) Respiratory capacity impairment and exacerbated H_2O_2 c) Complex I inhibition and high lactate accumulation	Rescued PD phenotypes	Oliveira Souza et al., 2019

					d) Loss of ELAV (embryonic lethal abnormal vision) and α-spectrin proteins for neuronal viability and synaptic stability		
13	Oregon R	3 days old, Male	4 hrs starvation, 20mM PQ for 48 hrs on filter paper	Dietary supplementation of 0.05% quercetin (Que), 0.02% curcumin (Cur), 0.05% *S. officinalis*, and 0.025% *Z. rhizome* extract for 48 hrs in diet	a) Mobility defects b) Reduced survival c) Elevated OS markers *viz.*, Increased ROS, CAT, SOD, and AchE levels	Rescued PD phenotypes	Park et al., 2012
14	Oregon K	9-10 days old	30mM PQ for 48 hrs on filter paper	*saffron* methanolic extract SME (0.05 and 0.1%) or *crocin* CR (10 and 25 mM)-enriched medium for 10 days on filter paper	a) Mobility defects b) Increased mortality	Rescued PD phenotypes	Rao et al., 2016
15	Oregon R	5–7 days old	20 mM PQ for 24 hrs on filter paper	Dietary supplementation of *L. microcarpa* fruit pulp extract (LMFE): 0.25 and 0.5% LMFE for 6 days in diet	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Increased MDA, PC, CAT, SOD, GST, AchE levels, depletion of GSH d) Fragmentation of DNA	Rescued PD phenotypes	Oloyede et al., 2020
16	Harwich	1-5 days old	0.44 mg/g of PQ in diet for 7 days	Caffeic acid (CA): 0.25, 0.5, 1 and 2 mg/g of CA in diet for 7 days	a) Increased mortality b) Reduced cell viability c) Elevated OS markers *viz.*, Increased free Fe (II) content and LP, reduced NPSH level d) Mitochondrial dysfunction	Rescued PD phenotypes	Nunes et al.,2019
17	Oregon K	6 days old, Male and Female	5.375 of PQ for 24 hrs, alternatively for 8 days	Dietary supplementation of *Decalepis hamiltonii (*DHA-I), Nicotinamide, Ellagic acid, and Quercetin: 0.02% for five days in the diet	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Increased MDA, ROS, AchE, CAT, SOD: Cu/Zn-SOD, Mn-SOD levels, GSH depletion	Rescued PD phenotypes	Niveditha et al.,2017
18	*D. melanogaster*	Male	3.5mM PQ for 4 days in the diet	Methanolic extract of *D. salina* (200µg/mL) in diet for 4 days	a) Mobility defects b) Increased mortality c) Depleted brain dopamine d) Elevated OS markers *viz.*, Enhanced MDA level	Rescued PD phenotypes	Salim et al., 2021
19	*D. melanogaster*	1- 4 days old, Male	3.5 mM PQ for 4 days in the diet	*Bougainvillea glabra Choisy* leaf extract (BG extract): 40, 120 and 200 µg/mL in food for 4 days	a) Mobility defects b) Increased mortality c) Depleted brain dopamine d) Elevated OS markers *viz.*, Enhanced AchE, ROS, LP	Rescued PD phenotypes	Soares et al., 2017

20	Oregon R-P2	3 days old	20mM PQ for 48 hrs in the diet	*Bacopa monnieri* extract (BME): 0.1% of BME for 48 hrs in the diet	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Increased LPO, H_2O_2 content, CAT, SOD levels d) Mitochondrial impairment e) Reduced ATP levels f) DAergic neuronal loss	Rescued PD phenotypes	Srivastav et al., 2018
21	Oregon R	5 days old	20 mM PQ for 24 hrs on filter paper	*Piperine* (*Piper nigrum and Piper Longum*):10 µMPiperine-coated gold Nanoparticles ($AuNPs^{piperine}$) supplementation for 24 hrs	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Increased LPO, CAT activity, Reduced GSH level, d) DAergic neuronal loss	Rescued PD phenotypes	Srivastav et al., 2020
22	Canton S	5 and 20 days old	10 mM PQ for16 hrs on filter paper	Diet supplementation of *Cistanche tubulosa* (CT): 5.4, 10.8, and 21.6 mg/ml for 5 and 20 days	a) Mobility defects b) Increased mortality c) Reduced cognitive behaviors d) Reduced reproductive capacity	Rescued PD phenotypes	Lin et al., 2017
23	Canton S	15 days old	0.2mg/ml PQ in food diet	*Piranhea trifoliata* bark extract*: P. trifoliata* extracts (0.1 mg/ml) for 10 days in the diet	a) Elevated OS markers *viz.*, increased PC and AchE levels b) Elevated citrate synthase activity	Rescued PD phenotypes	Alencar et al., 2023
24	Canton S	2 days old, Male and Female	1 mM PQ for 5 days on filter paper (Co-treatment) and 10mM PQ for 60 hrs (Pre-treatment)	*C. coriaceum*, Pequi aqueous extract of the leaves (AEL), and Pequi pulp oil (PPO): Pre-treatment for 7 days (1, 5, or 10 mg/g in diet) and co-treatment for 5 days (1 and 5 mg/ml)	a) Mobility defects, b) Increased mortality c) Elevated OS markers *viz.*, Increased ROS, LPO, CAT, and GST d) Elevated thioredoxin reductase and *Keap-1* levels (Stress-related genes)	Rescued PD phenotypes	Duavy et al., 2019
25	w^{1118}	10 days old, Females	18mM PQ in diet for 24 Hrs	Jujube (*Ziziphus Jujuba Mill*.) fruit powder: Diet supplemented with 30 mg/ml or 150 mg/ml for 10 days	a) Enhanced starvation stress b) Reduced lifespan	Rescued PD phenotypes	Ghimire and Kim. 2017
26	Canton S	20 days old, Male and female	10mM PQ for 0-16 hrs on filter paper	*Cistanche tubulosa* (CT) water extract: 5.4, 10.8, and 21.6 mg/ml of CT for 20 days in the food diet	a) Enhanced starvation stress b) Reduced lifespan c) Reduced memory formation	Rescued	Lin et al., 2017
27	Harwich	1-4 days old, Male and Female	5mM PQ on filter paper for 3 days	*Anacardium microcarpum* hydroalcoholic extract (AMHE), methanol (AMMF) and acetate (AMAF) fraction of *A.*	a) Mobility defects b) Reduced survival c) Elevated OS markers *viz.*, Increased ROS levels	Rescued PD phenotypes	Müller et al., 2017

				microcarpum: 1 and 10 mg/ml for 5 days in standard medium			
28	*D. melanogaster*	1-4 day old	3mM PQ for 4 days	*Chaptalia nutans* C. nutans root extract: 1, 5, and 10 mg/mL for 4 days (Co-treatment)	a) Mobility defects b) Increased mortality	RescuedPD phenotypes	Souza et al., 2020
29	*D. melanogaster*	24-day-old, Male and Female	2 hrs starvation, 20mM PQ on filter paper	*L. barbarum* polysaccharide (LBP) and two derived fractions, LBP-1 and LBP-2: 20 mg in diet supplementary for 24 days	a) Increased mortality	Rescued PD phenotypes	Tang et al., 2019
30	W^{1118}	20 days old	2 hrs starvation, 20mM PQ on filter paper	*Astragalus membranaceus;* Aqueous extract from Astragali Radix dry root (ARE): 1.25% in the basal diet for 15 to 20 days	a) Increased mortality	Rescued PD phenotypes	Zhang et al., 2022
31	w^{1118}	1 day old, Male	250 µM Rotenone (ROT) in media for 7 days	*Prunus avium L*; Anthocyanin-rich extract from Sweet Cherry (ACE): 50 µg/mL for 24 hrs in food media	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Increased ROS levels	Rescued PD phenotypes	Filaferro et al., 2022
32	*D. melanogaster*	2-8 days old, Male	500 µM ROT for 7 days in a cotton-soaked flask	Acteoside (ACT): 500 µM ACT for 7 days in cotton-soaked flasks	a) Mobility defects b) Reduced survival c) Impaired mitochondrial morphology and synaptic vesicles	Rescued PD phenotypes	Aimaiti et al., 2021
33	Oregon K	8-10 days old, Male	500 µM ROT in media for 7 days	Low Molecular Weight Chitosan (LMWC): 5 and 10mg/ml in basal media for 7 days	a) Mobility defects b) Depleted brain dopamine c) Elevated OS markers *viz.*, Elevated ROS, AchE levels d) Mitochondrial complex impairment	Rescued PD phenotypes	Pramod and Prashanth, 2020
34	STR5	1 day old, Male	24 hrs starvation, 500 µM ROT on filter paper for 24 hrs	L-dopa reduced (<0.1%) *M. pruriens* seeds extract (MPE): 40 µg/mL, Pre-treatment for 10 days on food media	a) Mobility defects	Rescued PD phenotypes	Johnson et al., 2018
35	w^{1118}	1, 20, and 30 days old, Male and female	Young flies: 200 µM of ROT for 1,10, 20, 30 days in food media Ages fly (20 days old): 200 µM of ROT for 3 and 5 days in food media	Resveratrol: Concentration of 1 µM for 24 hrs in food media	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Enhanced ROS levels d) DAergic neuronal loss e) Induces *dSARM* and enhanced *Eiger* and *Relish* expression f) Upregulation of *JAK/STAT* genes	Rescued PD phenotypes	Sur et al., 2018

36	Canton S	8-10 days old, Male	500 µM ROT for 7 days in food media	Myricetin a flavanol isolated from the brown seaweed *Turbinaria ornate*: Flies were pre-treated with 314 mM of myricetin (0.1.% effective dose) for three hrs	a) Mobility defects b) Impaired muscular coordination, Gait disturbances, Memory decline c) Depleted brain dopamine d) Elevated OS markers *viz.*, LP, SOD, CAT, and GPx activity, reduced GSH levels e) DAergic neuronal loss f) Reduced anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2) and Enhanced Bcl-2-associated X protein expression	Rescued PD phenotypes	Dhanraj et al., 2018
37	Harwich	1-3 days old, Male and Female	250 µM ROT for 7 days in diet	Kolaviron from *Garcinia kola* seeds: (100–500 mg/kg diet) throughout the lifespan and 200 mg/kg for 7 days in the diet	a) Mobility defects b) Reduced emergence and lifespan of flies c) Elevated OS markers *viz.*, Elevated H_2O_2, NO levels, reduced total thiols, AchE activity, Inhibited CAT and GST activities	Rescued PD phenotypes	Farombi et al., 2018
38	Oregon K	10 days old, Male	500 µM ROT for 7 days in diet	Curcumin and curcumin bioconjugate curcumin monoglucoside (CMG): Pre-treatment of either CMG or curcumin through diet for 5 days	a) Mobility defects b) Increased mortality c) Depleted brain dopamine, DOPAC, HVA, and Elevated DA turnover d) Elevated OS markers *viz.*, Increased ROS, H_2O_2 levels, GSH depletion	Rescued PD phenotypes	Pandareesh et al., 2016
39	Oregon K	9–10 days old, Male	500 µM ROT for 7 days	Saffron methanolic extract (SME) and its active constituent, crocin (CR): SME (0.05 and 0.1%) or CR (10 and 25 mM) enriched medium for 7 days	a) Mobility defects b) Increased mortality c) Depleted brain dopamine d) Elevated OS markers *viz.*, Enhanced ROS, HP, NO, PC, AchE levels, Reduced levels of reduced GSH and TSH e) Mitochondrial dysfunctions *viz.*, MTT reduction, Inhibition of complexes I–III	Rescued PD phenotypes	Rao et al., 2016
40	Harwich	1-5 days old, Male and Female	500 µM ROT for 7 days in diet	γ-orizanol (ORY) from rice bran oil: 25µM ORY for 7 days in the diet	a) Mobility defects, b) Depleted brain dopamine c) Elevated OS markers *viz.*, Elevated ROS, HP, MDA levels, Inhibition of SOD, CAT, GST AchE activity, decreased reduced TSH level d) Reduced MTT cell viability and mitochondria viability	Rescued PD phenotypes	Araujo et al., 2015

41	Oregon K	8-10 days old, Male	500 µM ROT for 7 days in diet	*Withania somnifera* (Ashwagandha, WS); WS root extract powder (0.005, 0.01, 0.05%) for 5 days in diet	a) Mobility defects b) Increased mortality c) Depleted brain dopamine d) Elevated OS markers *viz.*, Elevated ROS, LP, HP levels, Enhanced AchE and BchE activity, Reduced GSH and non-protein thiols e) Mitochondrial dysfunction *viz.*, Inhibition of Complex I–III and Complex II–III, Altered SDH and MTT activity levels	Rescued PD phenotypes	Manjunath and Muralidhara, 2015
42	*D. melanogaster*	1-3 days old, Male and Female	500 µM ROT for 7 days in diet	*V. officinalis* aqueous extract; 10 mg/mL in the food for 7 days	a) Mobility defects b) Increased mortality, Reduced exploratory activity c) Elevated OS markers *viz.*, Reduced total thiol, Elevated H_2O_2 levels, Elevated mRNA expression of antioxidant enzymes SOD, CAT and TH gene	Rescued PD phenotypes	Sudati et al., 2013
43	Oregon K	9-10 days old, Male	500 µM ROT for 7 days in the diet	*Selaginella delicatula* aqueous extract (SDAE): SDAE enriched diet (0.05, 0.1, and 0.2%) for seven consecutive days	a) Mobility defects b) Increased mortality c) Depleted brain dopamine d) Elevated OS markers *viz.*, Elevated ROS, PC and HP levels, Elevated activities of SOD, GSH, GST, Reduced AchE activity e) Reduced activity of NADH cytochrome c reductase and SDH	Rescued PD phenotypes	Girish and Muralidhara, 2012
44	Oregon K	10 days old, Male	500 µM ROT for 7 days in diet	*Bacopa monnieri* (BM) and Brahmi capsule (BC) powder; (0.05 and 0.1%) for 7 days in the diet	a) Mobility defects b) Increased mortality c) Depleted brain dopamine d) Elevated OS markers *viz.*, Elevated MDA, HP, PC, CAT, SOD, GST levels, Reduced GSH levels	Rescued PD phenotypes	Hosamani and Muralidhara, 2009
45	Albino Wistar	5 to 6 months old, Male (280–300g)	2.5 mg/kg ROT intraperitoneally for 4 weeks	α- Bisabolol (BSB), natural monocyclic sesquiterpene alcohol in ornamental flowers: 50 mg/kg injected intraperitoneally 30 min before ROT administration, once daily for 4 weeks	a) Elevated OS markers *viz.*, Elevated MDA, NO, and MMP-9 levels, Reduced GSH, CAT, SOD levels b) DAergic neuronal loss, Reduced DAergic transporters c) Increased activated glial cells d) Enhanced release of IL-1β, IL-6, and TNF-α e) Elevated levels of COX-2 and iNOS f) Reduced Bcl-2 level, Elevated Bax, Cleaved caspase-3 and 9 levels	Rescued PD phenotypes	Javed et al., 2020

					g) Mitochondrial dysfunction *viz.*, Inhibition of cytochrome-C release, Reduced ATP levels		
46	Sprague Dawley	1,7,14 and 24 days old, Male (250 ± 20 g)	2 mg/kg ROT subcutaneous for 21 days	α Mangostin (Polyphenolic from *Garcinia mangostana Linn)* :10mg/kg intraperitoneally for 21 days (Co-treatment)	a) Mobility and Cognitive defects b) Elevated OS markers *viz.*, Elevated MDA, NO levels, Reduced GSH levels c) DAergic neuronal loss d) Enhanced phosphorylated α-synuclein levels	Rescued PD phenotypes	Parkhe et al., 2020
47	Sprague Dawley	3 months old, Male (200-250 g)	6 µg/µL ROT intranigral for 24 hrs	Apigenin (AGN): 10 and 20 mg/kg intraperitoneally for 14 days	a) Altered muscle coordination and grip strength b) Elevated OS markers *viz.*, Elevated MDA, NO, AchE levels, Reduced GSH, SOD CAT, GPx, GR, GST activity c) Reduced brain TH and D2R protein levels d) Inhibition of Complex -I activity, Na^{+}/k^{+} ATPase, and Ca^{2+} ATPase activity e) Reduced BDNF and GDNF gene expression, Upregulation of TNF-α, IL-6, NF-κB and iNOS-1 mRNA expression f) Elevated α- synuclein protein g) DAergic neuronal loss	Rescued PD phenotypes	Anusha et al., 2017
48	Swiss albino	9-10 weeks old, Male	1 mg/kg/48h, ROT subcutaneously for 18 days	Biochanin A (BioA): 10 mg/kg intraperitoneally for 18 days	a) Mobility defects b) Depleted brain dopamine c) Elevated OS markers *viz.*, Elevated MDA, Reduced GSH levels d) Upregulation of astrocytes (GFAP protein) and elevated levels of cytokines. e) Reduced phosphorylation of PI3K/Akt/mTOR and autophagy-related protein beclin-1 f) DAergic neuronal loss	Rescued PD phenotypes	El-Sherbeeny et al., 2020
49	Wistar	8 weeks old, Male	2.5 mg/kg ROT intraperitoneally for 5 days	*Centella asiatica (*ECa233) :30 mg/kg orally for 20 days	a) Mobility defects b) Elevated OS markers *viz.*, Elevated MDA level, Reduced SOD and CAT expression levels c) Inhibited complex-I activity d) DAergic neuronal loss	Rescued PD phenotypes	Teerapattarakan et al.,2018
50	Sprague Dawley	One week old, Male (280–320 g)	2 mg/kg/day ROT subcutaneously for 10 days	Safflower (*Carthamus tinctorius*a. L.); standardized safflower flavonoid extract (SAFE), 35 or 70 mg/kg/day orally for 24 days	a) Reduced weight, rearing behavior, and grip strength b) Depleted brain dopamine, DOPAC, and HVA levels c) Elevated OS markers *viz.*, Elevated AchE levels d) Reduced brain TH protein levels	Rescued PD phenotypes	Ablat et al., 2016

					e) Reduced dopamine transporter and DJ-1 protein expression		
51	Swiss albino	3-4 months old, Male (28 ± 2 g)	3 mg/kg ROT orally for 60 days	Curcumin and mitochondria-targeted curcumin (MTC): 30 mg/kg orally for 60 days	a) Mobility defects b) Elevated OS markers *viz.*, Elevated MDA, NO, Reduced AchE, GSH, SOD, and CAT activity c) Irregular, damaged Purkinje cells and perineuronal vacuolation	Rescued PD phenotypes	Hasan et al., 2020
52	Wistar	Male (225-250 g)	2.5 mg/kg/day ROT intraperitoneally for 45 days	Demethoxycurcumin DMC:10 mg/kg orally for 45 days	a) Motor and non-motor activity defects b) Reduced brain TH protein expression c) Enhanced expression of Bax, Caspase-3, 6, 8 and 9 d) Reduced Bcl-2 expression	Rescued PD phenotypes	Ramkumar et al., 2019
53	Wistar	3 months old, Male (270–320 g)	0.5 mg/kg ROT subcutaneously for 21 days	(−) Epigallocatechin-3-gallate (EGCG) green tea catechin: 100 or 300 mg/kg intraperitoneally 60 min prior to ROT administration for 21 days	a) Reduced motor and non-motor activities b) Depleted brain dopamine, NE, 5-HT,5-HIAA, and elevated DOPAC and HVA levels c) Elevated OS markers *viz.*, Elevated NO, LP, Reduced GSH, SOD, and CAT levels d) Mitochondrial dysfunction *viz.*, Reduced activity of SDH, ATPase, and inhibited Complex-I-III e) Elevated levels of TNF-α, IL-1β, IL-6, caspase-3	Rescued PD phenotypes	Tseng et al., 2020
54	Wistar	6-7 months old, Male	2.5 mg/kg ROT intraperitoneally for 4 weeks	Ferulic acid (FA): 50 mg/kg, 30 minutes prior to ROT administration intraperitoneally for 28 days	a) Elevated OS markers *viz.*, Elevated MDA, Reduced GSH, CAT, SOD levels b) Elevated activated glial cells (GFAP & Iba-1) c) Increased release of IL-1β, IL-6, and TNF-α d) Elevated levels of COX-2 and iNOS e) DAergic neuronal loss	Rescued PD phenotypes	Ojha et al., 2015
55	Wistar	6 weeks old, Male (250–300 g)	1.3 mg/kg ROT subcutaneously for 35 days	Pomegranate (Punica granatum L.) juice (Gallic acid/ ellagic acid): 500 mg/kg intra-gestational for 45 days (10 days pre-treatment and 35 days combined treatment with ROT)	a) Reduced motor and non-motor activities b) Elevated OS markers *viz.*, Elevated MDA, Reduced mitochondria ALDH2 activity, Reduced CAT, GPx, GSH, and GST activity c) Reduced Bcl-xL expression d) Elevated α-synuclein expression e) DAergic neuronal loss	Rescued PD phenotypes	Kujawska et al., 2019
56	Wistar	Male (240-280 g)	3 µg/µl ROT intranigral infusion for 24 hrs	*Garcinia* kola seeds extract Kolaviron (KV): 200 mg/kg orally for 6 weeks (Pre-treated with KV for 3 weeks and post-	a) Altered motor and non-motor behaviors b) Elevated OS markers *viz.*, Elevated MDA, GST level, Reduced GSH, CAT, AchE activity c) Reduced protein levels of GRP78/Bip and XBP1 d) DAergic neuronal loss	Rescued PD phenotypes	Farombi et al., 2020

				treated with KV for another 3 weeks)			
57	Wistar	11-12 months old, Male (280–300 g)	2.5 mg/kg ROT intraperitoneally for 4 weeks	Lycopodium (Lyc): 50 mg/kg orally once daily for 4 weeks 30 mins before ROT administration	a) Elevated OS markers *viz.*, Elevated MDA, NO, Reduced GSH, CAT, SOD levels b) Increased activated glial cells (GFAP & Iba-1) c) Elevated release of IL-1β, IL-6, and TNF-α d) Elevated protein expression of MMP-9 and MMP-3 e) Elevated α-synuclein protein expression f) Elevated levels of COX-2 and iNOS g) DAergic neuronal loss	Rescued PD phenotypes	Jayaraj et al., 2019
58	Sprague-Dawley	12–15 months old, Male (300–350 g)	1 µg/1 µl ROT intracerebroventricular into SNpc	Ethyl acetate extract of *Morinda citrifolia* (MCE): 150 mg/kg pre-treated orally for 30 days and another 30 days post-recovery from stereotaxic surgery	a) Mobility defects b) Altered posture (catalepsy) c) Inhibition of Complex-I, IV activities d) Enhanced mRNA and protein expression of Bax, Caspase-3, 9 e) Reduced mRNA and protein expression of Bcl-2 f) DAergic neuronal loss	Rescued PD phenotypes	Kishore Kumar et al., 2017
59	Wistar	6-8 weeks old, Male (250–300 g)	2 µl ROT intracerebroventricular for 1 day into SNpc	Naringin: 80 mg/kg intraperitoneally for 14 days	a) Climbing defects b) Impaired posture (catalepsy) c) Elevated OS markers *viz.*, Reduced GPx, GR activities d) Reduced brain TH protein expression e) Depleted brain dopamine, DOPAC, and HVA, Elevated dopamine turnover f) Mitochondrial dysfunction *viz.*, Reduced MMP, Inhibited Complex-I, II, IV, V activities, Reduced ATP g) Enhanced protein expression of Cytochrome-c, Caspase-3, 9 h) DAergic neuronal loss	Rescued PD phenotypes	Garabadu and Agrawal, 2020
60	Albino	Male (180-200 g)	2 ml/kg/day ROT intraperitoneally for 4 weeks	Quercetin: 50 mg/kg/day intraperitoneally for 4 weeks	a) Reduced motor activity, muscle coordination, and grip strength b) Impaired posture (catalepsy) c) Depleted brain dopamine and TrxR activity d) Elevated OS markers *viz.*, Elevated MDA and DNA fragmentation, CHOP levels e) Reduced Beclin-1level f) DAergic neuronal loss	Rescued PD phenotypes	El-Horany et al., 2016

61	Wistar	Male (180-250 g)	2 mg/kg ROT subcutaneously for 35 days	Resveratrol (RSV) and Resveratrol nanoparticles (NRSV): 40 mg/kg orally for 35 days	a) Altered rearing behavior, Reduced muscle coordination, and grip strength b) Elevated OS markers *viz.*, Elevated MDA, Reduced GSH, and CAT levels c) Mitochondrial dysfunction *viz.*, Reduced SDH, Citrate synthase, aconitase, and inhibited Complex- I activity d) DAergic neuronal loss	Rescued PD phenotypes	Palle and Neerati, 2018
62	Wistar	6-8 months old, Male (280-300 g)	2.5 mg/kg ROT intraperitoneally for 4 weeks	Thymol (dietary monoterpene Phenol):50 mg/kg intraperitoneally 30 min prior to ROT for 4 weeks	a) Elevated OS markers *viz.*, Elevated MDA, Reduced GSH, CAT, SOD levels b) Increased activated glial cells (GFAP & Iba-1) c) Elevated release of IL-1β, IL-6, and TNF-α d) Elevated levels of COX-2 and iNOS e) Reduced TH fiber intensity f) DAergic neuronal loss	Rescued PD phenotypes	Javed et al., 2019b
63	Swiss albino	Male, (15-20 g)	1 mg/kg per 48 hrs ROT subcutaneously; 9 times	*Vicia faba* methanolic extracts of seeds or sprouts:200, 400, and 600 mg/kg/day orally for 17 days	a) Mobility defects b) Reduced brain dopamine c) Elevated OS markers *viz.*, Elevated MDA level d) Elevated release of IL-1β, NF-κB, and TNF-α e) DAergic neuronal loss	Rescued PD phenotypes	Abdel-Sattar et al., 2021
64	albino	8 weeks old, Male (20–25 g)	10 mg/kg PQ intraperitoneally twice a week for 3 weeks	Pomegranate (*Punica granatum L.*) seed extract (PSE) and juice (PJ): 500 mg/kg/day, by gavage for 2 weeks before PQ injection and then continued along with PQ treatment for another 3 weeks 5 ml of 1:40 dilution of PJ daily by gavage for 2 weeks prior to Pre-treatment and then administrated daily with PQ treatment for further 3 weeks	a) Reduced brain TH protein b) Depleted brain dopamine, DOPAC c) Elevated OS markers *viz.*, Elevated MD, Reduced GPx, CAT, SOD levels, d) Reduced ATP level e) Enhanced release of IL-1β, IL-6, and TNF-α f) Reduced IL-10 release g) Elevated *NF-κB* gene expression h) Elevated TGF-β, CD11b, and reduced GDNF protein levels	Rescued PD phenotypes	Fathy et al., 2021
65	CFT-Swiss	4 weeks old, Male (22–26 g)	15 mg/kg PQ intraperitoneally for 3 hrs	*Bacopa monnieri* (BM) standardized extract: 200 mg/kg orally for 4 weeks	a) Reduced body weight b) Depleted brain dopamine c) Elevated OS markers *viz.*, Elevated ROS, MDA, and HP levels, Elevated AchE, BchE activities d) Mitochondrial dysfunction *viz.*, Inhibited Complex I-III, II-III, SDH, MMT activity	Rescued PD phenotypes	Hosamani et al., 2014
66	Sprague-Dawley	6-8 weeks old, Male, (140-200 g)	10 mg/kg PQ intraperitoneally on day 8-12th of	*Zingiber zerumbet* ethyl acetate extract: 200 and 400 mg/kg orally for 19 consecutive days	a) Elevated tremors b) Elevated OS markers *viz.*, Increased MDA, PC levels, Reduced GPx, GSH, SOD, CAT activities	Rescued PD phenotypes	Ibrahim et al., 2018

			the treatment regime		c) DAergic neuronal loss		
67	Wistar	Male (150 g ± 10 g)	10 mg/kg orally for 40 days	*B.cernua* ethanolic extract (BCE): 500mg/kg orally for 40 days	a) Mobility defects and altered exploratory behavior, Reduced neuromuscular strength, Elevated anxiety-related behaviour, Reduced cognition, and spatial memory b) Depleted brain dopamine and serotonin c) Elevated OS markers *viz.*, Elevated MDA, AchE activity, Reduced GPx CAT, SOD, GSH levels d) Elevated release of IL-6, and TNF-α e) DAergic neuronal loss	Rescued PD phenotypes	Saadullah et al., 2022
68	Long-Evans hooded	5 months old, Male	10 mg/kg PQ intraperitoneally 5 times in 20 days	Ubisol-Q10 and ethanolic root extract of ashwagandha (ASH): 2 mg/mL ethanolic ASH extract + 50 µg/mL Ubisol-Q10 orally for 4 months	a) Mobility defects b) Reduced brain TH protein c) DAergic neuronal loss d) Reduced CARP1 levels e) Elevated LP (4-HNE) f) Increased activated glial cells (Iba-1), Reduced activation of GFAP g) Reduced GDNF and pro-BDNF fluorescence level h) Reduced Beclin-1 expression	Rescued PD phenotypes	Vegh et al., 2021
69	Swiss albino	Male (25 ± 5g)	10 mg/kg PQ intraperitoneally twice a week for 3, 6, and 9 weeks	*Mucuna pruriens* aqueous extract (Mp): 100 mg/kg orally 1 week prior to PQ and twice a week for 3, 6, and 9 weeks	a) Mobility defects b) Elevated OS markers *viz.*, Increased NO, MDA, reduced CAT levels, c) Reduced brain TH neuronal fibers density d) DAergic neuronal loss e) Increased iNOS immunoreactivity f) Enhanced *iNOS/GAPDH* -mRNA expression and iNOS protein expression	Rescued PD phenotypes	Yadav et al., 2013; 2017
70	Swiss albino	Male (25 ± 5 g)	30 mg/kg MB &10 mg/k PQ intraperitoneally twice a week for 3, 6 and 9 weeks	*Withania somnifera* (Ashwagandha) Ethanolic root extract of WS): 100 mg/kg orally daily for 3, 6, and 9 weeks	a) Behavioral defects (Reduced hanging, Gripping, Walking error, Posture) b) Depleted brain dopamine, DOPAC, and HVA levels c) Elevated OS markers *viz* Elevated MDA, NO levels, reduced CAT activity d) Reduced protein expression of Bcl-2 e) Elevated iNOS, Bax expression, and mRNA *iNOS* expression f) Elevated activated astrocytes (GFAP) g) DAergic neuronal loss	Rescued PD phenotypes	Prakash et al., 2013; 2014

71	Swiss albino	Male	1mg/kg/48 hrs subcutaneously for 9 times	*Tribulus terrestris* standardized extract (TTE): 5 or 10mg per kg, by oral gavage) for 17 days	a) Motor defects b) Elevated DNA damage (Upregulated 8-OHdG and MTH1 expression) c) Upregulation of CD11b d) Reduced TH immunoreactivity e) DAergic neuronal loss	Rescued PD phenotypes	Alzahrani et al., 2018
72	albino	4 weeks old, Male, (25.3 ± 2.4 g)	1.0 mg/kg ROT intraperitoneally for 21 days	*Selaginella delicatula* aqueous extract (SDAE): 100 mg/kg, orally for 21 days	a) Mobility defects b) Reduced brain dopamine c) Elevated OS markers *viz* Elevated ROS, MDA, PC SOD, GST, HP, TR, Reduced activity of GPx, BchE, Elevated AchE activity d) Mitochondrial dysfunction *viz.*, Inhibited Complex-I, II, MMP, CS and ATPase activity	Rescued PD phenotypes	Girish and Muralidhara, 2013
73	Wistar	Male (100–150 g)	10 mg/kg PQ orally for 14 days	*Phyllanthus amarus* aqueous leaf extract; 200, 300, 400 mg/kg orally for 14 days	a) No mobility defects b) Elevated OS markers *viz* Reduced SOD levels c) DAergic neuronal loss	Rescued PD phenotypes	Enemali et al., 2024
74	CFT-Swiss	6 weeks old, Male	1.0 mg/kg ROT intraperitoneally for 21 days	Aqueous extract of tomato seeds (TSE); 100 mg/kg, orally 1 hrs before ROT for 21 days	a) Mobility defects b) Altered gait pattern, elevated Anxiety c) Depleted brain dopamine d) Elevated OS markers *viz* Elevated ROS, MDA, PC, HP levels, Reduced activity of SOD, GST, Elevated activity of BchE and AchE e) Inhibited Complex-I, II activity	Rescued PD phenotypes	Krishna and Muralidhara, 2016
75	Wistar	Male (180-200 g)	1.5 mg/kg ROT (11 injections) subcutaneous for 21 days	*Pulicaria undulata* essential oil (PUEO): 50, 100, and 200 mg/kg for 21 days (1 hrs before rotenone on the days of its injection)	a) Reduced coordination and motor activity b) Depleted brain dopamine c) Elevated OS markers *viz* Elevated MDA level, reduced GSH levels d) Reduced ATP levels e) Elevated expression of IL-1β, and TNF-α f) Upregulation of iNOS protein expression g) Elevated α-synuclein gene expression (DAergic neuronal loss)	Rescued PD phenotypes	Issa et al., 2020

Table 1.1: Summary of the environmental toxin-mediated animal models of PD and nutraceutical/ plant extracts based/associated therapeutic interventions.

S. No.	Fly (Gain of functionGOF; Loss of function – LOF)	Age/Sex	Neurotoxin treatment strateg	Plant extract intervention strategies	PD phenotypes scored	Remark(s)	Reference(s)
1	*a-synuclein* (*A30P* and *A53T*α-synuclein)	5, 25 days old, Male	3 hrs starvation, 10 mM PQ for 48 hrs in culture media	*Decalepis hamiltonii (Dh)* root aqueous extract: 0.1 or 0.5% in fly food medium for 21 days	a) Mobility defects b) Increased mortality c) Impaired circadian rhythm d) Elevated OS markers *viz.*, Elevated ROS and LP Levels, Reduced SOD and CAT activity	Rescued PD phenotypes	Jahromi, et al., 2015
2	*Parkin* (Knockdown (KD)	3 days old, Female	3 hrs starvation, 1 mM PQ for 48 hrs on filter paper	*Avocado Persea Americana (Pa)* peel methanol extract:1 or 5 mg/mL in fly medium	a) Mobility defects b) Shortened lifespan c) Elevated OS markers *viz.*, Elevated LP level d) Reduced Brain TH protein levels e) Reduced *parkin* protein levels f) No DAergic neuronal loss	Rescued PD phenotypes	Ortega-Arellano et al., 2011, 2019
3	*a-synuclein* (Elav-Gal4 overexpression)	24 days old, Male	---	Diet supplementation of Tangeritin: 5, 10, and 20 µM of tangeritin mixed in diet for 24 days	a) Mobility defects b) Loss of activity pattern c) Depleted brain dopamine content d) Increased MAO activity e) Elevated OS markers *viz.*, Elevated levels of LP, PC, GST, Reduced GSH level	Rescued PD phenotypes	Fatima et al., 2017
4	*a-synuclein*	6, 12 days old, Male	---	Grape (*Vitis Vinifera*)-0.16–0.64 mg grape extract in 100 g of fly culture medium	a) Mobility defects b) Reduced lifespan c) Elevated OS markers *viz.*, Elevated ROS LP and DNA damage	Rescued PD phenotypes	Long et al., 2009
5	*a-synuclein* (Elav-Gal4 overexpression)	21 days old, Male	---	Diet supplementation of Capsaicin: 0.1,0.5,0.75,1.0 µL/mL of capsaicin in the fly medium for 21 days	a) Mobility defects	Rescued PD phenotypes	Siddique et al., 2012

6	*a-synuclein* (Elav-Gal4 overexpression)	24 days old, Male	---	Diet supplementation: 20, 40, 80, and 100 µM of capsaicin for 24 days	a) Depleted brain dopamine b) Increased MAO activity c) Elevated OS markers *viz.*, Reduced GSH, Elevated PC, LP, and GST levels	Rescued PD phenotypes	Siddique et al., 2018
7	*a-synuclein*	24 days old, Male	---	Diet supplementation of Black tea: 20, 40, and 60µM extract of black tea in fly medium for 24 days	a) Mobility defects b) Loss of activity pattern c) Depleted brain dopamine d) Elevated OS markers *viz.*, Reduced GSH levels, Increased LP, GST levels e) DAergic neuronal loss	Rescued PD phenotypes	Siddique et al., 2017
8	*a-synuclein*, *Parkin* null mutant (LOF)	20 days old	---	Dietary supplementation of Decaffeinated coffee and nicotine-free tobacco: 0.15% decaffeinated coffee and 0.03% nicotine-free tobacco in fly medium	a) Mobility defects b) Increased mortality c) DAergic neuronal loss (PPL1)	Rescued PD phenotypes	Trinh et al., 2010
9	*a-synuclein* (Elav-Gal4 overexpression)	24 days old, Male	---	Diet supplementation of curcumin: 25, 50, and 100 µM in the fly medium for 24 days	a) Reduced lifespan b) Loss of activity pattern c) Elevated OS markers *viz.*, Enhanced PC, LP levels d) DAergic neuronal loss	Rescued PD phenotypes	Siddique et al., 2014
10	Tyrosine hydroxylase (TH) Gal4/UAS-X (RNAi Knockdown), Canton-S	3 days old, Female	3 hrs starvation, 1 mM PQ for15 days on filter paper	Gallic acid: 0.1 mM gallic acid for 15 days on filter paper	a) Climbing defects b) Increased mortality c) DAergic neuronal loss	Rescued PD phenotypes	Ortega-Arellano et al 2013
11	*PINK* 1^{B9} (LOF)	7, 15 days old, Male	.---	Dietary administration of Resveratrol and grape skin extract (GSE): 0.2 and 1mM for 12 hrs for 7 days in the food diet and 4%, 8%, and 16% w/v of GSE powder in the food diet for 7 days	a) Climbing defects b) Reduced lifespan c) Altered posture d) Flight muscle loss e) Elevated OS markers *viz.*, Increased ROS f) Mitochondrial dysfunction viz., Reduced ATP production and mitophagy Inhibited complex-I activity	Rescued PD phenotypes	Wu et al., 2018

12	*Parkin* (TH-GAL4; UAS-RNAi-parkin Knockdown)	3 days old, Female	3 hrs starvation, 1,5,20mM PQ for 15 days on filter paper	Propyl gallate (PG): 0.1 mM and epigallocatechin gallate (EGCG): 0.1, 0.5 mM for 15 days on filter paper	a) Climbing defects b) Increased mortality c) DAergic neuronal loss (PPL1)	Rescued PD phenotypes	Bonilla-Ramirez et al., 2013
13	*DJ-1β* (*DJ-1β*^{ex54}strain)	5 days old, Male	---	Vincamine (*Vinca minor*):10 µM Vincamine during development and 5 days after eclosion	a) Elevated OS markers *viz.*, Elevated ROS and PC levels	Rescued PD phenotypes	Sanz et al., 2022
14	*Pink1*^{B9} (LOF)	1st instar larvae, 20, 30 days old, Male	PQ	Dietary administration of Gastrodin *(Rhizoma Gastrodiae):* 2 mM gastrodin from the 1st instar larvae to 20 or 30 days	a) Climbing defects b) Increased mortality c) Depleted brain dopamine d) Elevated OS markers *viz.*, Increased ROS levels e) DAergic neuronal loss (PPL1)	Rescued PD phenotypes	He et al.,2021
15	*DJ-1β*$^{\Delta}$ 93, *Oregon R+*	Male, female	10, 20, and 30 mM PQ in the diet	Dietary supplementation of Spirulina (*Arthrospira platensis*) and its active component C- phycocyanin: 5% and 10% w/v in the food media	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Elevated SOD, CAT activity	Rescued PD phenotypes	Kumar et al., 2017
16	*Pink1*^{B9} mutant (LOF)	larvae, 5, 18, 30 days old, Male	20mM PQ in food media for 2 days	Ginseng total protein (GTP): 0.02, 0.04, and 0.16 mg/mL in the standard diet for larvae and adults, 0.04 mg/mL GTP administration at 3–5 (I), 15–18 (II) and 25–30 (III) days	a) Mobility defects b) Reduced lifespan c) Abnormal wing posture d) Depleted brain dopamine d) Elevated OS markers *viz.*, Enhanced ROS levels e) Mitochondrial dysfunction *viz.*, Inhibited Complex-I, II, Reduced ATP, mtDNA, and MMP f) DAergic neuronal loss (PPL1)	Rescued PD phenotypes during 3–5 (I), 15–18 (II) but failed during (25-30 III)	Liu et al., 2019
17	Canton-S, TH > dj-1-β-RNAi/+ (knocked down *dj-1-β*function)	3 days old, Female	3 hrs starvation, 1 mM PQ for 10 and 15 days on filter paper	Epigallocatechin-3-gallate (EGCG) (*Camellia sinensis):* 0.5 mM for 10 days and 15 days on filter paper; 5 days pre-feeding	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Enhanced LP d) Reduced levels of Dj-1-β protein e) No change in brain TH protein	Rescued PD phenotypes	Martinez-Perez et al., 2018

18	Oregon K, UAS-*sod1*-IR, UAS *cat*-IR, RNAi line	5 days old, Male and Female	10 and 8mM PQ for 24 hrs on filter paper	4-Hydroxyisophthalic acid (DHA-I) form roots of (*Decalepis hamiltoni):* DHA-I-0.02% (1.1 mM) supplemented in the diet for five days	a) Reduced lifespan b) Elevated OS markers *viz.*, Reduced CAT, and SOD 1 activity	Rescued PD phenotypes	Niveditha and Shivanandappa, (2022)
19	UAS-MitoGFP/Cy; Mef2	1 day old, Female	170 mg/kg body weight PQ for 3 days in diet	Dietary supplementation of eicosapentaenoic and docosahexaenoic acids (EPA and DHA, omega-3 long-chain fatty acids): Concomitant EPA/DHA 0.31/0.19 mg/kg body weight for 3 days	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Increased H_2O_2 levels and impaired AchE activity d) DAergic neuronal loss	Rescued PD phenotypes	De Oliveira Souza, et al., 2019
20	Canton-S, *Ddc-GAL4*	3 days old, Female	3 hrs starvation, 1 mM PQ for 15 days on filter paper	1% to 10% glucose for 15 days on filter paper	a) Mobility defects b) Increased mortality	Rescued PD phenotypes	Ortega-Arellano et al., 2011
21	*Parkin* knockdown	3 days old, Female	3 hrs starvation, 1 mM PQ for 15 days on filter paper	Methanolic Extract of Avocado *Persea americana* (var. Colinred) CRE Peel: 0.1 and 0.5 mM (1 mg/mL) and (5 mg/mL) for 15 days on filter paper	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Enhanced MDA levels d) No change in brain TH protein levels e) No DAergic neuronal loss	Rescued PD phenotypes	Ortega-Arellano et al 2019
22	*a-synuclein*-(Elav-Gal4 overexpression)	24 days old, Male	---	Diet supplementation of Geraniol: 10, 20, and 40 µM for 24 days in diet.	a) Mobility defects b) Loss of activity pattern c) Depleted brain dopamine d) Elevated OS markers *viz.*, Increased PC, LPO, and GSH depletion, GST activity e) DAergic neuronal loss	Rescued PD phenotypes but failed to protect DAergic neurons	Siddique et al., 2016
23	*a-synuclein*-(Elav-Gal4 overexpression)	24 days old, Male	---	Crosotebush (*Larrea tridentata*) Nordihydroguaiaretic acid (NDGA): 0.1, 0.5, and 1µl/ml in the diet for 24 days	a) Mobility defects	Rescued PD phenotypes	Siddique et al., 2012
24	*a-synuclein*-(Elav-Gal4 overexpression)	21 days old, Male	---	Acetone extract *Eucalyptus citriodora L.* leaf: 0.25, 0.50 and 1.0 µl/ml in diet for 21 days	b) Mobility defects c) Elevated OS markers *viz.*, Elevated LP	Rescued PD phenotypes	Siddique et al., 2013

25	*a-synuclein*-(Elav-Gal4 overexpression)	24 days old, Male	---	Epicatechin gallate (EG): 0.25, 0.50, and 1.0 μg/mL in diet for 24 days	a) Mobility defects b) Elevated OS markers *viz.*, Elevated LP c) DAergic neuronal loss	Rescued PD phenotypes	Siddique et al., 2014
26	*a-synuclein*-(Elav-Gal4 overexpression)	24 days old, Male	---	*Bacopa monnieri* leaf extract: 0.25, 0.50, and 1.0 μg/mL in diet for 24 days	a) Climbing defects b) Loss of activity pattern c) Elevated OS markers *viz.*, Increased PC, LP levels d) DAergic neuronal loss	Rescued PD phenotypes	Siddique et al., 2014
27	*a-synuclein*-(Elav-Gal4 overexpression)	21 days old, Male	---	*Ocimum sanctum* leaf extract: 0.042 8×10^{-4}, 0.87×10^{-4}, and 1.85×10^{-4} g·mL^{-1} in diet for 21 days	a) Mobility defects b) Elevated OS markers *viz.*, Enhanced LP	Rescued PD phenotypes	Siddique et al., 2014
28	*a-synuclein*-(Elav-Gal4 overexpression)	24 days old, Male	---	Diet supplementation of *Majun Baladur*: 0.0014, 0.0028, 0.0042, and 0.0056 g per 20 g in diet for 24 days	a) Mobility defects b) Activity pattern c) Increased mortality d) Elevated OS markers *viz.*, Reduced NPSH, Enhanced PC, LP, GST, CAT, and SOD e) Reduced brain TH activity	Rscued PD phenotypes	Siddique et al., 2019
29	*a-synuclein*-(Elav-Gal4 overexpression)	24 days old, Male	---	Myricetin: 10, 20, and 40 µM in diet for 24 days	a) Climbing defects b) Increased mortality	Rescued PD phenotypes	Ara et al., 2017
30	w^{1118}& $PINK1^{B9}$	21 days old	---	Ethanoic extract of propolis: 250, 500 mg/mL in diet for 21 days	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Enhanced CAT activity	Rescued PD phenotypes	Ayikobua et al., 2020
31	$PINK1^{B9}$	10-15 days old, Male	---	Methanolic extract of *M. pruriens* or *W. somnifera* at 0.1% w/w in larvae and adults (L+/A+) or in adults only (L2/A+)	a) Elevated OS markers *viz.*, Reduced GSH and SOD activity b) Unexpected longer telomeres	Rescued PD phenotypes	Baroli et al., 2019
32	*Hsp70Ba, Hsp70Aa, CecA1, Amy-p, and Drs (RNAi* knock-down*)*	3 and 20 days old Male	PQ	Blend of Curcumin and Broccoli Seed Extract (BSE): 0.8, 1.6, and 3.2 g/L in food diet	a) Increased mortality b) DAergic neuronal loss	Rescued PD phenotypes	Cheng et al., 2021

33	*Park13* or *DJ 1βDelta93*	Larvae, 1 and 35 days old, Male	6 hrs starvation, 10mM PQ for 24 hrs on filter paper	*Amalaki Rasayana* (*Phyllanthus emblica*, synonym *Emblica Officinalis*): 0.5% AR since the first instar stage to adult	a) Increased mortality b) Reduced heat tolerance c) Elevated OS markers *viz.*, Increased ROS and LP, Reduced activity of SOD	Rescued PD phenotypes	Dwivedi and Lakhotia, 2016
34	*DJ-1RNAi*	1, 10, and 25 days old	---	*Tripterygium wilfordii* (TW) celastrol: Dietary feeding of 1, 5, and 20 μg/ml for 25 days	a) Mobility defects b) Increased mortality c) Depleted brain dopamine d) DAergic neuronal loss	Rescued PD phenotypes	Faust et al., 2009
35	*DJ-1β* mutant	5 days old, female	---	*Vinca minor*; Vincamine (VIN):10 μM VIN during development and for 5 days	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Enhanced ROS and PC levels	Rescued PD phenotypes	Sanz et al.,2023
36	esg-Gal4 UAS-GFP	25-days old, Male	2 hrs starvation, 20mM PQ for 24 hrs on filter paper	*Ipomoea batatas* L; Purple Sweet Potato Extract (PSPE): 0.5, 2.0 mg/mL in diet for 25 days	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Increased MDA, Reduced SOD, CAT activity d) Reduced SOD and CAT gene expression e) Reduced autophagy pathway activation	Rescued PD phenotypes	Han et al., 2021
37	DDC-Gal4 < UAS-*syn* (overexpression of α-synuclein)		---	Double Stem CellR (DSC), Swiss apples (*Malus Domestica*) and Burgundy grapes (*Vitis vinifera*): 0.1, 10, or 100 mg/ml for 28 days in the diet	a) Mobility defects b) Increased mortality	Rescued PD phenotypes	Ishola et al., 2022
38	*(LRRK2-G2019S*	3 days old	---	Water extracts of GE (WGE) and its bioactive compounds, gastrodin and 4-HBA: 0.1, 0.5, or 1.0% WGE, 0.1 or 1.0 mM gastrodin (B), and 0.1 or 1.0 mM 4 hydroxybenzyl alcohol (4-HBA) for 1,2,3,4,5,6 week in food diet	a) Mobility defects b) DAergic neuronal loss (PPL1, PPL2, PPM1/2, and PPM3 clusters) c) Enhanced accumulation and hyperactivation of G2019S proteins d) Activated Nrf2 in glia	Rescued PD phenotypes	Lin et al., 2021

39	*α-synuclein*	30 days old	---	Tianma Gouteng Yin (TGY) water extract: 10,50, 100 mg in diet for 30 days	a) Mobility defects b) Depleted brain dopamine c) Reduced brain TH protein levels d) Enhanced α -α-synuclein protein and DAergic neuronal loss	Rescued PD phenotypes	Liu et al., 2015
40	*dUCH*-knockdown	larval stage, 5, 10 days old	---	Dietary supplementation of *Polyscias fruticose* leaves extract: 1, 2, 4, 8, and 16mg/mL during development and for 10 days	a) Mobility defects b) Increased mortality c) DAergic neuronal loss (PPL1and PPM3	Rescued PD phenotypes	Ly et al., 2022
41	*Park*^{25} (LOF)	1 day old	---	Aqueous extract of *Ashwagandha*-root (ASH-root extract): 0.6% w/v of ASH-root extract from larval stage till the last day of their survival (L^+/A^+) (1 to day 20)	a) Mobility defects b) Increased mortality	Rescued PD phenotypes	Murthy et al., 2024
42	*PINK1*^{B9}	Males	---	Mucuna pruriens methanolic extract (Mpe)-0.1, 1 and 10% w/w in standard diet) both in larvae and adults (L^+/A) for 25 days; Assayed on 3–6 (I), 10–15 (II) and 20–25 (III) days old; (0.1% w/w rescue dose for Assays)	a) Mobility defects b) Increased mortality c) Impaired olfactory behavior d) Reduced brain TH protein levels e) Mitochondrial dysfunction *viz.*, Reduced T-bars density in antennal lobes and thoracic ganglia	Rescued PD phenotypes	Poddighe et al.,2014
43	LRRK2^{WD40}	Males		Methanolic extract of *Withania somnifera* root (Wse): 0.1, 1and 10% w/w in standard diet) both as larvae and adults (L^+/A^+) for 25 days; Assayed on 3–6 (I), 10–15 (II) and 20–25 (III) days old; (1% w/w rescue dose for Assays)	a) Mobility defects b) Increased mortality c) Mitochondrial dysfunction *viz.*, Reduced T-bars density in antennal lobes and thoracic ganglia	Rescued PD phenotypes	De Rose et al., 2016
44	*park*^{13}	7, 15, and 20 days old, Males	10mM PQ for 24 hrs	*(Convolvulus pluricaulis)* Scopoletin (Sp)- First instar larvae orally administered with 2.5mM Sp 10 days	a) Mobility defects b) Increased survival c) Elevated OS markers *viz.*, Elevated ROS, LP levels d) DAergic neuronal loss	Rescued PD phenotypes	Pradhan et al., 2020

					e) Mitochondrial dysfunction *viz.*, Inhibited complex I activity, Reduced MMP		
45	*A53T α-syn* (Overexpression of A53T α-syn via the Gal4-UAS system)	9 to 19 days old, Females	---	Cinnamon extract precipitation (CEppt): 0.75 mg/mL CEppt administered in food diet from larvae to 19 days	a) Mobility defects b) DAergic neuronal loss	Rescued PD phenotypes	Shaltiel-Karyo et al., 2012
46	*PINK* 1^{B9} (LOF)	7, 12,18 days old, Males	---	*Lycium barbarum* ; Methanol extract of LB fruit (LBFE): 0.1% LBFE w/w in culture medium for 6, 12, and 18 days	a) Mobility and olfactory deficits b) Increased mortality c) Elevated atrophy d) Depleted brain dopamine e) Reduced ATP production f) Reduced mRNA levels of *SOD1*, *SOD2*, and *CAT* antioxidant genes	Rescued PD phenotypes	Bai et al., 2022

Table 1.2 Summarizes the *Drosophila* models of genetic PD and nutraceutical/ plant extracts based/associated therapeutic intervention.

1.4 Toxin models of PD

The majority of PD cases are believed to be sporadic in nature, suggesting an involvement of environmental factors. Studies indicate that individuals living in rural areas who use pesticides on crops, drink well water, and work in mining for an extended period are more likely to develop PD (Elbaz et al., 2009; Ritz et al., 2009). Several animal models have shed light on the pathophysiology, etiology, and molecular mechanisms underpinning the disease progression (Dovonou et al., 2023; Pingale and Gupta, 2020). Neurotoxins such as 1-methyl-1,2,3,6-tetrahydropiridine (MPTP), 6-hydroxydopamine (6-OHDA), agricultural pesticides, 1,1′-dimethyl-4,4′-bipyridinium dichloride (Paraquat; PQ), and rotenone that specifically damage the catecholaminergic system produces these animal models associated with PD and mimic PD symptoms. Despite the variations in their modes of action, all these neurotoxins eventually contribute to the degeneration of DAergic neurons in the *substantia nigra pars compacta* (SNpc)**,** which is evident by the presence of LB structure (Accumulation of α-syn protein, ubiquitin, and gliosis), depletion of DA and its metabolites (DOPAC, 3,4-Dihydroxyphenylacetic acid and HVA, Homovanillic acid), mitochondrial dysfunction, OS (ROS) generation, neuroinflammation, impaired ubiquitin proteasomal system (UPS) and autophagy. To test therapeutic intervention strategies intended to mitigate PD symptoms, neurotoxin models seem to be the most appropriate. The following sections describe some of the most widely used neurotoxin-mediated animal models of PD.

1.4.1 MPTP

MPTP is a by-product obtained during meperidine synthesis (Langston, 2017). In the early 1980s, a group of young drug abusers from northern California developed Parkinsonian syndrome after an inadvertent intravenous injection of a narcotic

meperidine analog (Langston et al., 1983). The discovery of MPTP is now widely used to study PD in various model systems. MPTP readily crosses the blood-brain-barrier (BBB) and oxidizes to form 1-methyl-4-phenylpyridinium ion (MPP^+) using MAO-B in glia to its active metabolites (Langston, 2017). MPP^+ is taken up by the DAergic neurons through DA Transporter (DAT) and is either stored in the cytoplasmic vesicles or released into the cytoplasm, where it inhibits the mitochondrial complex I, causing neurotoxicity by releasing the ROS, specifically superoxide radicals that can cause DNA damage (Rossetti et al., 1988) and reduces the levels of adenosine triphosphate (ATP) (Chan et al., 1991). MPP^+ also exhibits specific apoptotic cascade factors such as cytochrome C and caspases in *substantia nigra* (SN), leading to apoptosis (Przedborski and Vila, 2001) and inflammation by activating microglia and macrophages by increasing pro-inflammatory cytokines, tumor necrosis factor (TNF), and interferons (Lofrumento et al., 2011). The neurotoxic effects of this toxin result from the degradation of vesicular monoamine transporter-2 (VMAT-2), which subjected DA to auto-oxidation (Lohr et al., 2014). Gastrointestinal abnormalities are another significant PD characteristic brought on by MPTP (Poirier et al., 2016). Pre-treatment with DAT inhibitors, such as difluoropine, or MAO-B inhibitors, such as selegiline or rasagiline, can prevent MPTP-induced DAergic neurotoxicity. MPTP administration in non-human primates can cause changes in locomotor activity. In some cases, the MPTP-treated primates may show some features of bradykinesia, rigidity, abnormal posture, tremor, dyskinesia, and stereotypy (Porras et al., 2012). According to Langston (2017), MPTP-induced Parkinsonian symptoms closely resemble those of PD patients. Therefore, developing animal PD models with this neurotoxicant is appealing (Rai and Singh, 2020; Zeng et al., 2018). Although MPTP is more often used in mice models as compared to primates, mainly because of lower cost; primate models are preferred when testing drug treatment protocols before human studies

(Verhave et al., 2011; Antzoulatos et al., 2010; Rose et al., 1993; Langston et al., 1999). Rodents, especially mice and rats, are vertebrate models used extensively to study PD pathology and to perform drug screening (Collins-Praino et al., 2013; Podurgiel et al., 2013; Salamone et al., 2013; Lennington et al., 2011). MPTP is mainly administered via a systemic route through subcutaneous, intravenous, or intracarotid injections. In an acute model generated by injecting 20 mg/kg of MPTP intraperitoneally four times at two-hour intervals in 8-weekold male C57BL/6 J mouse-induced parkinsonian phenotypes such as motor symptoms, loss of DAergic neurons (Park et al., 2015). Similarly, studies conducted by Lau et al. (2005) on 8-10week-old C57BL/6 male mice exposed to MPTP hydrochloride treatment (30mg/kg) for five consecutive days subcutaneous exhibit the loss of striatal DA and DOPAC levels, enhanced TUNEL-positive cells & loss of TH^+ DAergic neurons and, reduced TH protein in SN & striatum. Likewise, in C57BL/6 male mice exposed to 18 mg/kg/week of MPTP twice for five weeks, intraperitoneally significantly manifests the loss of striatal DA and TH^+ DAergic neurons in *Substantia nigra* (Lai et al., 2018). Further, 10-week-old female mice exposed to 20 mg/kg/day of MPTP intraperitoneally for seven days persuade loss of locomotor dysfunction (Jiang et al., 2019). Whereas in young male Wistar rats exposed to intranigral administration of 1 μ mol of MPTP in 2 μL of saline on 1^{st} day, on the 7^{th} day, and on the 14^{th} day, significantly impaired/altered motor coordination, enhanced LP, inhibited mitochondrial complexes I, II, III & IV activities and neuroinflammation (IL-1β & TNF-α enhanced apoptosis), reduced grip strength & rotarod activity, gait abnormality in rats. Intranigral MPTP significantly decreases DA and its metabolites with impaired DAergic cell density in rat brains (Bisht et al., 2017). Similarly, in adult male Sprague-–Dawley rats exposed to 20 mg/kg of MPTP for 4 times at two-hour intervals for 15 days introduced motor dysfunction by reducing rotarod grasping activity, significant (65%) loss of DAergic

neurons, OS, and the activation of TLR/NF-κB signaling pathway (Cheng and Zhu, 2019). A study by Datta et al. (2020) on 2–3 month old Wistar albino male rats reported that repeated exposure to three doses of MPTP (0.1 mg/nostril) intranasally at a 7-day interval for 21 days triggered motor deficiencies and damage to the DAergic system. In addition, this treatment also caused liver dysfunction in the treated rats, correlating with the impairment of the DAergic system (Datta et al., 2020). Non-human primates (NHP) such as marmosets (*Callithrix jacchus*), cynomolgus macaques, and baboons, though there are limitations such as longer life span, extensive care, expensive maintenance as well as complex ethical issues, they are beneficial in analyzing the motor and nonmotor symptoms of PD and for preclinical studies of novel drug therapies (Konnova and Swanberg, 2018). In 6.1-year-old Rhesus monkeys (*Macaca mulatta*) exposed to MPTP, 0.3 mg/kg in 0.9% saline twice a week for three months, significantly exhibited PD symptoms like reduced TH^+ cells & dendrites of DAergic neurons in SNpc, and autophagy by expressing CDK5 levels (Su et al., 2015). Similarly, in another study, 4-year-old male macaques (*Macaca fascicularis)* exposed to 0.25–0.5 mg/kg/week of MPTP in saline till stable PD development by exhibiting loss of nigral DAergic neurons and mitochondrial dysfunction (Carmona-Abellan et al., 2019). The NHP models are also helpful in studying other features like activity counts or the duration of L-DOPA anti-parkinsonian action (Huot et al., 2011; Johnston et al., 2011). The major limitation of the MPTP model(s) is that they do not reproduce the progressive nature of neurodegeneration and LB pathology. According to Meredith and Rademacher (2011), rats resist MPTP, but mice exhibit varying sensitivity levels to this toxin. Furthermore, rat's resistance to MPTP has not been replicated in clinical studies intended to create PD therapies (Lindholm et al., 2016).

1.4.2 6-OHDA

The 6-OHDA is another widely used neurotoxin that selectively destroys catecholaminergic neurons. It is a hydroxylated form of DA, synthesized endogenously in lesser quantities in the brain (Hernandez-Baltazar et al., 2018). The 6-OHDA does not cross the BBB, so it is directly injected into the striatum to introduce neuronal lesions in the animal models (Kostrzewa and Jacobowitz, 1974). In the striatum, through DAT, 6-OHDA is taken up and accumulates in the cytoplasm of DAergic neurons and is oxidized by molecular oxygen to form the superoxide anion, hydrogen peroxide, and 2-hydroxy-5-(2-aminoethyl)-1,4-benzoquinone (p-quinone) causing neurotoxicity. 6-OHDA also inhibits the mitochondrial respiratory chain complex I and IV and depletes ATP levels, increasing the iron levels and eventually leading to neuronal cell death (Hernandez-Baltazar et al., 2018; Glinka and Youdim, 1995). The most common lesion produced by 6-OHDA is through unilateral injection into the animal brain. The degeneration starts first in the tyrosine hydroxylase-containing DAergic neuron terminals in the SNpc, along with DA depletion (Sauer et al., 1994). When injected directly into the striatum to test the retrograde degeneration, the DAergic neuron terminals die before DAergic neurons in the SNpc, replicating the pathological process of PD in humans (Sauer et al., 1994). Indeed, some evidence suggests that the initial site of pathology in PD may start from the striatum and that “dying-back” axonopathy results in retrograde degeneration and neuronal loss in SN (Chu et al., 2012). Many investigations have shown the potential of 6- OHDA to reproduce Parkinsonian features in rodent models (Kostrzewa and Jacobowitz, 1974). A 16 μg of 6-OHDA for four weeks, when injected bilaterally in Male C57BL/6 mice, induces mild loss of DAergic neurons in the striatum, hippocampus, and cortex with no substantial motor impairment and OS (Branchi et al., 2010). When female Swiss mice were given 50 μg of 6-OHDA for three days through intracerebroventricular and bilateral

injection, they exhibited defects in motor movement and loss of DAergic neuronal cells in the SN & VTA. The intracerebroventricular injection of 6-OHDA in mice provides a unique model for screening antiparkinsonian drugs (Didonet et al., 2014). Similarly, Adult C57BL/6 mice and Sprague Dawley rats exposed to 4 µg and 8 µg of 6-OHDA unilaterally for 3-weeks and 4- weeks exhibit the loss of DAergic neurons in the SN & striatum, and microglial activation, iron inclusions in SN (Park et al., 2018; Olmedo-Díaz et al., 2017). Concentrations starting from 2 µg induced more than 70% damage in the nigrostriatal pathway, leading to the depletion of almost all the DAergic neurons, suggesting that adult C57BL/6 mice lesioned intraperitoneally could mimic the early or moderate stages of PD (Park et al., 2018). NHP, viz., marmosets (*Callithrix jacchus*) and rhesus monkeys (*Macaca mulatta*), were also utilized to model 6-OHDA mediated PD for analyzing the motor and nonmotor symptoms of PD and for preclinical studies of novel drug therapies. A study conducted by Eslamboli et al. (2003) injecting adult marmosets (*Callithrix jacchus*) unilaterally in the striatum with 4 mg/mL 6-OHDA observed the loss of TH immunoreactive neurons by 46% in SNpc and 68% in the striatum along with behavioral deficiencies (no climbing, no motor control) that were retained even after three and a half months from the date of the lesion (Eslamboli et al., 2003). Another research by Joers et al. (2014) in 5 to 17 years old adult rhesus monkeys (*Macaca mulatta*) treated intravenously with 50 mg/kg of 6-OHDA displayed no motor symptoms. However, it induced loss of DAergic neurons, reduced TH immunoreactivity in nigral and striatal neurons, and higher amounts of soluble α-syn (Joers et al., 2014). 6-OHDA does not produce LB-like inclusions in the nigrostriatal pathway nor cause progressive DAergic neuronal degeneration. Despite its limitations, the 6-OHDA lesion in the striatum produced more stable Parkinsonian features to study new therapeutic strategies in PD (Eslamboli et al., 2003).

1.4.3 Rotenone

The first use of ROT was done in 1985 when Heikkila et al. (1985) demonstrated in female Sprague-Dawley rats that when injected with 2 µg/µL exhibited about 80% of the total DAergic neuronal loss in the SNpc (Heikkila et al., 1985). ROT is a pesticide that increases the risk of PD in humans when ingested or inhaled. It has been shown to induce Parkinsonian features in animal models (Cannon et al., 2009). ROT crosses the BBB as it is a lipophilic compound and does not depend on DAT to enter DAergic neurons and exerts neurotoxicity by inhibiting mitochondrial complex I, generation of ROS, depletion of catecholamine levels (DA, DOPAC, HVA), GSH levels, elevated LP, microglial activation, α-syn aggregation and ubiquitin dysfunction causing DAergic neuronal loss (Ayajuddin et al., 2022; Bisbal and Sanchez, 2019; Cannon et al., 2009). The chronic administration of ROT in rat models exhibits DAergic neurodegeneration, LB-like cytoplasmic inclusions in the DAergic neurons, elevated OS, enhanced iron deposits, microgliosis, and behavioral phenotypes, such as bradykinesia, rigidity, postural instability, unsteady gait, and tremor (Ryu et al., 2002; Betarbet et al., 2000). The ROT-mediated reduction of DAergic neurons is associated with rigidity and decreased motor activity that can be prevented/altered by L-DOPA interventions (Alam et al., 2004). When mice were orally administered 30mg/kg of ROT for 28 days, they exhibited mild loss of DAergic neurons, reduced immunoreactivity /upregulation of α-syn in SNpc, and motor dysfunction. However, it did not activate astrocytes and microglia, suggesting that this model mimics the early-onset PD (Inden et al., 2007). Similarly, locomotor deficits, tremors, abnormal posture, and nigrostriatal DAergic neuronal degeneration were induced by subcutaneously injecting 2.5, 4, and 5 mg/kg of ROT in C57Bl/6 male mice for 30 to 45 days (Richter et al., 2007). ROT treatment in male Sprague–Dawley rats through intraperitoneal injection with 1mg/kg/day for six weeks induced endoplasmic

reticulum (ER) stress and apoptosis in the midbrain (Tong et al., 2016). DJ-1, DAT, and tyrosine hydrolase (TH) protein levels were altered in SN, and DA and its metabolites reduction in the striatum after a 10-day subcutaneous injection of ROT at a dose of 2 mg/kg/day in male Sprague-Dawley rats with motor dysfunction (Ablat et al., 2016). whereas 5 mg/kg, ROT triggered α-syn aggregation in C57/BL6J mice administered orally for four months (Arnhold et al., 2016). Oral administration to C57BL/6 mice with 30 mg/kg/week ROT for 4 weeks induced motor abnormalities, gastrointestinal dysfunction, change in fecal microbiota, increased TLR2 and inflammatory cytokine expression in the colon, and α-syn aggregation in the gut (Yang et al., 2018). In male Lewis rats, chronic intravenous injection of ROT at 2, 2.5, 3.5, or 5 mg/ kg for 21 days induced motor dysfunction, postural instability, microglial activation with 24% loss of DAergic neurons, and mitochondrial dysfunction (Fleming et al., 2004). However, systematic administration of ROT in rats causes high mortality and, somehow, is challenging to recreate. *Drosophila melanogaster* is one of the most appreciated and extensively used animal models of PD that is accessible for investigating the genes and proteins associated with the disease pathophysiology and developing noble therapeutic interventions. ROT-mediated exposure to *Drosophila* (500 μM) for a week prompted severe DAergic neuronal loss, motor defects, and mitochondrial complex I inhibition, yet no α-syn aggregation (Coulom and Birman, 2004). This study concluded that this chronic ROT-mediated *Drosophila* model could be used to study the neuro-pathophysiological mechanism of PD as it recapitulates the significant features of sporadic PD (Coulom and Birman, 2004). When 10-day-old adult male OK flies were exposed to ROT at 500 μM concentrations, resulted in reduced DA content, GSH levels, elevated ROS, and mitochondrial complex I-III inhibition (Hosamani et al., 2010). Similarly, another study conducted by Farombi et al. (2018) observed that 250 μM of ROT exposure to 3 days old

wild-type Harwich flies for 7 days exhibited a defect in negative geotaxis ability, and reduced acetylcholine levels, OS mediated elevated hydrogen peroxide levels, reduced catalase, GST, and thiol levels in the treated files (Farombi et al., 2018). Additionally, ROT-mediated exposure at 500 μM induced time and dose-dependent motor deficits, reduced survival, elevated malondialdehyde (MDA) levels, and reduced DA and its metabolites in the *Drosophila* PD brain (Siima et al., 2020; Pandareesh et al., 2016). Recently, an adult life stage-specific (ALSS) ROT-mediated *Drosophila* PD model demonstrated that during the health phase (4-5 days old flies; 500 μM) and transition phase (50-55 days old flies; 10 μM), when exposed to ROT exhibits dose-dependent locomotor dysfunction and susceptibility, mobility defects, inhibited mitochondrial complex -I activity, DAergic neuronal dysfunction (no loss of DAergic neurons; however reduction of rate-limiting enzyme tyrosine hydroxylase protein (TH) synthesis), and altered level of DA and its metabolites viz., DOPAC and HVA (Ayajuddin et al., 2022).The drawbacks of the ROT-mediated animal models are the non-availability of well-documented ROT-induced human PD cases with which it can be compared (Bové et al., 2005) and its low specificity to DAergic neurons (Blesa et al., 2012). Another disadvantage of this model is its ability to show motor impairments without DA neuronal loss in rats (Hisahara and Shimohama, 2010). Overall, ROT-mediated PD models exhibit all the pathological features of PD and are, thus, an attractive model of PD.

1.4.4 Paraquat

PQ is a herbicide that suppresses weeds and grass growth (Richardson et al., 2005). Since its discovery, PQ has been used as a neurotoxin to model PD because of its molecular structure similarities to MPP^+, the active metabolite of MPTP. PQ is analogous to MPP^+, which cannot cross the BBB but enters the brain through an amino acid transporter called systemic l-amino acid transporter. In the neuron, it is taken up by DAT, where it is either

stored in the cytoplasmic vesicles or released into the cytoplasm and induces impairment of intracellular antioxidant systems such as glutathione and thioredoxin which increases OS and damage the lipids, proteins, DNA, and RNA structure (Niso-Santano et al., 2010). It is also transported by organic cationic transporter-3 found in non-DAergic neurons of SN (Rappold et al., 2011). The PQ-mediated PD models involve many biochemical pathways, such as OS *viz.*, increased ROS, elevated LP, reduced GSH levels, reduced antioxidant enzymes, such as CAT, SOD, and glutathione peroxidase (GPx), elevated SOD, mitochondrial dysfunction, reduced mitochondrial membrane potential ($\Delta\Psi m$), ER stress, alteration in DA catabolism, inactivation of TH, and decrease in the brain-derived neurotrophic factor (BDNF), ultimately manifesting the death of DAergic neurons in the SNpc (See et al., 2022, 2023; Das, 2022; Phom et al., 2014). PQ-mediated PD model also induces the expression and aggregation of α-syn and LB-like structures in SNpc DAergic neurons, which are primarily affected in human PD (Manning-Bog et al., 2002). Thus, using PQ-mediated models, which directly or indirectly contribute to the pathogenesis of PD, may provide a larger picture to develop new therapeutic targets in the near future. Rats are exceptional models of PD that reproduce and replicate the PD phenotypes successfully. When long Evans hooded rats were assigned five intraperitoneal injections of 10 mg/kg PQ once every five days for 20 days, they exhibited a 50% loss of DAergic neurons (Muthukumaran et al., 2014). Furthermore, when C57BL/6 mice were exposed to 10 mg/kg/ PQ for three weeks, induced locomotor dysfunction, reduced levels of SOD, activated NADPH oxidase I enzyme leading to OS, death of DAergic neurons, and microgliosis (Nixon et al., 2018). Meanwhile, 8-week old C57BL/6 male mice exhibited intense hypokinesia and motor deficits through the intranasal infusion of 20 or 30 mg/kg of PQ mice for 30 days. However, the loss of DA and its metabolite DOPAC in the striatum was not severe (Rojo et al., 2007). Another study by Peng et al. (2007) used four

different age groups (2, 6, 12, and 24 months) of C57BL/6 male mice exposed intraperitoneally with 10 mg/kg twice a week for three consecutive weeks manifests age-independent PQ toxicity, and death of DAergic neurons in all the age groups (Peng et al., 2007). Breckenridge et al. (2013) reported that when C57BL/6 J male mice were injected intraperitoneally with PQ in a concentration of 10, 15, or 25 mg/kg/ week for three weeks did not alter the DA and its metabolites levels in the striatum and DAergic neurons in SNpc, with no activation of microglia and astrocytes (Breckenridge et al., 2013). In a Wistar rat model, when 2.5 mg/kg PQ was administered using osmotic minipumps every day for eight weeks, it induced a 40% reduction in DA levels in the striatum and a 41% reduction in DAergic neurons (Cristovao et al., 2020).

PQ effectively promotes the degeneration of DAergic neurons and increases ROS levels, cellular dysfunction, and motor deficits; as a result, excitement about employing it to replicate PD in animal models, such as *Drosophila*, has risen over the past twenty-five years (Das, 2022; Soares et al., 2017; Phom et al., 2014). When mutant flies fed with 20 mM of PQ for 24 hours exhibited an alteration in catalase activity, higher male susceptibility to the toxin than females, reduced life span, and selective loss of DAergic neurons (Chaudhuri et al., 2007). Similarly, when 7- to 10-day-old adult Canton-S (CS) female flies were exposed to 20 mM of PQ for 72 hours, a reduced survival rate by 60 % after 24 hours, and death of DAergic neurons in the ventral nerve cord was observed (Cassar et al., 2015). Five days old CS flies starved for 6 hours before exposure to 5 mM PQ for 48 hours exhibited reduced survival, locomotor dysfunction, selective loss of PPL1 and PPM subset of DAergic neurons, reduced TH activity, increased ROS, reduced SOD, CAT, GSH activity, increased LP, PC levels, reduced Complex I, III activity, reduced MMP intensity, ATP levels, reduced L-DOPA production, reduced DA content (Chaouhan et al., 2022; Maitra et al., 2021). Similarly, when 2-days old Oregon K (OK)

flies were exposed to 15-20 mM PQ for 48 hours manifested increased mortality, impaired climbing ability, GSH depletion, enhanced MDA and AchE levels, enhanced activities of CAT, SOD (Jahromi et al., 2013). Further, when 3-day-old flies were exposed to 5mM PQ for 24 hours prior to 4 hours starvation, exhibited increased mortality, impaired climbing ability, GSH depletion, enhanced MDA and ROS, enhanced activities of CAT, SOD, and elevated levels of AchE (Mahesh et al., 2022). A study conducted by Soares et al. (2017) demonstrated that PQ exposed in a concentration of 3.5 mM for four days exhibits higher mortality, reduced locomotor capacity, reduced DA levels, and enhanced AchE activity, ROS production, and LP (Soares et al., 2017). PQ exposure at 10-or-20-mM concentrations for 12 and 24 hours exerted toxic effects by increasing ROS production, reduced antioxidant activity, loss of DAergic neurons, increased LP, and motor deficiency in time and concentration-dependent manner in 5-day-old male flies (Shukla et al., 2016). PQ-induced damage in mitochondria and death of DAergic neurons in *Drosophila* exposed with 20mM for 24 hours also exhibited impaired motor activity, increased ROS, catalase activity, acetylcholine activity, decreased GSH Mn- & Cu/Zn SOD activity (Shivanandappa and Niveditha, 2018). Recently, PQ has been utilized to induce PD in *Drosophila* in an adult life stage-specific (ALSS) manner *viz.,* health phase (HP) and transition Phase (TP) (Phom et al., 2014) and demonstrated impaired climbing, reduced dose and time-dependent survival, DAergic neuronal dysfunction, and altered level of DA and its metabolites *viz.*, DOPAC and HVA and change in the brain-specific molecular targets (Das, 2022; Phom et al., 2014). This herbicide provides an excellent opportunity to investigate environment/gene-environment interaction associated with pathogenesis and therapeutic neuroprotective interventions through rapid screening. Nonetheless, PQ has drawbacks, such as a lack of specificity and a high animal mortality rate, severely limiting their use in PD research. However, all the studies highlighted

elucidate that the fly PD model mimics the human PD condition. Hence, it can be used as a powerful tool to study the neuropathogenesis of the disease and disease-modifying therapeutic intervention. Tables 1.1 and 1.2 summarize PQ-mediated *Drosophila* models of PD.

1.5 The importance of adult life-stage-specific animal models for late-onset NDDs

PD is a diverse and complicated progressive NDD distinguished by tremor, rigidity, and bradykinesia, with postural instability emerging later in life as the disease advances. The severity of PD has risen in the last 25 years. In 2019, global estimates indicated that there were over 8.5 million people with PD. According to current estimates, PD caused 5.8 million disability-adjusted life years (DALYs) in 2019, an 81% rise since 2000, and 329,000 deaths, a more than 100% increase since 2000. PD is expected to impact 17.5 million people globally by 2040, owing to population aging, enhanced longevity, reduced smoking, and increased industrialization (WHO, 2023; GBD, 2016; Chen et al., 2001). Furthermore, recent investigations have connected COVID-19 to neurodegenerative illnesses, particularly in COVID-19 patients who developed encephalitis (Shen et al., 2022; Brundin et al., 2020), and it has been suggested that COVID-19 may accelerate brain aging (Mavrikaki et al., 2021). As a result, more people may develop PD than previously anticipated. Several animal models have been established to investigate the pathophysiology of DAergic neurodegeneration, susceptible genes, and PD pathways in response to neurotoxin, as well as biomarkers and therapeutic strategies. However, translating preclinical discoveries from the laboratory to the bedside remains inadequate, and effective disease-modifying medications have yet to be discovered. Even though aging is the most important risk factor for developing idiopathic PD, most research

employs young animals in their experimental systems, neglecting age-related cellular and molecular pathways. Consequently, investigations on young animals may not accurately reflect human PD, limiting translational outcomes. Preclinical studies on PD should imitate the disease characteristics, such as late-onset, to develop therapeutic strategies. Most of the previous investigations on the neuroprotective efficacy of nutraceuticals and small compounds have focused on young animals (**Table 1.1 &1.2**). Researchers have also developed *Drosophila* PD model employing young (3–30 days) animals even though it is a late-onset NDD (Zhang et al., 2022; Chaouhan et al., 2022; Fathy et al., 2021; Mahesh et al., 2021; Srivastav et al., 2020; Duavy et al., 2019; Nunes et al., 2019; Sur et al., 2018; Ibrahim et al., 2018; Niveditha and Shivanandappa, 2018; Oliveira Souza et al., 2018; Soares et al., 2017; Ghimire and Kin, 2017; Jahromi et al., 2013). Three distinct stages characterize *Drosophila's* adult life: the health phase (HP), which corresponds to the adult young life stage with no apparent mortality; the transition phase (TP), which corresponds to the middle adult life stage with 10% mortality; and the senescence phase (TP), which corresponds to the old adult life stage with a steady decline in survival (Arking et al., 2002). *Drosophila* aging has been associated with alteration of 23% change in the genome-wide transcription profile (Pletcher et al., 2002). Bordet et al. (2021) conducted a transcriptome analysis utilizing microarrays to evaluate the gene expression patterns of various life stages of *Drosophila melanogaster*. The results indicated that 1184 genes exhibited significant variations in expression levels between the young age flies (3 days old) and elderly age group flies (45 days old). *Drosophila's* changes in genome-wide transcription profile over distinct life phases are comparable to those of humans, where substantial changes in gene expression associated with aging are potent risk factors for an array of diseases, including PD (Yang et al., 2015; Kumar et al., 2013). Our laboratory developed an ALSS *Drosophila* model of PD using environmental risk factors pesticides

viz., PQ and ROT (Mitochondrial complex I inhibitor) and used it to demonstrate the neuroprotective efficacy of curcumin (CU). This was done while keeping in mind the crucial factors that aging is a potential risk factor for late-onset NDD like PD and that there is a lack of therapeutic intervention data on the later phase of life span, which is an important paradigm in finding the therapeutic strategies for PD (Ayajuddin et al., 2022; Phom et al., 2014). It has been shown that during the HP and TP of a *Drosophila's* life, exposure to neurotoxicant PQ and rotenone can cause PD-associated motor impairments and depleted brain DA and tyrosine hydroxylase (TH) protein levels. However, only during the HP, but not during the TP, CU intervention could restore the reduced TH protein level, impaired motor function, and reduced DA levels (Ayajuddin et al., 2022; Das, 2022; Phom et al., 2014). Additionally, our research group has recently concluded that CU-mediated neuroprotection is HP-specific in an ALSS *Drosophila* model of PD produced by the neurotoxin PQ and ROT. These findings indicate that the DAergic neuroprotective effect of CU is stage-specific in adult life, underscoring its limitations as a therapeutic agent in PD during the transition phase, which is a later stage of *Drosophila* life. Furthermore, findings from our laboratory work indicate that factors such as life stages and their physiological impact on disease progression should be taken into account while modeling a disease *in vivo* to match the onset and progression of a disease as precisely as possible to the human condition (Ayajuddin et al., 2022; Phom et al., 2014). This could be a potential cause for failure to translate preclinical study results into a clinical setting, as well as using young animal models that do not correspond with the relevant life phases of disease in humans. Therefore, it's essential to investigate phytochemicals/nutraceuticals/PE neuroprotective efficacy in the later stage of the model(s) organism. Recently, it has been demonstrated that aged animals in PD research are more susceptible to developing pathology and neurodegeneration and present with a

more disseminated and accelerated disease course compared to young animals (Das, 2022; Klæstrup et al., 2022; Phom, 2018; Phom et al., 2014). Therefore, utilizing the established hypothesis, I will be evaluating the neuroprotective efficacy of certain plant extracts with particular emphasis on *Leea asiatica* (L.), employing the adult transition stage-specific *Drosophila* model of PD (Das, 2022; Phom, 2018; Phom et al., 2014).

1.6 *Leea asiatica* (LA) in ethnomedicinal culture and pharmacological preclinical studies

LA have been used by several ethnic communities in the healthcare system. The efficacy of LA varies from primary healthcare to treatment of some complications such as snake bites (Nair et al., 2014), bone setting (Bhandary et al., 1995), worm infection, eye diseases, gastrointestinal disorders treatment of heart disorders, liver disorders, and diabetic condition (Sen et al., 2011). LA as a whole plant has been used in ethno-medicine by various ethnic groups of India (detailed description in **Table 1.3**). Recent laboratory studies have shown the presence of terpenoids, phenolic, flavonoid compounds, triterpines, and glycosides in methanolic, ethyl acetate, and petroleum ether extract of LA leaves (Kil et al., 2019; Nair et al., 2014; Sen et al., 2013). The scientific validation on how LA might impart therapeutic intervention are still in progression, yet few laboratory studies have provided experimental validation. A study conducted by Sen et al, (2012) explored that the methanol and ethyl acetate extract of LA leaves have significant anthelminthic activity against Indian adult earthworms (*Pheretima posthuma*) by scavenging free radical responsible for OS. Specifically, the worms were paralyzed by the ethyl acetate fraction of LA leaves, while the methanol fraction of LA leaves led to their death. The evaluation of antioxidant efficacy was assessed by quantifying diphenyl-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging, nitric oxide radical scavenging, LP assay, and through the ferric thiocyanate

method (Sen et al., 2012). The nephoprotective effect in cisplatin-induced toxicity in albino mice and Wistar rats was also assessed using methanol, ethyl acetate, and petroleum ether extract of LA leaves by evaluating *in vitro* and *ex vivo* antioxidant activity by measuring scavenged DPPH$^{\cdot}$ responsible for LP inhibition, superoxide anion radical ($O_2^{let^-}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide radical ($NO^{\cdot}$), hydrogen peroxide (H_2O_2), metal chelating ability, and total antioxidant activity. The methanol extract prevents OS induced damage by scavenging, $O_2^{let^-}$, $OH^{\cdot}$, $NO^{\cdot}$, H_2O_2 levels (Sen et al., 2013). Another laboratory study provides evidence on the hepatoprotective efficacy of LA in acetaminophen-induced hepatotoxicity in Wistar rats. The methanol extract of LA showed dose-dependent increase in antioxidant activity by significantly increasing levels of endogenous antioxidant enzymes such as SOD, CAT, GPx, and GSH and by lowering the level of serum SGOT (serum glutamic oxaloacetate transaminase), SGPT (serum glutamic pyruvic transaminase), ALP (alkaline phosphatase), total bilirubin, total cholesterol and triglyceride (Sen et al., 2014). Increased levels of GSH in serum and liver combined with enhanced activity of antioxidant enzyme SOD, CAT suggests that the LA leaves extract have multiple ways to impart protection via manipulating the anti-oxidant defense mechanism. Nair and colleagues have observed LA methanolic extract from whole plant have potent anti-inflammatory and wound healing activity in male albino Wistar rats (Nair et al., 2014). The efficiency of wound healing was reported to be quite impressive, although not on par with standard wound healing compounds like 10% boric acid solution. Also, rats that were induced inflammation in the paw by carrageenan injection (*Carageenan induced paw oedema in rats* by Winter et al., 1962), when fed water with LA extract and aspirin showed marked anti-inflammatory activity, adding to the efficiency as par with marketed drugs. Nair et al, (2014) also tried to address the issue of dermal toxicity and oral toxicity of the extract *in vivo* in a dose-dependent manner and found no side effects caused by the

LA. Another major study investigated the potential application of ZnO NRs (Zinc oxide nanorods obtained *via* green synthesis approach utilizing the leaf extract of *Leea asiatica*) as an effective anticancer drug. The anticancer activity of ZnO NRs was assessed through MTT (3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, XTT((2,3-bis (2-methoxy-4-nitro-5- sulfophenyl) -5- [(phenylamino) carbonyl]-2H-tetrazolium hydroxide)) assay, NRU (Neutral Red uptake: Mitochondrial lactate dehydrogenase and lysosomic cytotoxic assessments), and LDH (Lactase dehydrogenase release: for apoptotic and necrosis cell death) assay, concluding that the cytotoxicity properties of ZnO NRs are time and concentration-dependent. The authors further demonstrated that ZnO NRs cause no mortality and toxicity in zebrafish embryos, which established ZnO NRs as a safe potential drug for human screening. Further, When ZnO NRs exposed against the breast cancer cell line (MCF-7) resulted in necrosis and apoptosis through ROS production, which can cause DNA damage leading to apoptosis in the breast cancer cell line (MCF-7) (Ali et al., 2021).

All these studies reported that LA extract has potent radical scavenging and metal chelating activity as efficient as standard drugs. These antioxidant and anti-inflammatory properties of LA may be primarily responsible for the demonstrated anthelminthic, nephroprotective, and hepatoprotective efficacy, which may contribute to imparting therapeutic cures to many other diseases that have not been scientifically validated yet treated by ethnic communities in India. All these studies related to the scientific and preclinical validation of LA under multiple disease conditions are summarized in **Table 1.4**.

S.No.	Ethnic Groups	Ethnomedicinal uses	Part of the Plant	Nature of Treatment	Reference(s)
1	Siddis of Uttara Kannada district, Karnataka, India	Healing Bone fractures	Whole plant	Whole plant is grounded into a paste and applied over the fractured area as a thick layer	Bhandary et al., 1995
2	Tribes of Uttar Pradesh, India	Antidote to Snake Bite	Roots, along with the bark of *Boswellia serrata*	Pounded and taken orally	Nair et al., 2014
3	Tribes of Hazaribag, Bihar, India	Antidote to Snake Bite	Roots, along with the bark of *Boswellia serrata*	Paste applied on the site of the snake bite	Nair et al., 2014
4	Ethnic people in West and South district of Tripura, India	Anthelminthic	Roots	Pounded to make into a paste and consumed orally	Sen et al., 2011
		Liver disorder	Leaves	Juice of the leaves consumed orally	
		Heart Disorder	Whole plant	Whole plant is boiled in water and consumed orally (Decoction)	
		Diabetes	Leaves	Leaves kept in the water overnight and consumed next day	
5	Ethnic people in North Andaman, India	Treatment of Boil and blisters	Roots	Pounded to make a paste and applied externally on the site	Prasad et al., 2008
6	Chiru Tribe of Manipur, India	Washing of Hair	Leaves	Leaves juice mixed with water and applied externally on hair	Nair et al., 2014
7	Tribes of Madhya Pradesh, India	Joint disease	Bulbs	Bulb Paste with water and applied on the joints	Wagh and Jain, 2013, 2014

Table 1.3: Overview of ethnomedicinal uses of *Leea asiatica* (L.) among different tribal communities of the Indian subcontinent.

S. No.	Ethnomedicinal use	Scientific Validation			Reference(s)
		Type of extract (Solvent)	Mechanism of action (Demonstrated activity)	Animal Model (s)	
1	Anthelmintic evaluation (Treatment of worm infection)	1. Methanol extract of *Leea asiatica* leaves 2. Ethyl acetate extract of *Leea asiatica* leaves	1. Anti-oxidant activity (free radical scavenging activity) 2. Lipid peroxidation inhibition activity 3. Anthelmintic activity against Indian adult earthworms	1. *In vitro* 2. Wistar rats 3. *Pheretima posthuma*	Sen et al., 2011; 2012
2	Nephoprotective evaluation (Treatment of Kidney disorder)	1. Methanol extract of *Leea asiatica* leaves 2. Ethyl acetate extract of *Leea asiatica* leaves	1. Antioxidant activity (free radical scavenging activity) 2. Lipid peroxidation inhibition activity 3. Nephroprotective activity	1. *In vitro* 2. Albino mice 3. Wistar rats	Sen et al., 2013
3	Hepatoprotective evaluation (Treatment of liver disorder)	1. Methanol extract of *Leea asiatica* leaves	1. Antioxidant Activity (free radical scavenging activity) 2. Hepatoprotective activity	1. Wistar rats	Sen et al., 2014
4	Wound healing activity evaluation (Treatment of wounds/cuts and snake bites)	1. Methanol extract of the whole plant of *Leea asiatica*	1. Anti-inflammatory activity 2. Wound healing activity	1. Wistar albino rats	Nair et al., 2014
5	Anticancer Activity evaluation	1. Zinc oxide nanorods (ZnO NRs) obtained *via* green synthesis approach utilizing *Leea asiatica* leaf extract	1. Anti-oxidant activity (free radical scavenging) 2. Apoptotic and Necrosis pathways activation (Genotoxicity assay)	1. Breast cancer cell line (MCF-7) 2. Zebrafish embryos	Ali et al., 2021

Table 1.4: Overview of scientific validation done on the ethnomedicinal value of *Leea asiatica* (L.).

1.7 Conclusion

PD is a late-onset disease associated with neurodegeneration, incurable and disturbing conditions that ultimately lead to neuronal death. Therefore, it is essential to understand the disease progression and therapeutic effectiveness at a later stage of the organism(s)/animal(s), the age equivalent to disease onset in humans. The DAergic neurons of the nigrostriatal pathway are more susceptible to late-onset driven dysfunction than other neuronal populations in the central nervous system (CNS) owing to several age-

dependent changes, including DA metabolism, DA uptake and synthesis, receptor sensitivity, mitochondrial function, calcium dynamics, iron concentration, and proteostatic function (Surmeier, 2018; Collier et al., 2017; Zucca et al., 2017; Reeve et al., 2014). This is true even though aging is the leading risk factor for developing idiopathic PD; most studies exploring the modulatory effect of prospective or potential therapeutic compounds are restricted to young animal models. Consequently, studies in young animals may not accurately reflect human PD, limiting translational outcomes. However, studies established by Phom et al. (2014; 2018) and Ayajuddin et al. (2022), where they developed an ALSS *Drosophila* model of PD using environmental risk factors in the form of pesticides *viz.,* PQ and ROT (Mitochondrial complex I inhibitor) and used it to demonstrate the neuroprotective efficacy of CU, showing relevance to life stages where neurodegenerative disease such as PD sets in, which is pertinent for disease modeling. Also, mechanistic insights were provided by Das, (2022), employing the ALSS *Drosophila* model and demonstrated/deciphered the molecular basis of CU efficacy. Review of literature relating to traditional knowledge of Indigenous communities revealed the potential therapeutic efficacy of LA for multiple human disease conditions. Therefore, utilizing the robustness and uniqueness of the PQ-mediated PD model, I decided to employ a transition stage-specific *Drosophila* model of sporadic PD to screen the protective efficacy of LA leaf extract. Therefore, in the absence of knowledge in that aspect, the objectives of the present study are as follows:

CHAPTER II:
Preparation of Leaf Aqueous Extract of *Leea asiatica* (L.)

CHAPTER III:
Understanding *Leea asiatica's* Dopaminergic (DAergic) Neuroprotective Efficacy in the Adult Transition Stage *Drosophila* Model of Parkinson's Disease

CHAPTER IV:

Leea asiatica Mediated Regulation of Brain Dopamine (DA) Metabolism in the *Drosophila* Model of Parkinson's Disease: Implications to its therapeutic efficacy

CHAPTER V:

Oxidative Stress-mediated DAergic Neurodegeneration and its Sequestration by *Leea asiatica* (LA) in the *Drosophila* Model of Parkinson's Disease

Chapter 2

Preparation of Leaf Aqueous Extract of

Leea asiatica (L.)

Graphical overview

Steps involved in the preparation of aqueous extract from the *L. asiatica* leaves

2. Introduction

The exceptional chemical variety of natural commodities such as plant extracts, whether in the form of pure chemicals or standardized extracts, presents a plethora of options for discovering novel therapeutics (Jamtsho et al., 2024; Cos et al., 2006). Plant-based products are expected to be worth $83 billion US globally, and this sector is still expanding (WHO, 2011). Moreover, it is believed that up to 60% of anticancer medications and about 25% of contemporary drugs are derived from natural sources (Newman and Cragg, 2012; Brower, 2011). There are almost 20,000 medicinal plants in 91 nations, including 12 mega-biodiversity countries, and over 80% of people globally rely on traditional medicine (Ekor, 2014; WHO, 2013; Sasidharan et al.,2011; Raskin et al., 2002; Farnsworth et al., 1985) to address their fundamental medical needs.

The World Health Organization (WHO) Global Report on Traditional and Complementary Medicine (2019) lists acupuncture, herbal remedies, homeopathy, indigenous traditional medicine, traditional Chinese medicine, naturopathy, chiropractic, osteopathy, ayurvedic, and Unani medicine as some of the many traditional medical systems practiced worldwide.

The usage of herbal remedies in Asia and India reflects a long history of human interactions with nature. Men turned to ethnopharmacology/herbal remedies after experiencing unpleasant effects and microbial resistance to chemically manufactured medications. Plants used in traditional medicine include a diverse spectrum of chemicals that can treat chronic and infectious disorders (Halder and Jha, 2023; Anand et al., 2019; Duraipandiyan et al., 2006). Numerous studies have attempted to characterize the potential health advantages of plants, including their anti-inflammatory, anti-tumoral,

antidiabetic, and antioxidant properties (Roleira et al., 2015; Ekor, 2014; Jung et al., 2006; Watt and Breyer-Brandwijk, 1962). They discovered thousands of phytochemicals from plants that are both safe and effective, with fewer side effects. Many assert that consuming particular natural or herbal products has significant health benefits. However, preclinical studies followed by clinical trials are required to confirm the efficacy of a bioactive chemical(s) to verify the traditional claim. Clinical trials aimed at understanding the pharmacokinetics, bioavailability, effectiveness, safety, and medication interactions of newly created bioactive substances and their formulations (extracts) must be carefully assessed. Clinical trials are meticulously structured to protect participants health while answering specific research questions by assessing immediate and long-term adverse effects. Their findings are recorded before the treatment is widely distributed to patients.

The initial steps in utilizing physiologically active compounds from plant resources are extraction, pharmacological screening, bioactive compound isolation and characterization, toxicological evaluation, and clinical evaluation.

Extraction is the critical initial stage in medicinal plant analysis since removing the desired chemical components from the plant materials is required to separate and characterize them. The essential procedure includes processes such as pre-washing, drying plant materials or freeze drying, grinding to generate a homogeneous sample, often optimizing the kinetics of analytic extraction, and increasing sample surface contact with the solvent system. Proper procedures must be followed to ensure that possible active ingredients are not lost, altered, or destroyed during the extraction of plant materials. Suppose the plant was chosen for its traditional applications (Fabricant and Farnsworth, 2001), in that scenario, to correctly mimic the traditional 'herbal' medication, the extract

must be made as per the instructions provided by the traditional healer. The type of the bioactive chemical compound present is largely determined by the solvent system taken under consideration. Various solvent solutions are available for extracting bioactive compounds from natural sources. Plant samples are extracted using a variety of processes, including sonification, heating under reflux, and soxhlet extraction (United States Pharmacopeia and National Formulary, 2002; Japanese Pharmacopeia, 2001; Pharmacopoeia of the People's Republic of China, 2000). Menstruum is another name for the solvent used in medicinal plant extraction. The type of plant/ portion of the plant to be extracted, makeup of the bioactive chemicals, and the solvent's availability all influence the choice of solvent. According to studies by Sasidharan et al. (2011), Pandey and Tripathi, (2014), and Altemimi et al. (2017), polar solvents like water, methanol, and ethanol are typically used to extract polar molecules, while nonpolar solvents like hexane and dichloromethane are used to extract nonpolar chemicals. The most polar solvent, water, is employed in the extraction of numerous polar substances. Water dissolves a large variety of substances and has the advantages of being inexpensive, nontoxic, nonflammable, and highly polar. These properties make it an excellent solvent. Drawbacks include the fact that it encourages the growth of mold and bacteria, that it may cause hydrolysis, and that a significant amount of heat is needed to concentrate the extract (Tiwari et al., 2011; Das et al., 2010).

LA's a perennial shrub or small tree belonging to the family Vitaceae. It is a folk medicinal plant that inhabits tropical and subtropical areas native to India, Bhutan, Bangladesh, Southeast Asia, Malaysia, Laos, Vietnam, Australia, and South Africa (Zhang et al., 2015; Soejima et al., 2006). It has many local names in the Indian continent, such as Banchalita (Bengali), Nalugu, Manippiranda, Nedeel (Malayalam), Kaadumari

drakshi (Kannada), Koknal (Manipuri), Kumala, and Murkhur (Hindi), Galeni, and Lahasune, (Nepali), (ENVIS, 2020). In modern times, conventional remedies and ethnomedicinal plants have emerged as key sources of new drug development. Almost half of the chemical medications approved by the FDA between the 1940s and at the end of 2014 for the treatment of human diseases were either inspired by or derived from natural sources (Newman and Cragg, 2016, 2012)

LA has been exploited by various ethnic communities of India for the treatment of a broad spectrum of diseases, including wound healing, eye diseases, diabetes, gastrointestinal disorders, asthma, constipation, cough and cold, snakebite, parasitic intestinal worms, blood coagulation, bone fracture, hepatic disorder, osteoarthritis, hair fall, stress-related disorders (Jain and Pachaya, 2015; Sen et al., 2014). The tribes of Karnataka have used it to treat bone fractures. The roots of the plant are pounded and applied on boils and blisters by North Andaman tribal communities, whereas the leaves have been used to treat worm infections and liver disorders by the tribes of Tripura, India. In addition, the leaves are taken orally to treat headaches and rheumatoid arthritis. It has also been used as an active ingredient to treat snake bites, bone setting, and liver disorders, and cancer (Ali et al., 2021; Sen et al., 2011; Prasad et al., 2008; Bhandary et al., 1995). To date, few published reports have described the biological effects of LA extracts, including anthelmintic and antioxidant-related nephroprotective, hepatoprotective activities, and anticancer activity (Ali et al., 2021; Sen et al., 2014, 2013, 2012). Recently through detailed phytochemical constituent(s) studies on the aerial part of LA revealed a total of twenty-four compounds, out of which one noble compound identified as 4-hydroxyphenol-β-D-{6-O-[4-O-(7S,8R-guaiacylglycerol-8-yl)-3-methoxybenzoyl]}-β-D-glucopyranoside,seven

triterpenoids, eight flavonoids, two phenolic gluocosides, four diglycosidic compounds, and two miscellaneous compounds (Kil et al., 2019).

Identifying the phytochemicals of herbal medicine is very important because it can be used as primary data for predicting biological effects, providing safety information, and clarification of medicinal use in the healthcare system. Therefore, in this chapter, I have described an optimized method/procedure for generating aqueous extract from the leaves of LA by employing rotary evaporating equipment, which works on a simple principle that a revolving evaporation flask is connected to a round-bottomed flask containing the sample to be evaporated. To reduce the pressure inside the flask, a vacuum is introduced into the system, and the flask is heated using a water bath. The solvent evaporates from the sample when heated and exposed to low pressure as the flask rotates. After that, the solvent vapor is collected in a receiving flask and condensed in a different condenser.

2.1 Taxonomical classification of *Leea asiatica* (L.)

Kingdom: Plantae

Phylum: Tracheophyta

Class: Equisetopsida

Subclass: Magnoliopsida

Order: Vitales

Family: *Leeaceae (Vitaceae)*

Genus: *Leea* (Royen ex L. ex L.)

Species: *asiatica* (L.)

2.2 Materials and Methods

The following materials and types of equipment were used during the processing and preparation of the aqueous extract of LA leaves.

Spinwin™ 50mL tube conical bottom (Tarsons, WB, India, catalognumber:500041), Gloves (Tarsons, WB, India, catalog number: 370120), Sterilized 2 mL centrifuge tubes (Tarsons, WB, India, catalog number: 500010), Parafilm™ wrapping film (Bemis, WI, USA, catalog number: PM996), Conical flask (Borosil, Maharashtra, India, catalog number: 4980024), Magnetic stirrer bar #8 mm × 40 mm (Tarsons, WB, India, catalog number: 4113), SPINNOT™ digital magnetic stirrer hotplate (Tarsons, WB, India, catalog number: 6090), Sterilized microtips (Tarsons, WB, India, catalognumber:521010), Freshwrapp aluminum foil 9–11µm (Hindalco, Maharashtra, India, catalog number: HV2241), Whatman™ filter paper (GEHealthcare, Buckinghamshire, UK, catalog number:1001917), Micropipette,i.e.,1,000µL (Gilson, WI, USA,catalognumber:30040), -80°Cultra-low temperature freezer (New Brunswick Innova, Hamburg, Germany, model: U101-86), BOD incubator (BIOMATRIX, Telangana, India, model: TH-382), Powder Strainer/Sieve **(**HOMETALES, Harayana**,** India, model: 8905723113520), Slicer Chopper Cutter (Avenn, Delhi, India, model: Q500),Transparent Polythene bags (Zygoma, Gujarat, India, model: SVP-001), Mortar & Pestle #8 Inch (Elecopto, Maharashtra, India, model: EO-350), Evaporating flask B-29 #1000mL (EQUITRON ROTEVA, Maharashtra, India, catalog number: #S1315-25), Receiving flask S-35 #1000mL(EQUITRON® ROTEVA, Maharashtra, India, catalog number: #S1316-25), Digital Vacuum controller (EQUITRON® ROTEVA, Maharashtra, India, catalog number :8920.DVC.MBX.AED.006), ROCKYVAC™ Vacuum Pump

(Tarsons, WB, India, catalog number: 7010), Rotary Vacuum Evaporator (63)-V (EQUITRON® ROTEVA, Maharashtra, India, catalog number: 8763RVO.AED.002), Refrigerated Chiller #04L (EQUITRON® ROTEVA, Maharashtra, India, catalog number: 8502.ROT.000.AED.001), Vacuum Trap w/SS support rod, clamp & base (EQUITRON® ROTEVA, Maharashtra, India, catalog number: 8841.CT0.000), Water bath (EQUITRON® ROTEVA, Maharashtra, India, catalog number:TD11AR.11033)

2.3 *Leea asiatica* (L.) collection, identification, propagation, and processing

The LA plant sapling was collected from Nagaland University, Lumami campus, Zunheboto, Nagaland, India. It was authenticated from the Botanical Survey of India Eastern Regional Centre Shillong, Meghalaya, India, under the identification reference number **"BSI/ERC/Tech./PlantIden./2015/244"**. The identified sapling was planted in a greenhouse to regulate the temperature and humidity of the environment inside, that is situated at the Department of Zoology, Lumami campus. The fresh and mature LA leave samples were harvested using sharp tools sterilized at the beginning and after use. The leaves were washed with tap water, and soil particles, contaminants, and other foreign materials were removed from the leaves by gentle brushing. The leaves were dried in the shade at room temperature of 22±2°C for about a week. The collected leaves were placed in loosely sealed plastic bags in a moisture-free atmosphere. Next, the dried leaves were chopped into smaller pieces and crushed into powder using a mortar and pestle. The leaves were powdered gently to avoid heat generation. The powder was then passed through an aluminium sieve (1mm) to get uniform particle size. The tools used for chopping and grinding were also disinfected using sterilization. Finally, the dried and

crushed leaves powder was stored in plastic bottles at room temperature 22±2°C until aqueous extraction.

2.4 Preparation of *Leea asiatica* (L.) leaf aqueous extract

2.4.1 Procedure

1. The aqueous extracts of LA leaves were prepared by separately soaking 20 grams (g) of powder in 200 ml of autoclaved double distilled water within a conical flask.
2. Then, the conical flask was vigorously shaken and transferred with a magnetic stirrer onto the hotplate with a temperature of 40 °C and moderate stirring at 430 rpm for 48 hours.

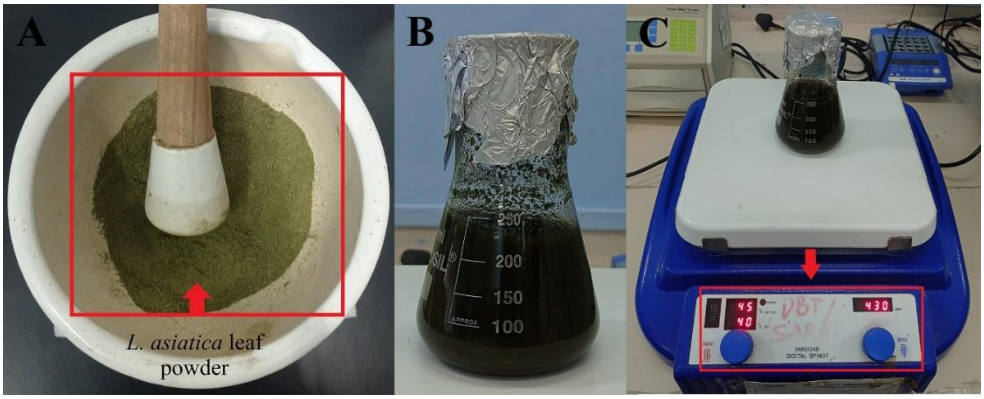


Figure 2.1. Pulverization of *Leea asiatica* (L.) leaves and aqueous solution preparation. Leaves are grounded in fine powder particles using mortar and pestle (A). For aqueous solvent preparation, 20 grams of powder dissolved in 200mL of autoclaved distilled water and left on the magnetic hotplate stirrer at 40°C with 430rpm stirring speed for 48 hours (B&C).

3. The suspended aqueous solution was then subjected to rotary evaporation using a rotary evaporator. To initiate the rotary evaporation, a round *bottom flask*, also known as an *evaporating flask*, was removed from the base of the condenser connected directly above the hot water bath. The solution was loaded into the cleaned round *bottom flask*.

4. The *round bottom flask* was then connected to the rotary evaporator condenser using a "*Keck clamp"*.

Critical: Inspect the flask to ensure it is clean. Use *grease* or *oil* to avoid air leakage while connecting the bottom flask to the condenser. Use a "*Keck clamp"* to secure the round *bottom flask* to the condenser.

5. Use the "*joystick knob"* on the rotary evaporator apparatus to lower the *bottom flask* into the water bath so that the flask containing the LA solution is partially submerged.

Critical: Make sure the flask is not so low that the "*Keck clamp*" joint is in the water.

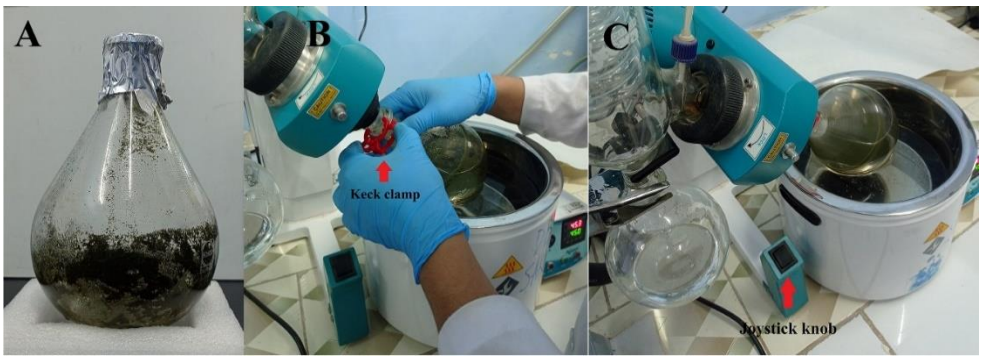


Figure 2.2. Storing solution in the *evaporating flask* and securing it with the *Keck clamp* and *joystick knob*. Solution stored in the *evaporating flask* (A) and secured with

"*Keck clamp"* (B) and positioned as such so that the level of the solution matches the water levels in the water bath using a *"joystick knob"* (C).

6. The chilled water circulator was then switched ON, located on the control panel of the water circular, and the temperature was set at 4 °C and left for 3-5 minutes for it to start, and then the compressor was turned ON.

Critical: Ensure the tubing is securely connected to the back of the chilled water circulator and the condenser. The chilled water circulator and condenser should be filled with water, and confirm that the rod inside the chilled water circulator is submerged in the water.

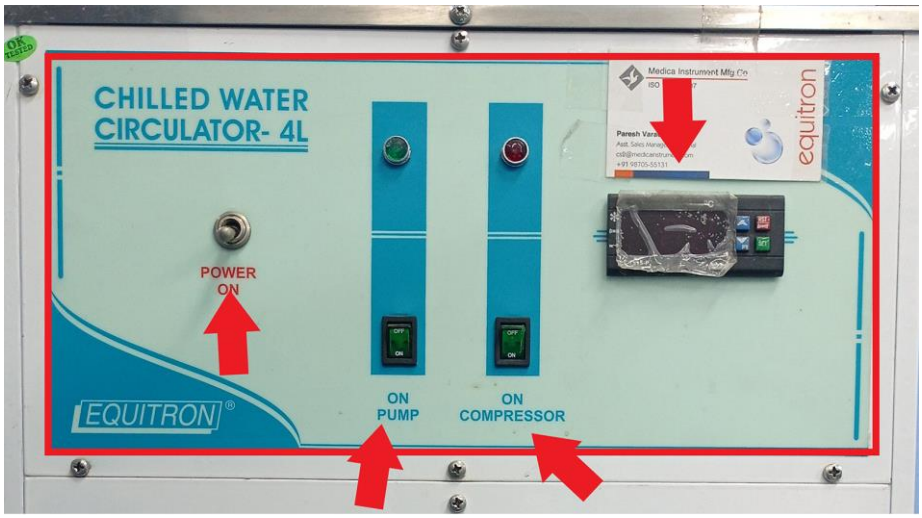


Figure 2.3. Switching ON the chilled water circulator and compressor. Switch ON the chilled water circulator and set the temperature at 4°C, then wait for 3-5 minutes before switching ON the compressor.

7. Turn ON the water bath using the switch on the right side of the bath panel and set the temperature to 45°C.

Critical: Fill the water bath with enough water for the round *bottom flask* to sit in. To lower the entire setup, press the "*joystick knob"* on the rotary evaporator control panel. Use the Up and Down key on the "*joystick knob"* to raise or lower the setup.

8. The rotary evaporator system was then turned ON by pressing the power button on the control panel. Set the vapor pressure at 35.7°C, start rotating the bottom flask at a medium rate, and adjust the "*rotation notch"* to bring the rotation to 45 rpm.

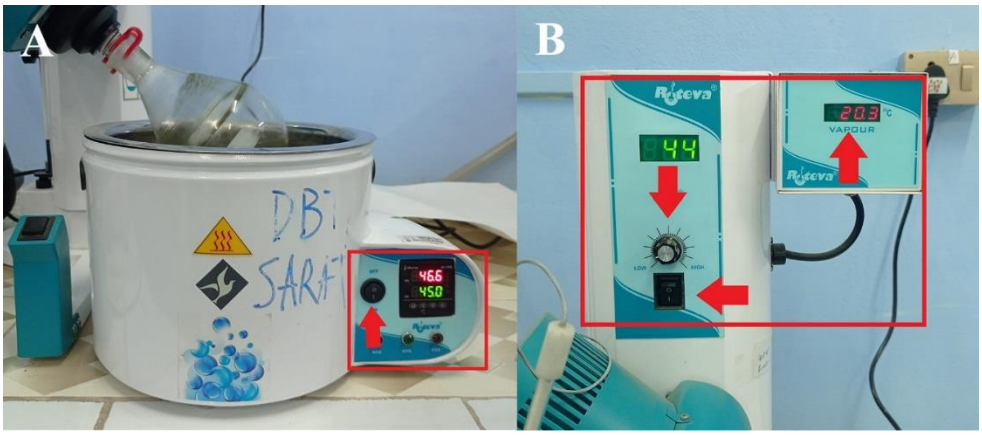


Figure 2.4. Switching ON the water bath and rotary evaporating system and setting up the rotation speed of the flask and vapor pressure. Turn ON the water bath by switching on the power button on the tub's control panel and set the temperature at 45°C (A). For rotary evaporation, on the control panel, switch on the power button, set the rotation speed of the evaporating flask at 45 rpm, and wait for some time so that the vapor pressure can climb/reach up to 35.7°C (B).

9. Turn on the vacuum pump and vacuum controller and set the vacuum pressure to 171-173 mbar by slowly turning the *knob*.

Critical: Ensure that the vacuum pump is connected to the condenser. Ensure the release valve at the top of the condenser is turned to the closed position.

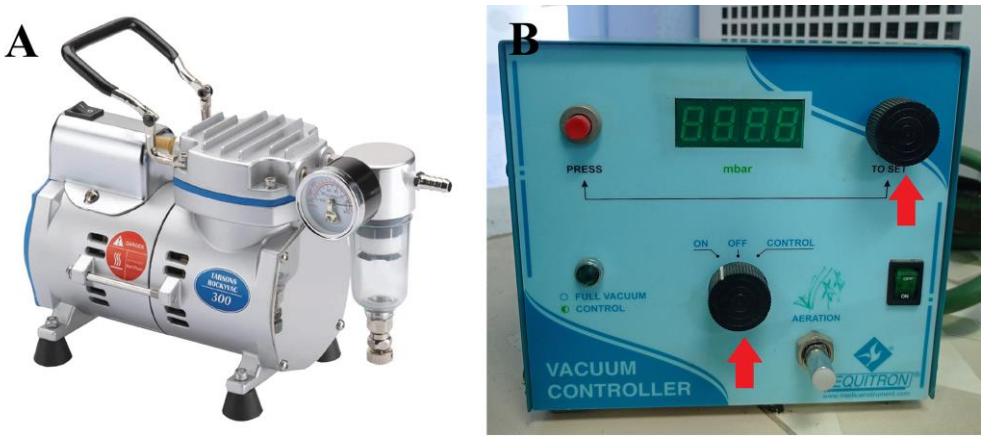


Figure 2.5. Details of the vacuum pressure for the rotary evaporator. Turn on the vacuum pump (A) and set up the vacuum pressure to 171-173 mbar using the vacuum controller by rotating the "To Set knob" (B).

10. Allow the solution to evaporate. The distilled aqueous solvent was collected in the large *receiving flask*, also known as a round-bottomed *flask reservoir*. There is no set amount of time required for complete evaporation. Keep the suspended liquid solution for evaporation until the fluid level is no longer changing. Evaporation can also be tested by lifting the flask from the water bath, drying the outside with paper towels, and feeling the flask with your hand as it rotates. If the solvent continues to evaporate, the flask will be frigid.

11. When the sample has evaporated, allow it to remain in the reduced pressure system for a few extra minutes to remove any remaining solvent residue.

12. To stop the evaporation, reverse all the previous steps: Turn off the water bath, stop the rotation of the *bottomed flask*, lift the *bottomed flask*, turn off the vacuum

pump, release the vacuum pressure by pressing down the *Aeration button*, turn off the vacuum controller and then refrigerated chiller.

13. Collect the distilled leaves aqueous extract of LA from the *receiving flask* and, aliquot it in 2 mL of the Eppendorf tube and store it at -80°C until further use.

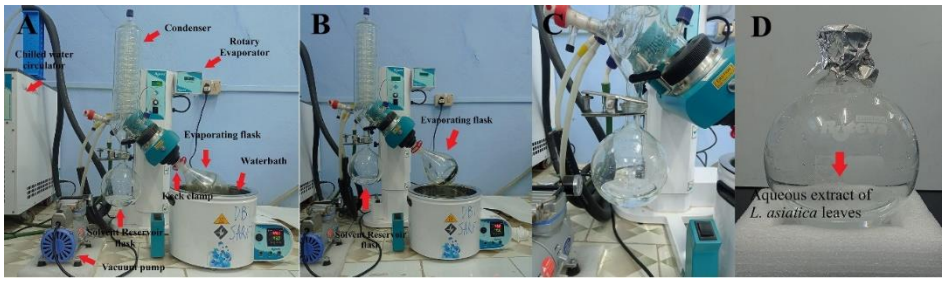


Figure 2.6. Running a rotary evaporator system with secured functioning components and storing the aqueous extract in the *receiving flask/solvent reservoir.* Let the rotary evaporator system evaporate (A&B). The aqueous extract was stored in the receiving flask and aliquot in 2 mL of tube from there and kept at -80°C (C&D).

2.4.2 Precautions and Recommendations

1. The fresh and tender leaves of LA should be ground into a fine powder and extracted. Infected or premature leaves should be discarded.

2. The leaves should be shade-dried with no sunlight and humidity between 60% - and 70%until crisp. Care should be taken to avoid any fungal growth in the dried leaves. The leaves should be shifted/switched onto a fresh blotting paper every week to prevent this.

3. Proper care should be taken while crushing the leaves with a motor and pestle so as not to generate heat.

4. The materials/instruments used for harvesting the leaves, powdering the leaves, storing them, and for rotary evaporation should be sterilized and cleaned before use.

5. To avoid air leakage, Grease or oil should be used while connecting the *bottom flask* to the condenser. Use a "*Keck clamp*" to secure the round *bottom flask* to the condenser.

6. The *bottomed flask* should not be positioned so low that the joint with the "*Keck clamp"* is in the water. It should be partially submerged so that the levels of the suspended solution in the *bottomed flask* and water bath match.

7. All the components of the rotary evaporator system should be connected and secured correctly before collecting the distilled extract solution. The chilled water circulator and condenser should be filled with water so that the rod inside the water circulator is submerged in the water.

8. The tube connection from the chilled water circulator to the condenser should be properly secured from leakage.

9. After the extraction process, all the openings of the rotary evaporator system should be closed and covered with parafilm and aluminum foil.

10. Wear eye protection appropriate for the chemicals used (*safety goggles or glasses*). Wear a lab coat and proper shoes/pants.

11. Wear appropriate gloves while transferring chemicals and touching equipment.

12. The same setting should be reused while repeating the process of rotary evaporation.

2.6 Preparation of different concentrations of *Leea asiatica* (L.) leaf aqueous extract for experimental studies

2.6.1 Preparation of *Leea asiatica* (L.) extract stock

To prepare the LA extract stock, 20 grams of LA leaf powder were dissolved in 200 mL of autoclaved double distilled water. The prepared powder solvent was then subjected to the rotary evaporator to acquire the distilled aqueous extract. The obtained distilled aqueous extract of LA concentration was 10%. Different concentrations of LA extract were achieved through serial dilution with autoclaved double distilled water/or 5% sucrose solution, as shown in **Table 2.1**

Standard	**Autoclaved double distilled water/5% Sucrose solution**	**Concentration**	**Stock Name**
20 grams	200 mL	10%	R10
225 µL R10	75 µL	7.5%	R7.5
150 µL R10	150 µL	5%	R5
75 µL R10	225 µL	2.5%	R2.5
50 µL R10	250 µL	1.25%	R1.25
25 µL R10	275 µL	0.625%	R.625

Table 2.1: Preparation of multiple concentrations of aqueous extract of *Leea asiatica* (L.).

2.6.2 Precautions

1. The LA leaf aqueous extract stock (10%) should be stored/kept at -80^{o}C in order to prevent sample degradation.

2. Keep the stock solution at room temperature to melt and then prepare multiple concentrations (Avoid heat generation to prevent degradation)

3. All the concentrations were made in the autoclaved double distilled water to prevent contamination by molecules that could spoil the stock solution.

4. Fresh pipette tips were used to prepare serial dilutions of the LA stock.

Chapter 3

Understanding *Leea asiatica's* Dopaminergic (DAergic) Neuroprotective Efficacy in the Adult Transition Stage *Drosophila* Model of Parkinson's Disease

Chapter 3

3. Introduction

Drosophila, dopaminergic (DAergic) system is involved in locomotion (Ayajuddin et al.,2023; Pendleton et al., 2002), sleep, and arousal (Foltenyi et al., 2007; Kume et al., 2005; Andretic et al., 2005), courtship behavior (Koza et al., 2021; 2023; Liu et al., 2008; Neckameyer, 1998), inhibition of startle-induced hyperexcitability (Friggi-Grelin et al., 2003), saliency-based decision making (Zhang et al., 2007), and associative learning (Schwaerzel et al., 2003; Tempel et al., 1984). In addition, the DAergic system influences how light perception, circadian entrainment, and social spacing are regulated in flies (Kasture et al., 2018). The flies with tyrosine hydroxylase (TH) deficiency, and consequently dopamine (DA) deficiency, have hypoactivity, longer sleep duration, decreased alertness, a lack of desire for sucrose, defective olfactory aversive learning, and locomotor impairments that tend to get worse with age (Cichewicz et al., 2017; Riemensperger et al., 2011). TH, expressed by the *ple* gene in flies, converts tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). The loss of the *Drosophila* TH gene results in embryonic lethality (Neckameyer and White 1993). TH-null lethality can be reversed by restoring hypoderm-specific TH expression, but not CNS-specific TH expression (Riemensperger et al., 2011). When L-DOPA and carbidopa are fed to TH-deficient flies, brain levels of DA improve (Cichewicz et al., 2017). Male-male courting behaviour in flies is markedly increased by over-expression of TH (Liu et al., 2008). Hypoactivity, sugar preference, and unpleasant learning are restored in TH-deficient flies by feeding them L-DOPA, a precursor to DA. Similarly, TH-deficient mice model displayed hypoactivity and reduced eating behaviour, and these symptoms can be improved with L-DOPA therapy (Kasture et al., 2018; Zhou and Palmiter, 1995).

Complete deletion of TH activity causes heart failure in mice and, most likely, in humans (Zhou et al., 1995; Kobayashi et al., 1995). According to Blanchard-Fillion et al. (2001), Nagatsu et al. (1990), and Ng et al. (2015), early loss of TH activity followed by a drop in TH protein is thought to contribute to DA deficiency and phenotypic manifestation in PD, L-DOPA-responsive dystonia, and/or infantile parkinsonism in mammals. All these exciting results highlight the relevance and importance of animal models for human NDDs such as PD.

The *Drosophila* models of PD recapitulate the characteristic pathophysiological features of human PD, such as locomotor defects, DAergic neurodegeneration, and reduced brain DA (Ayajuddin et al., 2023; Chaouhan et al., 2022; Phom et al., 2014; Shukla et al., 2014; Chaudhuri et al., 2007; Feany and Bender, 2000).

The misexpression of human α-syn in the central nervous system of *Drosophila* causes degeneration of DAergic neurons, disruption of eye-ommatidial, formation of filamentous aggregates that are structurally similar to LB inclusions, and progressive age-dependent locomotor dysfunction identical to the clinical manifestations of PD in human (Feany and Bender, 2000). Since the inception of the first *Drosophila* PD model by Feany and Bender, (2000), many laboratories have started employing the same animal model to study the effects of mutations or over-expression of genes involved in PD. The PQ-mediated *Drosophila* PD model shows time and dose-dependent DAergic neurodegeneration, altered DA metabolism, and locomotor dysfunction that are accompanied by changes in neuronal appearance, such as the aggregation of cell bodies into circular shapes, fragmentation, and eventually the selective loss of subsets of

DAergic neurons from the particular cluster (Chaouhan et al., 2022; Maitra et al., 2021, 2019; Song et al., 2017; Shukla et al. 2014; Lawal et al., 2010; Chaudhuri et al. 2007).

Drosophila has approximately 280 DAergic neurons per brain. These DAergic neurons are dispersed among eight clusters per hemisphere, each consisting of four to thirteen individual neurons, except for the PAM cluster, which has nearly 100 neurons per hemisphere (Mao and Davis, 2009; Nässel and Elekes, 1992). However, the quantifiable DAergic neurons in the whole fly brain are PAL (4-5 neurons), PPL1 (11-12 neurons), PPL2 (6-7 neurons), PPM1/2 (8-9 neurons), PPM3 (5-6 neurons) and VUM (3 neurons) (PAL- Protocerebral anterior lateral; PPL- Protocerebral posterior lateral; PPM- Protocerebral posterior medial) that can be tagged with primary anti-TH antibody (Ayajuddin et al., 2023; Koza et al., 2023; Navarro et al., 2014; Whitworth et al., 2006; Friggi-Grelin et al., 2003; Nässel and Elekes, 1992; Budnik and White, 1988).

In this chapter, I attempted to understand the neuroprotective efficacy of *Leea asiatica* in the PQ-mediated transition stage-specific (TP) fly model of PD by employing both the pre-and co-feeding regimen through assessing mobility phenotypes and further by understanding the DAergic neurodegeneration and therapeutic neuroprotection under LA intervention through **a**) the quantification of DAergic neuronal number and **b**) through quantification of the levels of TH protein in DAergic neurons through quantification of the fluorescence intensity (FI) of secondary antibodies which targets the primary antibody anti-tyrosine hydroxylase (anti-TH). TH is the rate-limiting enzyme necessary for the synthesis of DA. FI of the secondary antibody targeting the primary anti-TH antibody represents TH abundance in the DAergic neuron.

Chapter 3

3.1 Materials and Methods

3.1.1 *Drosophila* stock and husbandry

The male Oregon K (OK) flies of the *D. melanogaster* were used in the present study procured from the National *Drosophila* Stock Center, Mysuru University, Mysuru, Karnataka, India. The flies were reared in a fly incubator at 22° ± 2° C temperature with 60% humidity, and 12:12 hours (Hrs) light/dark cycle (Percival, United States). A culture media constituting sucrose, yeast, agar-agar, and propionic acid was used to feed the flies (Phom et al., 2014; Luckinbill et al., 1984).

3.1.2 Collection and aging of adult male files

The collection of adult male flies was achieved by keeping the parental generation in freshly prepared media vials for laying eggs for 3 to 4 days and then removed. After 10-12 days, the flies that eclosed were lightly anesthetized to separate males and females. The flies were trapped /scored by giving them a few drops of diethyl ether to lightly sedate them. Each vial with fresh culture media contained not more than 25 flies. Every 3rd day, the collected flies were moved to a freshly prepared media vial. The aging was done according to the experiment requirements. The flies belonging to 50-55 days representing the transition phase (TP) of the adult life span were used to model the late-onset form of PD (Phom et al., 2014).

3.1.3 *Drosophila* life span assay

Life span curves were obtained from independent trials with a minimum of 100 flies per experiment. The experimental flies were shifted to freshly prepared culture media every third day until all the flies were recorded dead (Phom et al., 2014).

3.1.4 Chemicals for feeding and exposure

The required chemicals viz., Sucrose procured from Sisco Research Laboratory (SRL, Maharashtra, India, catalog number: 84973), Type I Agar Agar procured from HiMedia (Thane, India, catalog number: GRM666), Propionic acid procured from MERCK (Rahway, USA, catalog. number: 8006050-500-1730), and market available sugar tolerant dry yeast (Angel, instant dry yeast) were used for preparation of food media. Methyl viologen dichloride hydrate /Paraquat was procured from Sigma Aldrich (PQ; Sigma-Aldrich, St. Louis, MO, USA, catalog number: 856177). PQ was used for exposure methodology procedures on Whatman filter paper no.1 disc in a 30x100mm glass vial.

3.1.5 Paraquat susceptibility assay

The OK male flies aged 4-5 days old were utilized to explore the oxidative stress against the different concentrations of PQ (2.5, 5,10, 15, 20, and 40 mM). The different concentrations of PQ prepared in 5% sucrose were dispensed into a glass vial containing Whatman filter paper. At every 24 hours, the survival rate was observed and documented. Care was taken to avoid starvation of the flies before switching it to the different concentrations of PQ as starvation could lead to alteration of the cell survival pathways (Phom et al., 2014).

3.2 *Leea asiatica* (LA) pre- and co-feeding regimens in the adult transition stage *Drosophila* model of Sporadic PD

Two different treatment regimens were employed to comprehend the neuroprotective efficacy of LA in the *Drosophila* model of PD, i.e., the co-feeding and pre-feeding regimes. The concentration of PQ used for the experiment was 10 mM for the transition

phase flies, respectively. The 10mM PQ concentration was selected from the studies conducted/performed by Phom et al. (2014) with utmost care so that they did not affect the viability but caused mobility defects in *Drosophila*. The aqueous extract of LA concentrations was chosen in such a way as to exhibit no impairment in mobility and no effect on the survival of the fly.

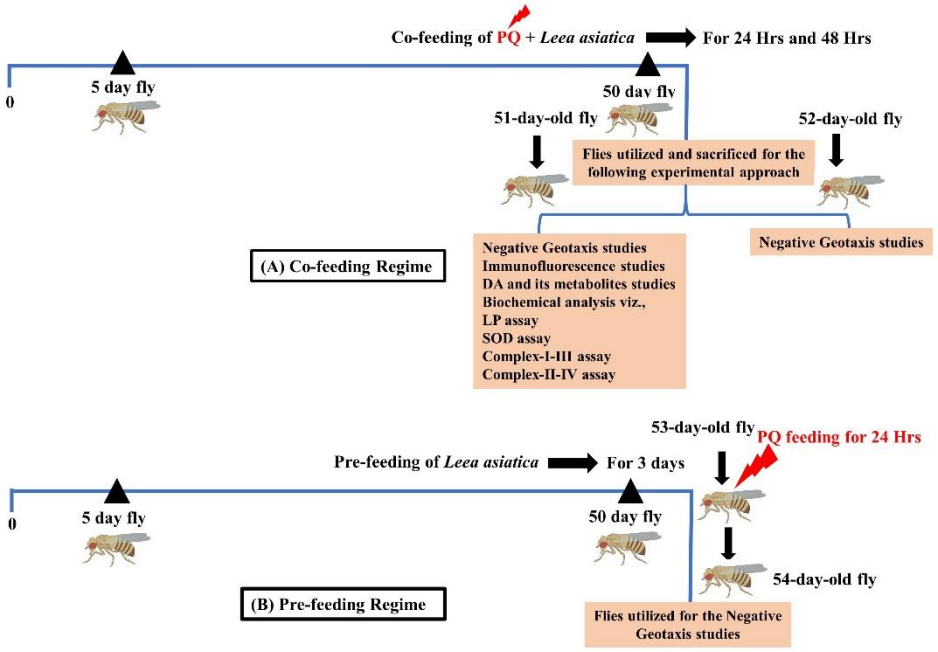


Figure 3.1: *Drosophila* feeding regimens: (A) **Co-feeding**: Flies were fed with PQ alone or PQ with different concentrations of aqueous extract of LA for 24 and 48 hours during the transition phase (50-55 days old). Control flies remained in 5% sucrose only, while the *per se* group was fed with LA extract only. The negative geotaxis assay (NGA) was performed after 24 and 48 hours of exposure to PQ in the transition phase flies, respectively. (B) **Pre-feeding regimen**: Flies of the transition phase were pre-fed with an aqueous extract of LA for 3 days. The control and group treated with PQ alone remained at 5% sucrose during this period. The flies were then exposed to PQ for 24 hours. Control

and *per se* group remained in 5% sucrose. After 24 hours of exposure to PQ, NGA was performed in the transition phase flies.

(A) Co-feeding regime: For the co-feeding regimen, the flies were fed with PQ alone for the PD treatment group, and a combination of PQ along with aqueous extract of LA (10%, 7.5%, 5%, 2.5%, and 1.25%) for the co-feeding group. The preparation of aqueous extract of LA with PQ for co-feeding was achieved by dissolving 10mM PQ in 1ml of LA extract with 5% sucrose (R10%). Then, from R10%, different concentrations of LA were prepared by dissolving 225 µl of R10% extract along with 75 µl of 10mM PQ for R7.5% only and by dissolving 150 µl of R10% extract along with 150 µl of 10mM PQ for 5% and, by dissolving 75 µl of R10% extract along with 225 µl of 10mM PQ for 2.5% and by dissolving 50 µl of R10% extract along with 250 µl of 10mM PQ for 1.25% respectively. Also, LA extract *per se* at the aforementioned concentrations (LA 10%) was achieved by dissolving 5% sucrose in 1 ml of aqueous extract of LA (10%). LA extract *per se* 7.5% was achieved by dissolving 225 µl of LA extract with 75 µl of 5% sucrose solution. Treatments were carried out by feeding the flies with 275 µl of 10mM of PQ (Induced PD group), 10mM PQ + 1mL of LA Extract (R10%), 10mM PQ +7.5% LA Extract (R7.5%), 10mM PQ + 5% LA Extract (R 5%), 10mM PQ + 2.5% LA Extract (R 2.5%), 10mM PQ +1.25% LA Extract (R1.25%) (LA co-feeding group), and 5% sucrose + 1mL LA extract (10% LA *perse* group), 5% sucrose + 7.5% LA extract (7.5% LA *perse* group) respectively on Whatman filter paper No.1 in disc-feeding experiments. The control flies were only kept in a 5% sucrose solution on filter paper. Climbing ability was assessed after 24 and 48 hours of co-feeding with PQ during the transition phase flies (**Figure 3.1 A**).

(B) Pre-feeding regimen: For the pre-feeding regimen, the flies were fed with 5% sucrose alone to the PD treatment group and a combination of 5% sucrose along with aqueous extract of LA (10%, 7.5%, 5%, 2.5%, and 1.25%) to the pre-feeding groups for 3 days and then switched to PQ. The LA pre-feeding was achieved by dissolving 5% sucrose in 1 ml of extract (R10%). Then, from R10%, different concentrations of LA were prepared by dissolving 225 µl of R10% extract along with 75 µl of 5% sucrose for R7.5% only and by dissolving 150 µl of R10% extract along with 150 µl of 5% sucrose for 5% and, by dissolving 75 µl of R10% extract along with 225 µl of 5% sucrose for 2.5% and by dissolving 50 µl of R10% extract along with 250 µl of 5% sucrose for 1.25% respectively. Also, LA extract *per se* at the aforementioned concentrations (LA 10%) was achieved by dissolving 5% sucrose in 1 ml of aqueous extract of LA (10%). LA extract *per se* 7.5% was achieved by dissolving 225 µl of LA extract with 75 µl of 5% sucrose solution. Treatments were carried out by feeding the flies with 275 µl of 10mM of PQ (Induced PD group), 10mM PQ + 1mL of LA Extract (R10%), 10mM PQ +7.5% LA Extract (R7.5%), 10mM PQ + 5% LA Extract (R 5%), 10mM PQ + 2.5% LA Extract (R 2.5%), 10mM PQ +1.25% LA Extract (R1.25%) (LA pre-feeding group), and 5% sucrose + 1mL LA extract (10% LA *perse* group), 5% sucrose + 7.5% LA extract (7.5% LA *perse* group) respectively on Whatman filter paper No.1 in disc-feeding experiments. The control flies were only kept in a 5% sucrose solution on filter paper. Climbing ability was assessed after 24 hours of feeding with PQ for the transition phase (**Figure 3.1 B**). For the experimental group in the TP, a minimum of 100 flies were exposed to quantify LA-mediated neuroprotective efficacy.

3.3 Negative geotaxis assay

The mobility deficit was measured using a negative geotaxis assay (climbing assay), as described by Botella et al. (2004) and Phom et al. (2021). Briefly, the individual fly was placed into a plastic tube that was 1 cm in diameter and 26 cm long and was given 2 minutes to acclimatize. The fly was then taped to the tube's bottom, and the height the fly reached in 12 seconds was measured. Each fly followed the identical procedure thrice, and 10 flies from each group were scored. The time point where the fly exhibited significant mobility impairments, but no mortality was chosen/selected to assess the neuroprotective efficacy.

3.4 Whole brain immunostaining for Tyrosine Hydroxylase (TH):

3.4.1 TH Immunostaining

Sterilized 1.5 mL centrifuge tubes (Tarsons, WB, India, catalog number: 500010), Parafilm^{TM} wrapping film (Bemis, WI, USA, catalog number: PM996), Conical flask (Borosil, Mumbai, India, catalog number: 5100), Magnetic stirrer bar #8 mm × 40 mm (Tarsons, WB, India, catalog number: 4113), SPINNOT^{TM} digital magnetic stirrer hotplate (Tarsons, WB, India, catalog number: 6090), Sterilized micro tips (Tarsons, WB, India, catalog number: 521010), Freshwrapp aluminum foil 9–11 μm (Hindalco, Maharashtra, India, catalog number: HV2241), Glass plate (Suwimut, USA, catalog number: B08FRB2NTM), Fingernail polish (FacesCanada, Mumbai, India, catalog number: CC4403), Glass spacer (Borosil, Mumbai, India, catalog number: 9115S01), Microscopy slides #76 mm × 26 mm (ReliGlas, Haryana, India, catalog number: 7101), Gold-seal coverslips (22 mm^2) (Electron Microscopy Sciences, PA, USA, catalog

number: 63765-01), Whatman™ filter paper (GE Healthcare, Buckinghamshire, UK, catalog number: 1001917), Paraformaldehyde (PFA) pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA, catalog number: I58127), Phosphate buffered saline (PBS) pH 7.4 (HiMedia, Maharashtra, India, catalog number: ML023), Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA, catalog number: T8787), Normal goat serum (NGS) (Vector Labs, CA, USA, catalog number: S1000), Rabbit anti-tyrosine hydroxylase (anti-TH) polyclonal primary ab (Millipore, MA, USA, catalog number: Ab152), Goat anti-rabbit IgG H&L (TRITC-labeled) polyclonal secondary ab (Abcam, MA, USA, catalog number: Ab6718), VECTASHIELD® mounting medium (Vector Labs, CA, USA, catalog number: H1000), Fly head capsule handling items e.g., needles #31 G × 6 mm (Tentabe BD, Punjab, India, catalog number: 324902), Dissecting fine forceps (EMS, PA, USA, catalog number: 78620-4B), Brush (TEYUP, Delhi, India, model number: SR-1013), Delicate task Kim wipers (KIMTECH™, GA, USA, catalog number: 370080), Micropipette i.e., 1,000 µL, 50 µL, 10 µL, 2 µL (Gilson, WI, USA, catalog number: 30040), Frost-free refrigerator (Whirlpool, MI, USA, model number: FF26 4S), pH/mV meter (Hanna Instruments, RI, USA, model: HI2211-02), -20°C ES Series refrigerator (Thermo Scientific, MA, USA, model: 50616100444443250), -80°C ultra-low temperature freezer (New Brunswick Innova, Hamburg, Germany, model: U101-86), Stereo zoom microscope (Carl Zeiss, Jena, Germany, model: Stemi 305), Stereo zoom microscope (Leica, Wetzlar, Germany, model: E24), Fume hood (BIOMATRIX, Telangana, India), BOD incubator (Percival, IA, USA, model: DR-36VL), Test tube rotator (Tarsons, Rotospin, WB, India, catalog number: 3070) and disk for 24 × 1.5 mL tube (Tarsons, WB, India, catalog number: 3071), Axio Imager M2 fluorescence microscope fitted with 100W Mercury lamp (Carl Zeiss, Jena, Germany, catalog number: 430004-9902-000), AxioCam ICm1

monochromatic camera (Carl Zeiss, Jena, Germany, catalog number: 426553-9901-000), ZEN 2012 SP2 blue edition, version 2.0.14283.302 (Carl Zeiss, Jena, Germany), Microsoft Office Excel Worksheet 2007 (Microsoft Inc., WA, USA).

Recipes

1. **4% PFA solution (50 mL)**

PFA 2 g

1× PBS 50 mL

a. Add PFA in 1× PBS in a conical flask, cover it with parafilm, and shake it thoroughly for 10 min.

b. Transfer the flask with a magnetic stirrer on the hotplate for heating/boiling with a temperature ranging from 80 °C to 110 °C with moderate stirring at 150 rpm.

c. Keep the flask on the hotplate until the cloudy solution becomes transparent.

d. After this, switch off the hotplate but keep the stirring for 15 min. Allow the solution to cool down, aliquot it in a 1.5 mL centrifuge tube, and store it at -80 °C.

Critical: Do not store the solution for more than a week.

Caution: PFA is a potential carcinogen; hence, the whole process should be done under a fume hood. Wear hand gloves and a lab coat during handling and preparation of PFA solution.

2. 0.1% PBST (phosphate buffered saline and Triton X-100) (50 mL)

10× PBS 5 mL

Autoclaved enzyme-free water 45 mL

Triton X-100 50 µL

a. Add 5 mL of 10× PBS in 45 mL of autoclaved enzyme-free water.

b. Mix 50 µL of Triton X-100 and vortex it for 10 seconds. The solution can be stored at room temperature for one week.

3. 0.5% PBST (50 mL)

10× PBS 5 mL

Autoclaved enzyme-free water 45 mL

Triton X-100 250 µL

a. Add 5 mL of 10× PBS in 45 mL of autoclaved enzyme-free water.

b. Mix 250 µL of Triton X-100 and vortex it for 10 s. The solution can be stored at room temperature for one week.

4. 5% NGS blocking buffer solution (1 mL)

NGS 50 µL

0.5% PBST 950 µL

Add 50 µL of NGS in 950 µL of 0.5% PBST and mix it properly by vortexing for 10 s. The solution can be stored at room temperature for 1–2 hours.

5. **Anti-TH polyclonal primary ab solution**

Anti-TH polyclonal primary ab 5 µL

5% NGS blocking buffer 1,245 µL

Take 1,245 µL of 5% NGS blocking buffer and add 5 µL of anti-TH polyclonal primary ab (1:250 dilution). Mix it gently by inverting the tube slowly and place it on the ice until used.

6. **TRITC-labeled polyclonal secondary ab solution**

TRITC-labeled polyclonal secondary ab 5 µL

5% NGS blocking buffer 1,245 µL

Take 1,245 µL of 5% NGS and add 5 µL of TRITC-labeled polyclonal secondary ab (1:250 dilution). Mix it gently by inverting the tube slowly and store it on ice until used.

3.4.2 Characterization of DAergic neurodegeneration

The following four steps were taken into consideration to comprehend neurodegeneration in the fly model of sporadic PD:

A) Anti-TH immunostaining of the whole *Drosophila* brain.

B) Image acquisition.

C) Quantification of DAergic neurons.

D) Quantification of neurodegeneration through quantification of fluorescence intensity (FI) of DAergic neurons.

A) Anti-TH Immunostaining in the whole *Drosophila* brain:

The *Drosophila* brain was immunostained for fluorescence microscopy (Carl Zeiss, Axio Imager M2 with ZEN software, Germany) according to the protocol of Chaurasia et al. (2024); Ayajuddin et al. (2023); Koza et al. (2023). Elaborately, Anti–TH Immunostaining procedures were carried out as follows:

Methods:

1. The whole fly head tissue were fixed in 4% paraformaldehyde (PFA; pH 7.4) containing 0.5% Triton X-100 (TX-100) for 2 hours (Hrs) through mixing by using a test tube rotator with constant velocity (10 rpm) at room temperature (RT).

2. PFA was then removed after 2 Hrs of fixation by washing the fly brains with PBS that contains 0.1% TX-100 (0.1% PBST) three times after every 15 minutes at RT.

3. Dissection of brains was carried out in PBS (pH 7.4) under a stereo zoom microscope using fine forceps and needles to remove the head capsule and connecting tissues at RT.

4. The brains were then washed with 0.1% PBST for 5 times after every 15 minutes at RT.

5. The brains were blocked with 5% NGS in PBS containing 0.5% TX-100 (0.5% PBST) for 120 minutes at RT.

6. Then, the brains were incubated/probed with primary anti-TH polyclonal antibody in the dilution of **1:250** for 72 Hrs at 4°C through mixing by using a test tube rotator at constant velocity (10 rpm).

7. The excess primary antibodies were washed off by 0.1% PBST for 5 times after every 15 minutes at RT.

8. The brains were then incubated with a TRITC (Tetramethylrhodamine) labelled polyclonal secondary antibody in the dilution of **1:250** for 24 Hrs in the dark (**Critical**: Cover centrifuge tube containing brains with aluminum foil) by thorough mixing with a test tube rotator at a constant velocity (10 rpm) at RT.

9. Again, to eliminate excess polyclonal secondary antibodies, the brains were washed with 0.1% PBST for 5 times after every 15 minutes at RT.

10. The brains were mounted in VECTASHIELD® mounting medium and then topped with cover glass (Electron Microscopy Sciences). **Critical:** Glass spacers were placed around the VECTASHIELD® mounting medium to protect brains from being crushed by a coverslip.

Critical: Brains were scanned in a dorsoventral orientation.

11. Clear fingernail polish was used to seal the edges.

12. The samples were prepared for image acquisition.

Precautions and Recommendations:

1. During fixation, brains were thoroughly mixed using a circular rotator (Rotospin from Tarsons, India Cat: 3070) at a constant speed of 10 RPM.

3. Circular rotator was used for proper incubation/mixing of primary and secondary antibodies to the brain samples.

4. To prevent brains from being crushed, care was taken by keeping glass spacers while mounting the brain with a cover slip.

5. To prevent the drying of the samples, the edges were carefully sealed with nail polish.

6. In order to prevent bleaching, image acquisition was carried out on the same day.

B) Image Acquisition

The *ZEN 2012 SP2* software of fluorescence microscope equipped with a 100W Mercury lamp was used to capture brain images. Steps for acquisition of *Drosophila* brain Image for quantification of DAergic neurons and fluorescence Intensity (FI) using fluorescence microscope (Axio Imager 2, Carl Zeiss) with *ZEN 2012 SP2* software illustrates from **figure 3.2 to figure 3.12**.

Methods:

1. At a 40x objective lens of fluorescence microscope, prepared/stained brains were viewed/observed (**Figure 3.2**).

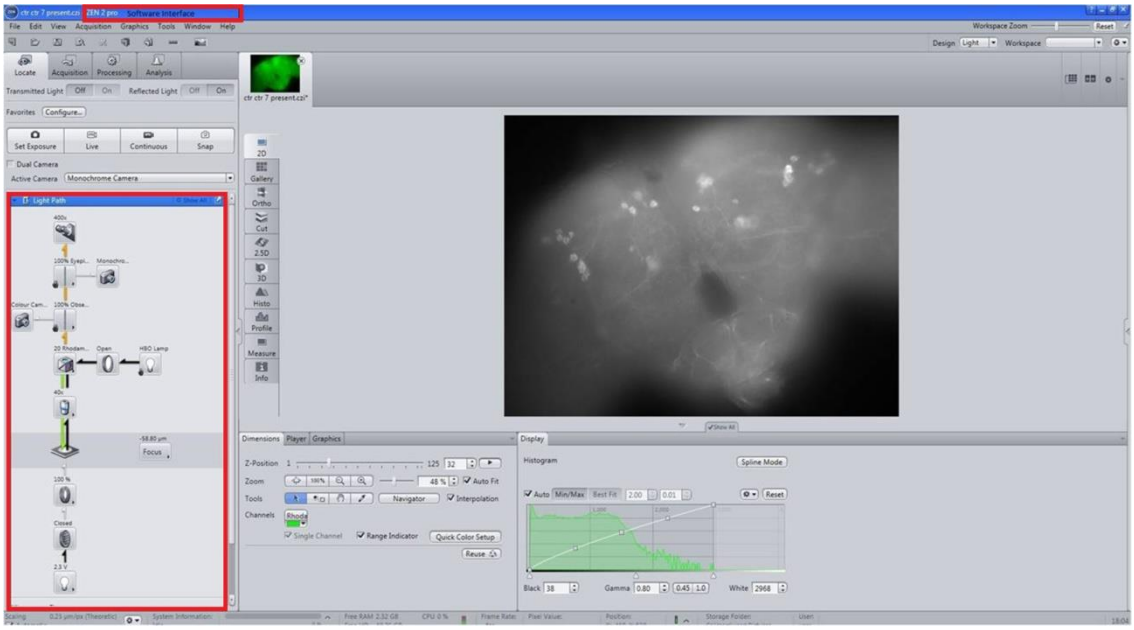


Figure 3.2. Scanning of the whole brain of *Drosophila*. Scan the anti-TH immunostained *Drosophila* brain using Carl Zeiss, Axio Imager M2 (40× objective lens) with ZEN 2012 SP2 software that interactively controls image acquisition, image processing, and analysis of the images.

2. Images were scanned and taken using a monochromatic camera with a Rhodamine fluorescence filter (**Figure 3.3**).

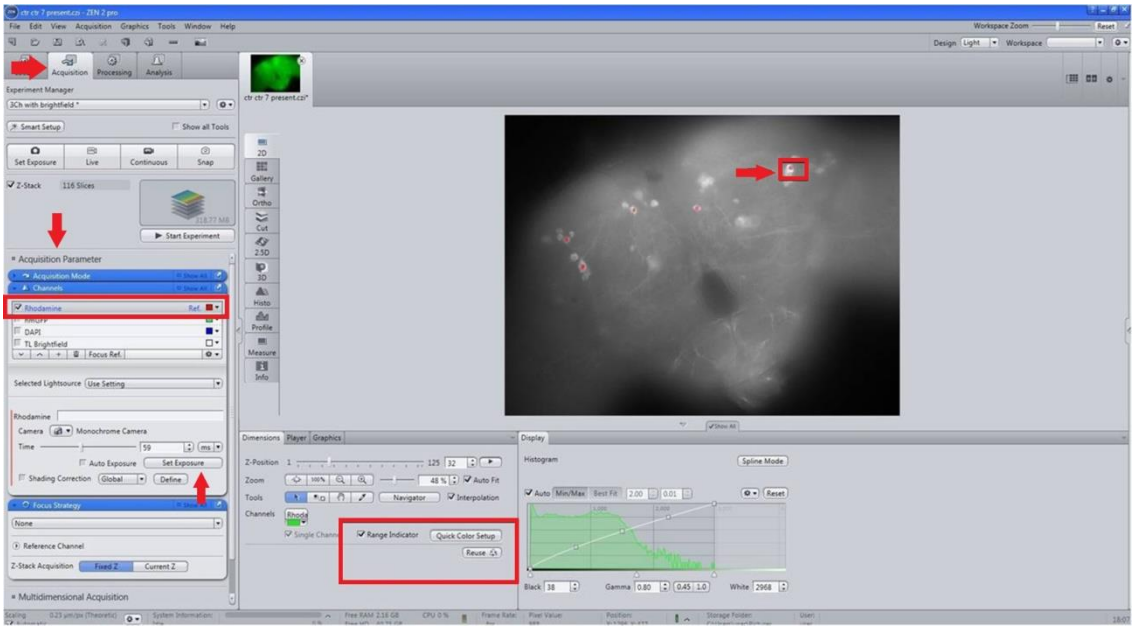


Figure 3.3. Image acquisition and performing the red dot test. For image acquisition, select a monochromatic camera with a Rhodamine filter. Perform a red dot test for visibility of dopaminergic (DAergic) neurons and assessing saturation using a brain, reusing the same exposure time for other samples.

3. A red dot test was performed in the control brain in the acquisition panel (select range indicator from *Dimensions* and set exposure from *Acquisition parameter*) for visibility of DAergic neurons and to assess the signal saturation during the image acquisition. Reuse the same exposure time for all brain samples (**Figure 3.3**).

4. Then, Z-stack programming was performed with constant interval of 1.08 µm for each image (**Figure 3.4**).

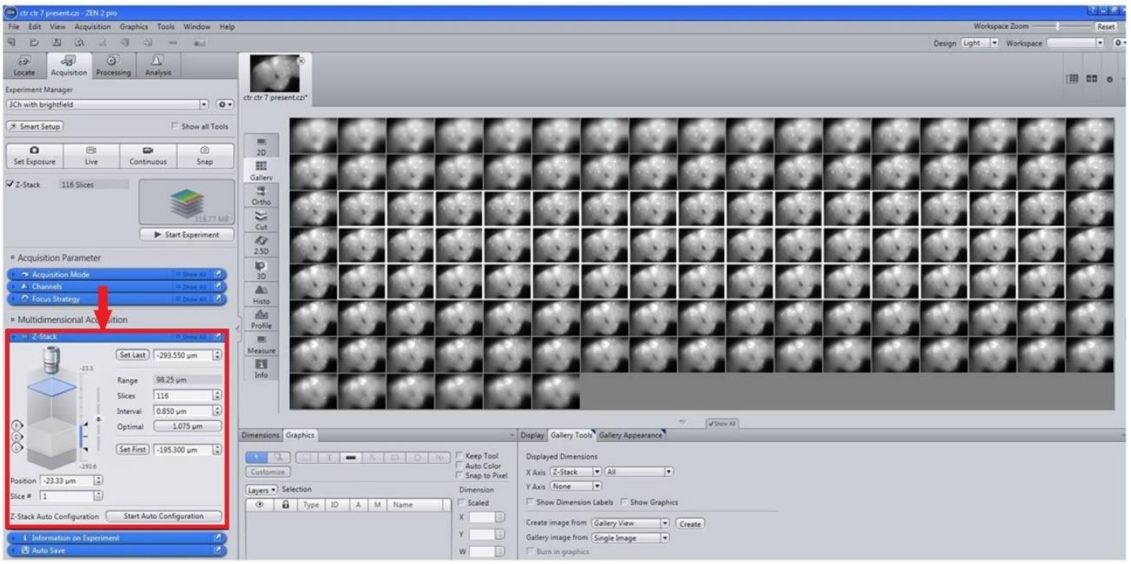


Figure 3.4. Selection of images and Z-Stacking.

5. For image processing/generating in 2D, on the method column apply *Ortho* and *Maximum intensity projection (MIP)* from *Ortho display* with *X–Y Plane* (**Figure 3.5**).

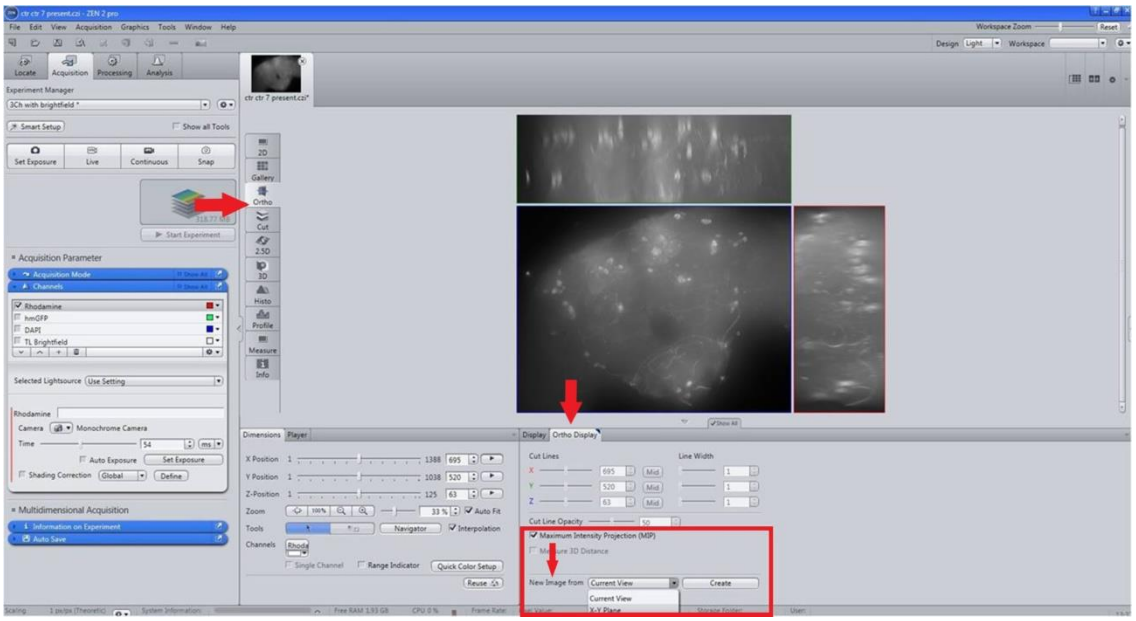


Figure 3.5. Creation of 2D image. For creating a 2D merged image, on the *Method* column, select *Maximum intensity projection (MIP)* with *X-Y Plane.*

6. The 2D image of the brain was exported in.jpg format for presentation (**Figure 3.6**).

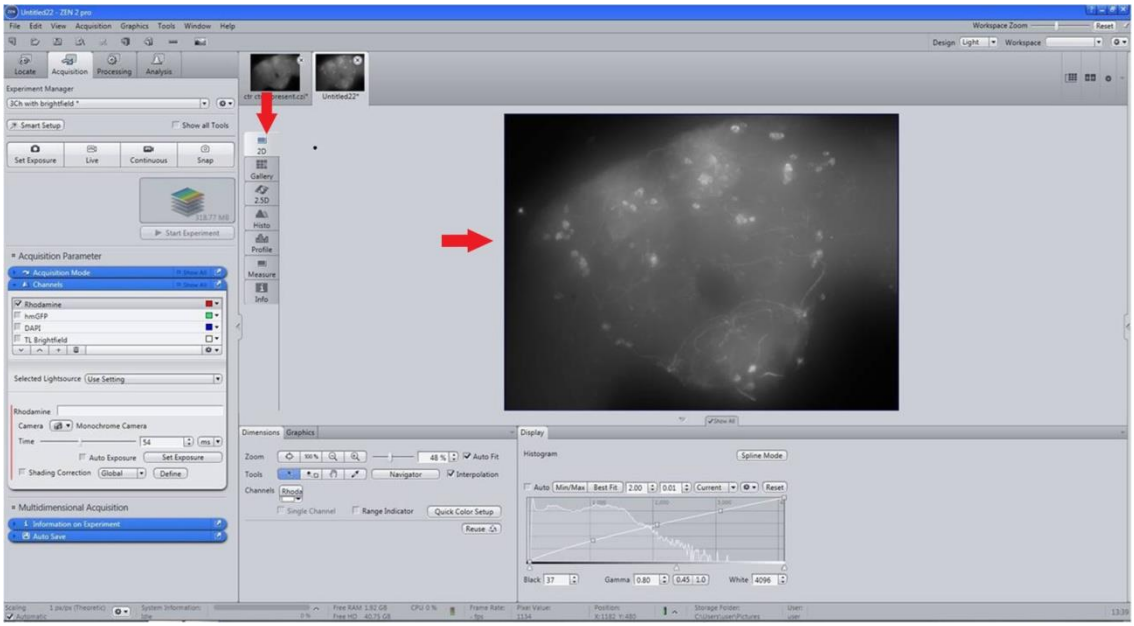


Figure 3.6. Export of 2D brain image to the required format

Precautions and Recommendations:

1. Special attention/care was taken during image acquisition for the same orientation of the brains.

2. The red dot test was carried out carefully.

3. The same setting was always reused for all the brain images.

4. Care was taken to ensure that all the DAergic neurons were covered and scanned while performing the Z-stack programming.

After the images were acquired through Z-stack programming, the subsequent steps were taken into consideration:

C) Quantification of the total number of DAergic neurons

In the past one and half a decade, to gain a better understanding, numerous *Drosophila* models have been reported elucidating the mechanisms of PD development, progression, and rescue strategies (Ayajuddin et al., 2022; Akinade et al., 2022; Navarro et al., 2014; Phom et al., 2014; Whitworth, 2011). The ground-breaking findings by Feany and Bender (2000), prompted the excitement surrounding this model that demonstrated the age-associated loss of DAergic neurons in the α-synuclein-mediated *Drosophila* PD model that were similar to human PD. The DAergic neuronal system and its placements in the *Drosophila* brain were described using DA and anti-TH immunoreactivity (Budnik and White, 1988; Nässel and Elekes, 1992). These studies led to the characterization of individual clusters, which were named according to their anatomical position in the brain (Monastirioti, 1999). The details of neurons anatomical location and numbers were presented in **Table 3.1**, and the *Drosophila* brain cartoon (**Figure 3.17A**) depicts the position of DAergic neurons in the fly brain.

Clusters	Abbreviated as	Number	Location	Remark
Protocerebral anterior medial	PAM	~100	Medial tips of and areas posterior to horizontal lobes	Not countable
Protocerebral anterior lateral	PAL	4-5	Optic tubercle, superior posterior slope, ventral medial protocerebrum	countable
Protocerebral posterior medial	PPM1	1-2	Ventrally along midline	countable, too close and usually clubbed together as PPM1/2
	PPM2	7-8	Subesophageal ganglion, ventral medial protocerebrum	
	PPM3	5-6	Central complex	countable
Protocerebral posterior lateral	PPL1	11-12	Mushroom bodies and vicinity, superior arch	countable
	PPL2	6	Calyx, lateral horn, posterior superior lateral protocerebrum, Lobula	countable
Ventral unpaired medial	VUM	3	Lower subesophageal	Easily countable
Protocerebral posterior deutocerebrum	PPD	0-1	Posterior slope	Too low or absent
Protocerebral posterior dorsomedial	PPM4	0-1	Central complex	Too low or absent
Protocerebral posterior lateral	PPL3	0-1	Superior posterior slope, dorsal edge of the lateral horn	Too low or absent
	PPL4	0-1		
	PPL5	0-1		

Table 3.1: The table briefs the anatomical location and number of DAergic neurons in the *Drosophila* brain, arranged in each hemisphere in different clusters. There are a total of 280 DAergic neurons in the *Drosophila* brain. While the majority of these clusters can be quantified, the PAM cluster cannot be counted/quantified using fluorescence microscopy. (Modified from Nässel and Elekes, 1992).

Method	**Paraffin section / light microscopy**		**Whole-mount / confocal microscopy**		**Reference(s)**
Cluster/ Model	PPL1	PPM1/2	PPL1	PPM1/2	
α-Syn	No	Yes	-	-	Feany and Bender, 2000
	Yes	Yes	-	-	Auluck et al., 2002
	-	Yes	-	-	Auluck and Bonini, 2002
	-	Yes	No	No	Auluck et al., 2005
	-	Yes	-	-	Chen and Feany, 2005
	-	-	-	No	Pesah et al., 2005
Parkin	No	No	-	-	Greene et al., 2003
	-	-	-	No	Pesah et al., 2004
	-	No	-	-	Yang et al., 2003
	-	-	Yes	No	Whitworth et al., 2005
	-	No	-	-	Cha et al., 2005
DJ-1α	-	-	No	No	Menzies *et al.*, 2005
	-	-	No	No	Meulener et al., 2005
	-	Yes	-	-	Yang et al., 2005
DJ-1β	-	-	No	No	Meulener et al., 2005
	-	-	No	No	Park et al., 2005
Rotenone	PPL1	PPM1/2	PPL1	PPM1/2	
50 µM	-	-	Yes	Yes	Wang et al., 2007
250 µM	-	-	Yes	No	Lawal et al., 2010
250 µM	-	-	Yes	Yes	Coulom and Birman, 2004
500 µM	-	-	Yes	Yes	Coulom and Birman, 2004
500 µM	-	-	No	No	Meulener et al., 2005
500 µM	-	-	No	No	Navarro et al., 2014
10 µM			No	No	Ayajuddin et al., 2022
500 µM					
Paraquat	PPL1	PPM1/2	PPL1	PPM1/2	
100 µM	-	-	No	No	Meulener et al., 2005

10 mM	-	-	Yes	No	Lawal et al., 2010
10mM			Yes	Yes	Inamdar et al.,2012
10mM			Yes	Yes	Shukla et al.,2014
10mM			No	No	Ayajuddin et al., 2023
20mM			Yes	Yes	Shukla et al.,2014
20 mM	-	-	Yes	Yes	Chaudhuri et al., 2007
20mM	-	-	No	No	Navarro et al., 2014
5mM			Yes	Yes	Chaouhan et al.,2022
5mM			Yes	Yes	Maitra et al., 2019; 2021
1mM			Yes	Yes	Ortega-Arellano et al., 2017

Table 3.2: Summarisation of variations in the loss of DAergic neurons in *Drosophila* models of PD (both genetic and sporadic) from different laboratories. (Yes: DAergic neuronal loss in individual clusters and/or total DA neuronal number; No: No DAergic neuronal loss in individual clusters and/or total DA neuronal number)

The quantification of the DAergic neurons was followed by articulating these steps:

Methods:

1. Clusters were identified from the Z-stack images/scans by obtained through Z-stack programming with constant intervals (**Figure 3.7**).

2. The image was enlarged to reveal the cell body/structure (**Figure 3.7**).

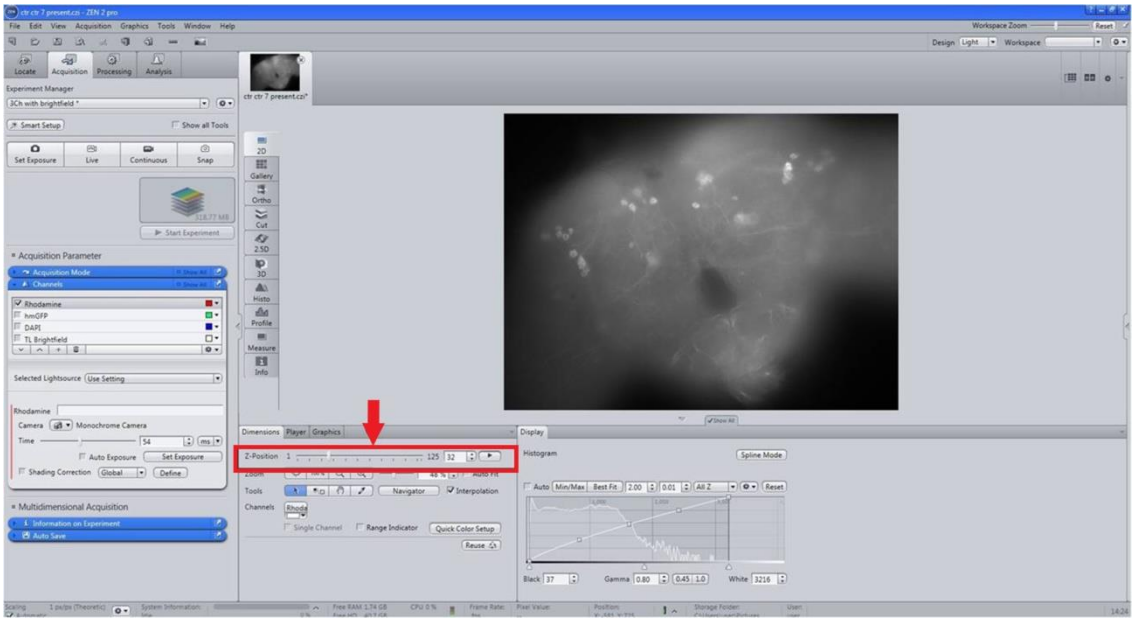


Figure 3.7. Quantification of dopaminergic (DAergic) neuronal number and fluorescence intensity (FI). For the quantification of DAergic neuronal number and FI, select 3D images/scans of Z-Stack with brain regions; PAL, PPL1, PPL2, PPM1/2, and PPM3 (PAL: Protocerebral anterior lateral; PPL: Protocerebral posterior lateral; PPM: Protocerebral posterior medial).

3. The number of DAergic neurons in each cluster was determined/counted in an unbiased manner.

4. For each group of treatments, a minimum of 5 to 6 brains were quantified.

D. Quantification of the fluorescence intensity of secondary antibodies to characterize neurodegeneration/neuroprotection

The loss of DAergic neurons was observed differently depending on the method adopted (Table 3.2). However, there are two methods widely used to quantify the DAergic

neurodegeneration, viz., immunostaining of the fly brain using anti-tyrosine hydroxylase (anti-TH) antibody and subsequently with secondary antibody and by tagging DAergic neurons with green fluorescent protein (GFP) using a TH-Gal4 driver line. The TH-Gal4 driven decrease in the fluorescence signal intensity of the GFP reporter correlates with the state known as "neuronal dysfunction" (Navarro et al., 2014), which underlies the decrease in TH and denotes DAergic degeneration. Hence, by taking advantage of the anti-tyrosine hydroxylase (anti-TH) antibody immunostaining method (Ayajuddin et al., 2023; Chaurasia et al., 2024) here, I attempted to investigate the DAergic neurodegeneration and LA mediated neuroprotection by measuring the FI of the fluorescently labeled secondary antibody targeted against the primary antibody (anti-TH) using *ZEN 2012 SP2* software from Carl Zeiss, Germany. *ZEN 2012 SP2*, Carl Zeiss software is a single user and a license must be acquired to utilize the imaging system to interactively control image acquisition, image processing, and analysis fluorescence microscope. The protocol for quantification of the FI is described below.

Methods:

1. Regions of the fly brain's PAL, PPL1, PPL2, PPM1/2, PPM3, and VUM (quantifiable DA neuronal clusters) were chosen from 3D scan images (**Figure 3.7**).

2. The brain images were enlarged to see the clear neurites (**Figure 3.8**).

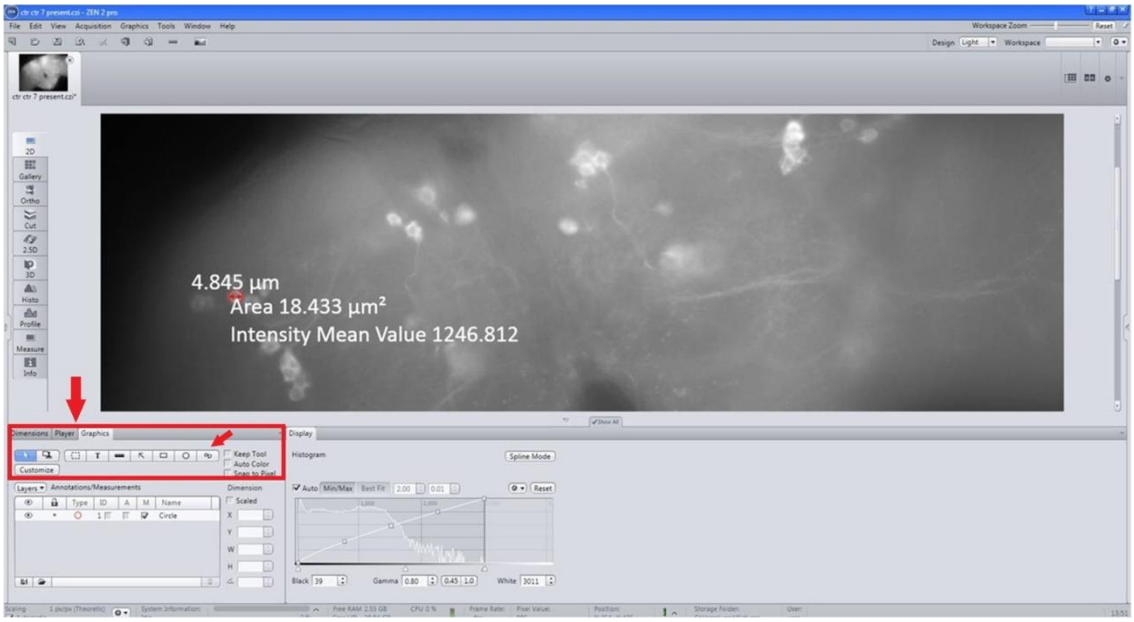


Figure 3.8. Details of the quantification of the fluorescence intensity (FI). Enlarge the images to see clear neurites, select appropriate tools, *draw spline contour* from graphics and draw a line around the neuron, and display intensity mean value and area.

3. The appropriate graphics tools '*draw spline contour'* was selected, and a line was drawn to encircle the neuron, giving intensity mean and area (**Figure 3.8**).

4. *More measurement options* were selected, and the *intensity sum* was chosen by right-clicking inside the neuron (**Figure 3.9**).

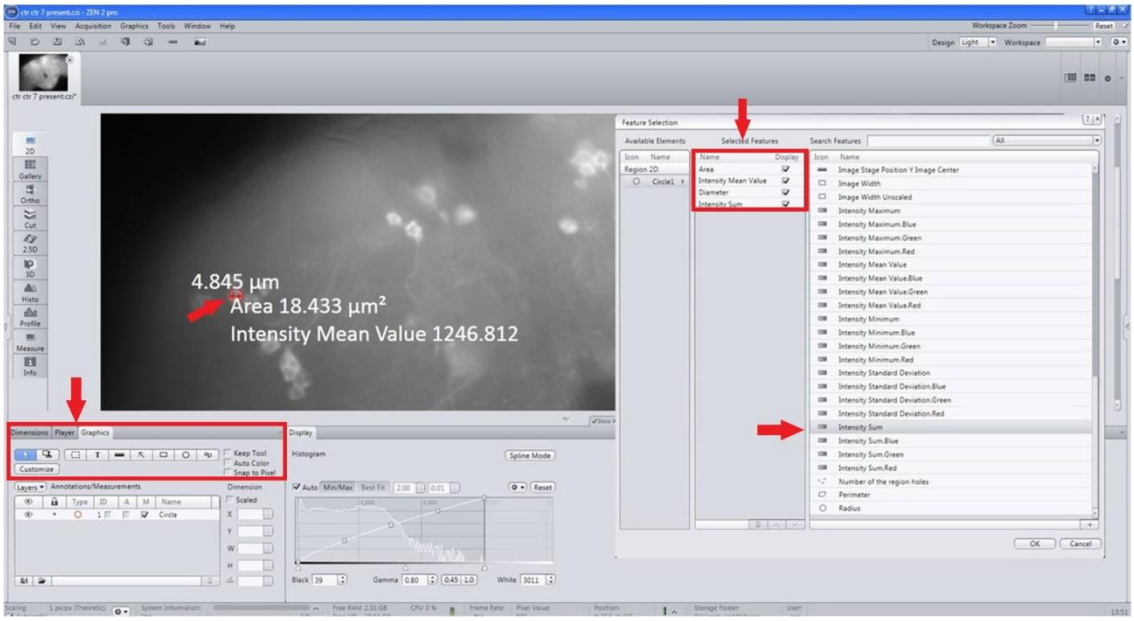


Figure 3.9. Measurement of fluorescence intensity (FI) sum. Select *intensity sum* by opting for *more measurement options* (software provides the pixel value upon right-clicking on the neuron).

5. *List*, *view all*, and *create document* were selected from the *measurement* tab on the left side of the panel (**Figure 3.10**).

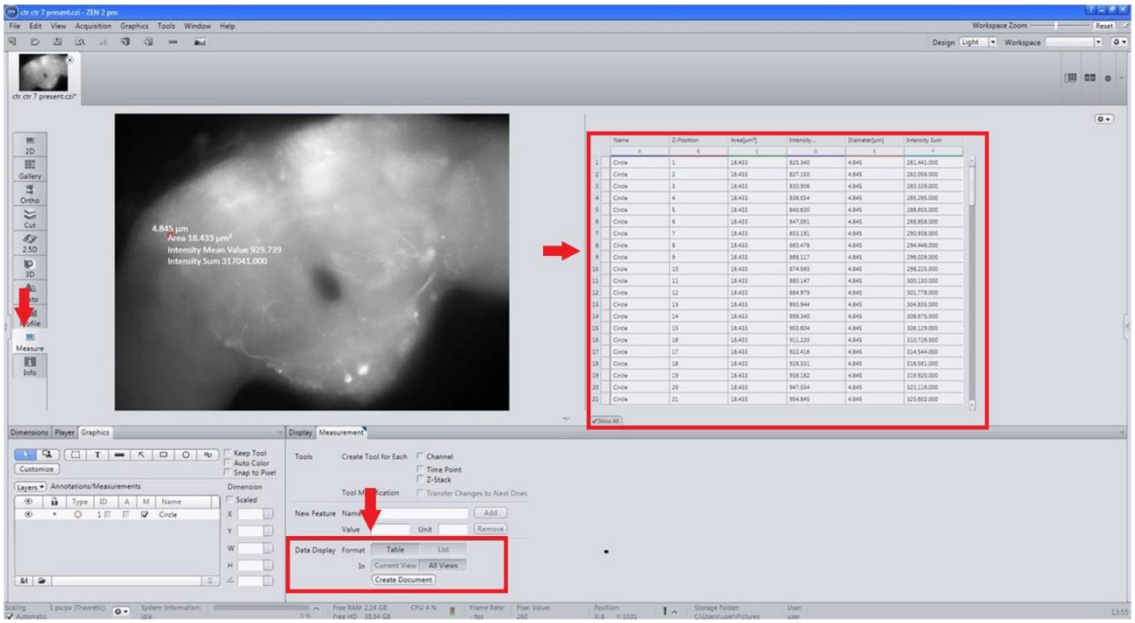


Figure 3.10. Fluorescence intensity (FI) compilation. From the *measurement* option select *list*, *All views*, and *create document*.

6. The area and FI sum were recorded for each scan of a neuron in .xml format (**Figure 3.11**).

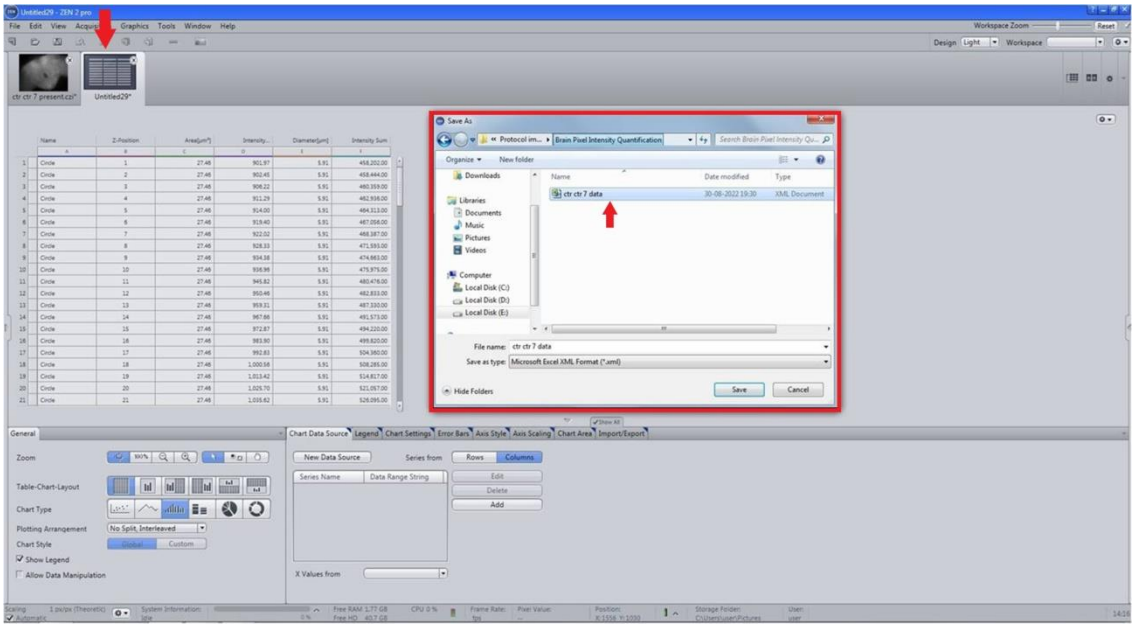


Figure 3.11. Measuring the FI sum for each scan of a neuron in .xml format

7. For quantification of FI of a single neuron, a total of eleven scans with an interval of 1.08 µm for each scan, meaning the cumulative of 11.88 µm width was considered (**Figure 3.12**).

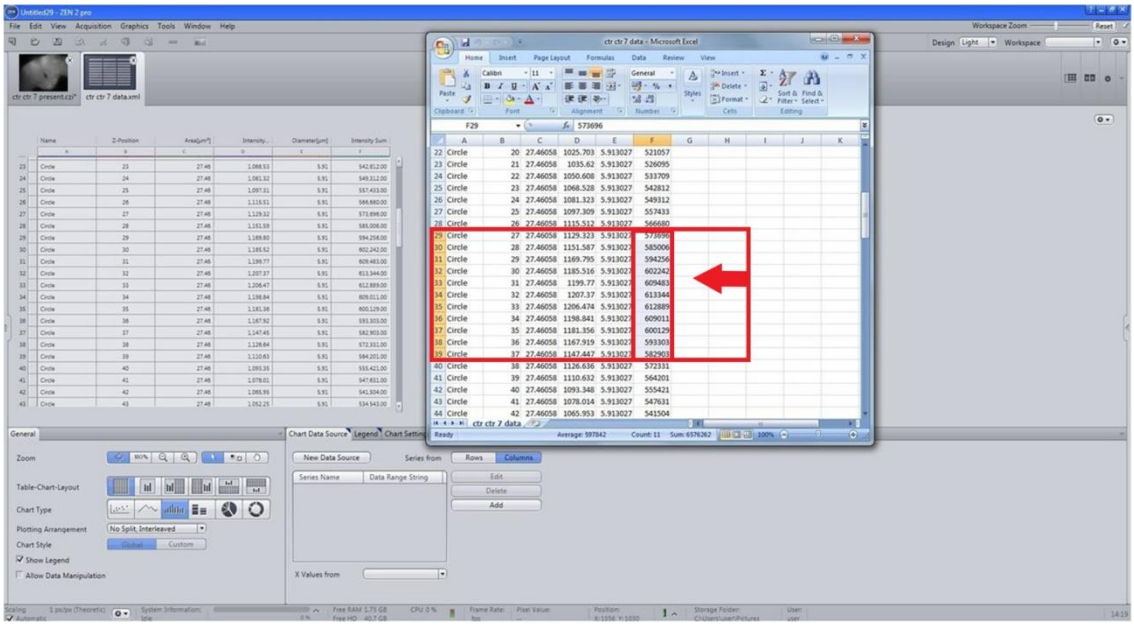


Figure 3.12. Compilation of fluorescence intensity (FI) of a single neuron and all the neurons of a cluster. For the characterization of FI of a single neuron, a total of 11 scans with an interval of 1.08 µm for each scan (cumulative 11.88 µm width) were considered. Take the average and find the standard error. Follow the same method/step(s) for all the dopaminergic (DAergic) neurons. The intensity sum of all the neurons in a specific cluster gives the total FI of that particular region (cluster-wise). The total FI is the sum of the FI of all the neurons belonging to all the DAergic neuronal clusters.

8. The intensity sum of all the neurons in a cluster gives the total fluorescence intensity (FI) of that particular region (cluster-wise).

9. Total FI is the sum of the FIs of all the neurons belonging to all the DAergic neuronal clusters.

3.5 Statistical analysis

Graphs were created using Graph Pad Prism 5.0 software (Graph Pad Inc., San Diego, CA, USA). Statistical analysis was completed, and results were expressed as the mean ± standard error of the mean (SEM). A Two-way ANOVA followed by Bonferroni post-test and one-way ANOVA followed by Newman-Keuls multiple comparison test was carried out to draw significance for DAergic neuronal number and TH-protein synthesis quantification. P-values < 0.05 were regarded as significant.

3.6 Results

3.6.1 Survivability of *Drosophila*

The adult life period of *Drosophila* has three stages: a health span, a transition span, and a senescent span (Arking et al., 2002). The adult health span is indicated by the period of life span where no natural deaths occur. The adult transition span is recognized when there is a visible but less significant decrease in the survival of adults. It is described as an instance accompanied by about 10% mortality and 90% survival. The adult senescent span/phase is defined by the slow and stable decrease in the number of live flies, as evident by a decline in the survival curve due to a gradual increase in mortality rate. It is illustrated by a gap between the last part of the transition stage and the greatest extent of the fly's prolonged

existence. In animal studies, the upper life span limit is usually considered the mean life span of the longest-lived 10% of a given group. The flies were cultured on the standard culture medium and transferred to freshly prepared culture media every third day, and mortality was recorded until all the flies were dead. Based on the survival proportions and longevity studies of the Oregon K strain of *Drosophila*, it was found that the health span extended up to 30 days; the transition span/stage was recorded from 31-60 days of the adult period, and the senescent span/period is from 61-120 days. The length of the fly life was recorded to be 121 days, while the median life duration was 95 days (**Figure 3.13**; Phom et al., 2014).

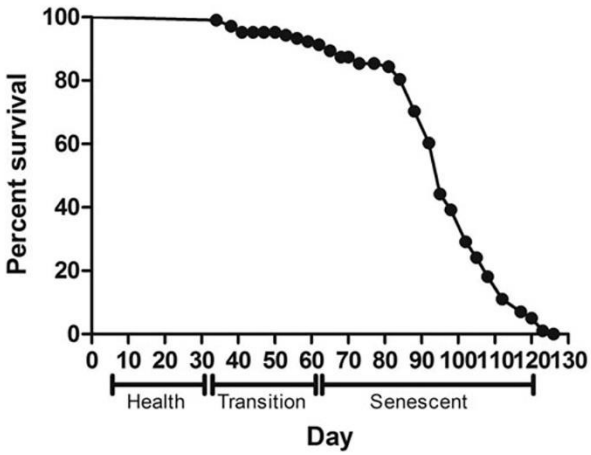


Figure 3.13. Survival curve of Oregon K male flies in standard culture media. The flies were transferred to fresh media on every 3rd day. The mortality was recorded until all of the flies died. Healthspan extends from day 4/5 to 30 days; the transition phase is 31–60 days of adult span, and the senescent span is 61–120 days. The maximum life span is 121 days, and the median is 95 days. (Adapted from Phom et al., 2014)

3.6.2 *Drosophila* susceptibility to PQ is concentration-dependent

The OK male flies aged 4-5 days old were utilized to explore the oxidative stress against the different concentrations of PQ (2.5, 5,10, 15, 20, and 40 mM). The different concentrations of PQ prepared in 5% sucrose were disposed of or transferred into a glass vial containing Whatman filter paper. The control flies were kept in a 5% sucrose solution. At every 24 hours, the survival rate of the flies was observed and documented until the most diluted solution of PQ, which was 2.5mM, showed 100% mortality. The response variation among all tested concentrations was significant, as evident through comparison of survival curves (log-rank [Mantel–Cox] test, $p < 0.0001$). After 72 hours, the survival rates were 95%, 79%, 48%, 15%, 8%, and 2% at the exposure doses of 2.5, 5, 10, 15, 20, and 40 mM, respectively. This suggests that the flies were highly affected by the PQ concentration above 10 mM in the method utilized to understand the susceptibility. Thus, I decided to subject the flies to 10 mM PQ to assess the mobility defects, DAergic neurodegeneration, and oxidative stress markers linked to PD after 24 hours of exposure, during which only 1%–2% of the flies died (**Figure3.14**; Phom et al., 2014; Phom, 2018).

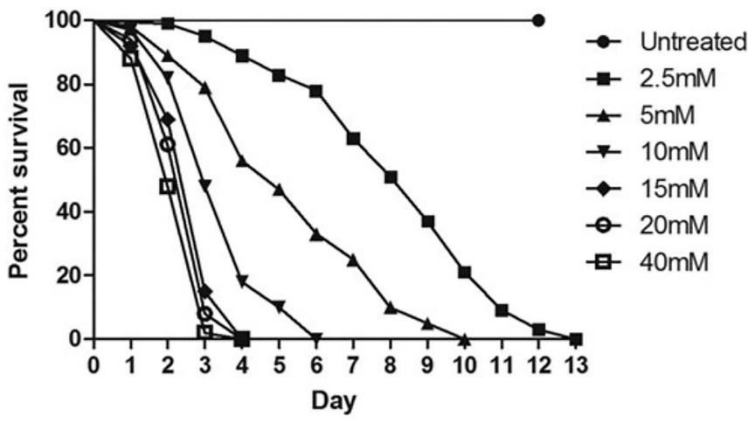


Figure 3.14: Concentration- and time-dependent mortality of *Drosophila melanogaster* (Oregon K) exposed to paraquat (PQ). Adult male flies (4–5 days) were exposed to six different concentrations of PQ (2.5, 5, 10, 15, 20, and 40 mM). PQ exposure induced concentration-dependent lethality. Comparison of survival curves reveals that the response difference among different tested concentrations was significant (log-rank [Mantel–Cox test, $p < 0.0001$]). (Adapted from Phom et al., 2014)

3.6.3 *Leea asiatica* (LA) leaf extract rescues the mobility dysfunctions induced by PQ under the co-treatment regime in the adult transition phase (TP) of the *Drosophila* model of PD

To evaluate the mobility dysfunction/impairments in the PQ-mediated *Drosophila* model of PD during the TP and whether LA could alter the mobility defects/rescue induced by PQ under co-feeding regimens. The fly's ability to climb upward in light for 12 seconds was evaluated after 24 and 48 hours of exposure to 10 mM PQ or PQ along with LA (10%, 7.5%, 5%, 2.5%, and 1.25%). After 24 and 48 hours of 10mM PQ feeding to flies, flies exhibited resting tremors and slow movement, as indicated by a significant reduction in the speed (bradykinesia), the characteristic clinical feature of PD in humans (**Figure 3.15 A&B ***P<0.001**). Some flies could not even climb the wall and failed to hold their grip. Flies also exhibited a restless tendency by showing excessive wing flipping. The speed of the flies was significantly improved/altered when PQ was fed along with LA (co-feeding regime) as compared to the flies that fed with PQ alone (**Figure 3.15 A&B; ***P<0.001**), suggesting the neuroprotective efficacy of LA (**Figure 3.15 A&B; ***P<0.001**) during the TP of *Drosophila*. LA extract *per se* fed flies has no adverse

influence on the mobility performance and has shown similar climbing ability as of the control (sucrose only) fed flies. These results suggest LA is a therapeutic compound in late-onset diseases like PD.

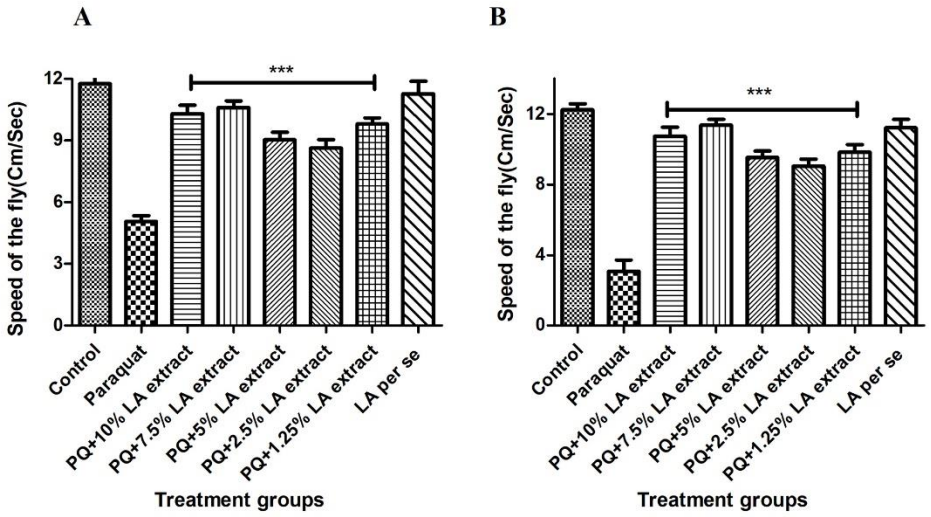


Figure 3.15: Negative geotaxis assay for co-treatment regime in 50-day-old flies. LA leaf extract rescues the mobility dysfunction/defects induced by PQ under a co-treatment regime during the transition span/phase (TP). 10mM PQ induces mobility defect in 50-day-old flies at different time points, 24 and 48 hours. The mobility defect was significantly altered when the flies were co-fed along with LA (R10% - 10mM Paraquat+10% LA Extract; R7.5% - 10mM Paraquat+7.5% LA Extract; R5% - 10mM Paraquat+5% LA Extract; R2.5% - 10mM Paraquat+2.5% LA Extract; R1.25% - 10mM Paraquat+1.25 % LA Extract for 24 hours (A), 48 hours (B). The ingestion of LA alone

does not cause any difference in the climbing ability of the fly when compared to the control. Feeding the flies with the different concentrations of LA did not significantly extend/alter the longevity (C) (CTR- Control; Td- Treated with 10mM Paraquat; *Perse*10% - Sucrose + *10%* LA extract alone). The significance was drawn using One-way ANOVA followed by the Newman-Keuls Multiple Comparison Test (***p<0.001; **p<0.01; *p<0.05; NS-Not Significant) when compared to the PQ (10mM) treated group.

3.6.4 *Leea asiatica* (LA) leaf extract rescues the mobility dysfunctions induced by PQ under the pre-treatment regime in the adult transition phase (TP) of the *Drosophila* model of PD

It is essential to prove that the neuroprotective effect of LA conferring is not due to antagonistic interaction with PQ. To disprove this possibility, the pre-feeding regime was ritualized, in which flies were fed with LA along with 5% sucrose for 3 days, and switched to 10 mM PQ treatment for 24 hours. In the pre-treatment regime, flies exhibited similar mobility defects after PQ ingestion (**Figure 3.16 A; ***P<0.001**) as shown during co-feeding, which was rescued when fed with LA, suggesting the observed rescue phenotype or neuroprotective efficacy of LA is not through physical interaction but due to sequestration of the PQ **(Figure 3.16 A; ***P<0.001)**. LA alleviates mobility defects in both the pre-and co-feeding regimens, as shown by a negative geotaxis assay during the transition phase **(Figure 3.15 A&B; Figure 3.16 A; ***P<0.001)**. Based on these findings, LA may be effective as a treatment for late-onset NDDs that manifest later in life, such as PD.

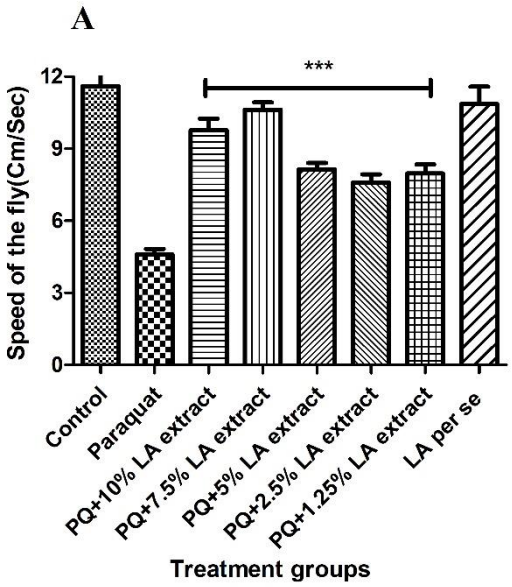


Figure 3.16: Negative geotaxis assay for Pre-treatment regime in 50-day-old flies. LA leaf extract rescues the mobility defects induced by PQ under the Pre-treatment regime during the transition phase. 10mM PQ induces mobility defects in the transition phase flies after 24 hours of treatment. The mobility defect was significantly altered when the flies were Pre-fed alone with LA (R10% - 10% LA Extract; R7.5% - 7.5% LA Extract; R5% - 5% LA Extract; R2.5% - 2.5% LA Extract; R1.25% - 1.25 % LA) Extract for 3 days prior to PQ treatment for 24 hours. The ingestion of LA alone does not cause any difference in the climbing ability of the fly when compared to the control. (CTR- Control; Td- Treated with 10mM Paraquat; *Perse*10% - Sucrose + *10%* LA extract alone). The significance was drawn using One-way ANOVA followed by the Newman-Keuls Multiple Comparison Test (***$p<0.001$; **$p<0.01$; *$p<0.05$; NS-Not Significant) when compared to the PQ (10mM) treated group.

3.6.5 Anti-TH immunostaining of the *Drosophila* whole-brain indicates that PQ does not cause a loss in the number of DAergic neurons but diminishes Tyrosine Hydroxylase (TH) protein synthesis in the TP-PD brain

The adult *Drosophila* brain consists of six quantifiable DAergic neuronal clusters in each brain hemisphere (**Figure 3.17A**) (Whitworth et al., 2006; Ayajuddin et al., 2023; Koza et al., 2023; Chaurasia et al., 2024). The DAergic neuronal clusters, including PAL, PPL1, PPL2, PPM1/2, PPM3, and VUM, contain 4-5, 11–12, 6–7, 8–9, 5–6, and 3 DAergic neurons, respectively. It was feasible to count the DAergic neurons using fluorescently-tagged secondary antibodies that were specific to the primary antibody directed against the DA synthesizing rate-limiting enzyme TH (**Figure 3.17B**).

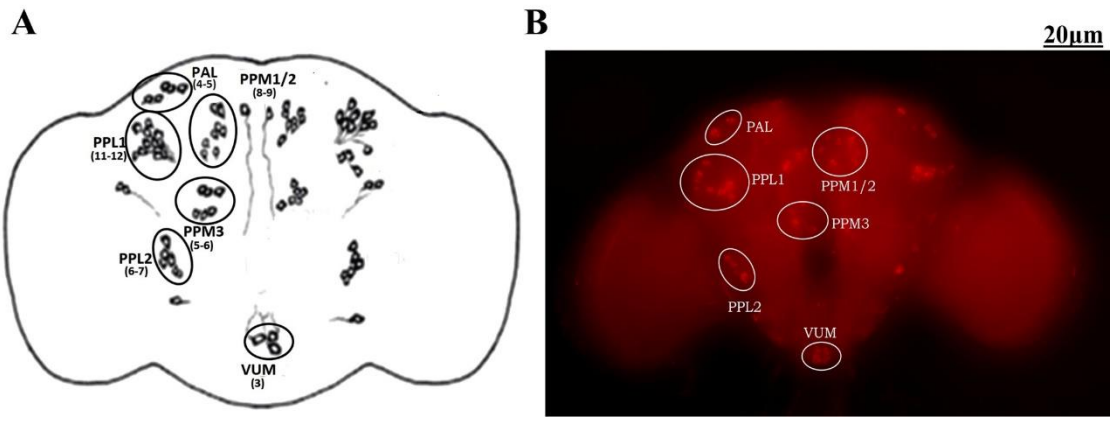


Figure 3.17: (A) Cartoon of *Drosophila* brain showing the position of different clusters of DAergic neurons. The brain of *Drosophila* has around 140 DAergic neurons in each hemisphere, which are arranged into six different clusters. Some of them are PAL (4-5 neurons), PPL1 (11-12 neurons), PPL2 (6/7 neurons), PPM1/2 (8/9 neurons), PPM3 (5-6

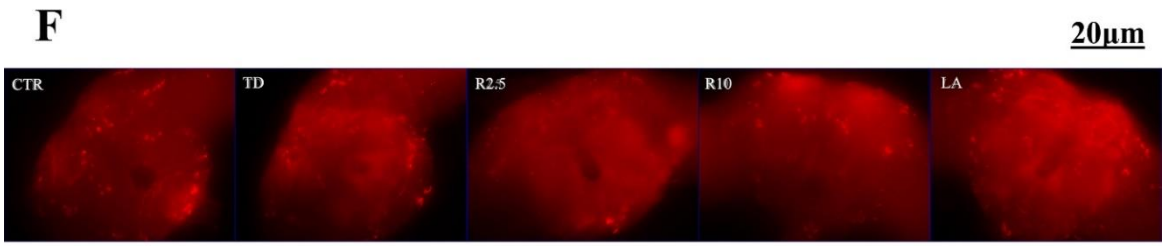
neurons) and VUM (3 neurons). These DAergic neurons are easily countable using a fluorescently labeled secondary antibody against the anti-TH primary antibody. There are other regions such as PAM with around 100 neurons which are not easily quantifiable. The Scale bar of the brain image in the panel is 20µm (PAM: Protocerebral Anterior Medial; PAL: Protocerebral Anterior Lateral; PPM: Protocerebral Posterior Medial; PPL: Protocerebral Posterior Lateral; VUM: Ventral Unpaired Medial). (B) Image of whole-brain mount of *Drosophila* captured using *ZEN software* of Carl Zeiss Fluorescence Microscope (Axio Imager 2, Carl Zeiss, Germany).

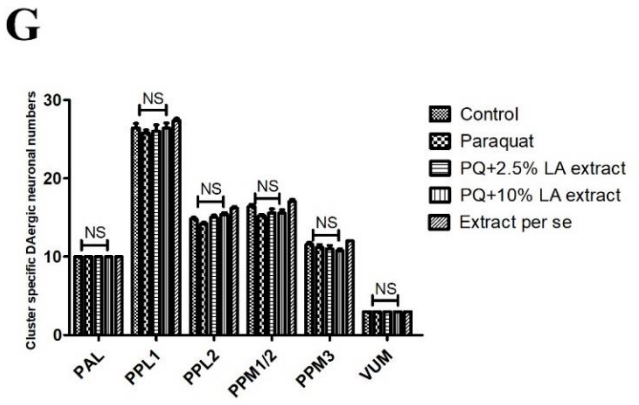
The images of the various experimental groups in the TP of *Drosophila* brain were shown in **Figures 3.18F**, respectively. Results indicate that there was no discernible change in the number of DAergic neurons quantified cluster-wise and brain-wise between all the treatment groups investigated throughout the TP (**Figure 3.18G&H**) as compared to the control group. In the TP life phase, the total number of DAergic neurons in the brains of the various treatment groups did not differ from the control group (**Figure 3.18H**). Further, natural aging of the brain did not alter the number of DAergic neurons, neither in the cluster-specific manner nor in the merged/group-wise fashion **(Figure 3.18 G &H; Das, 2022)**. The FI of the DAergic neurons was further assessed (a secondary antibody that is fluorescently labeled, tags the primary antibody anti-TH) to ascertain whether there was a difference in or reduction in the quantity of TH protein synthesis. Results indicate that the TH protein synthesis and FI directly correlates. In TP flies fed with 10 mM PQ alone for 24 hrous significantly reduced the FI in the PAL, PPL1, PPL2, PPM1/2, and PPM3 clusters, respectively, by 36.23% (*$p<0.05$), 40.05% (**$p<0.01$), 35.96%

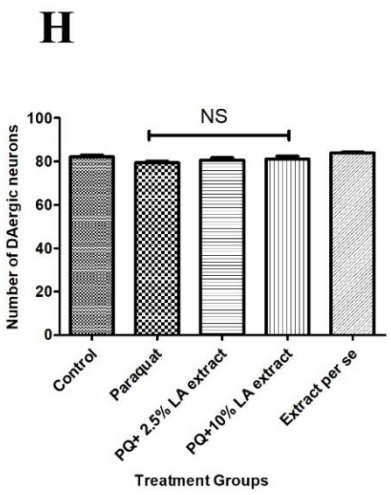
(*p<0.05), 31.30% (*p<0.05), and 37.36% (*p<0.05) (**Figure 3.18I**). This illustrates that feeding the flies with 10mM PQ alone considerably lowers the level of the TH enzyme (reduced level of TH protein synthesis). The findings are consistent with those of Navarro et al. (2014), Ayajuddin et al. (2023), and Das, (2022), where they measured and showed a reduction in the FI of GFP reporter protein rather than actual neuronal cell death, demonstrating that while TH protein synthesis level is decreased, DA neuronal structure (cell body) is not degenerated (thus no loss in the number of neurons). Further subsequent investigation was made by consolidating the FI of all the DAergic neurons belonging to all the neuronal clusters of a treatment group. Results indicate that neurotoxicant 10mM PQ exposure at TP led to depletion in the FI by around ~30%, (**P<0.01) (**Figure 3.18J**), indicating decreased levels of TH protein synthesis at TP-PD brain. Further, brain-specific aging-associated changes in FI were also characterized in a cluster-specific fashion and in a brain-wise manner. Results reveal that with natural aging in the TP brain, the levels of TH protein synthesis are down-regulated by about 40.15% (***p<0.001), 44.66% (***p<0.001), 65.05% (***p<0.001), 20.15% (NS), and 5.14% (NS), respectively, in all the quantifiable clusters *viz.,* PAL, PPL1, PPL2, PPM1/2, PPM3 when compared with HP brain (**Figure 3.19 C).** Similarly, group-wise merged FI of all the quantifiable DAergic neurons were also investigated, and results deduce that the levels of TH protein synthesis in the TP brain are altered by about 30~40 % (**p<0.01) when compared with the HP brain (**Figure 3.19 D).**

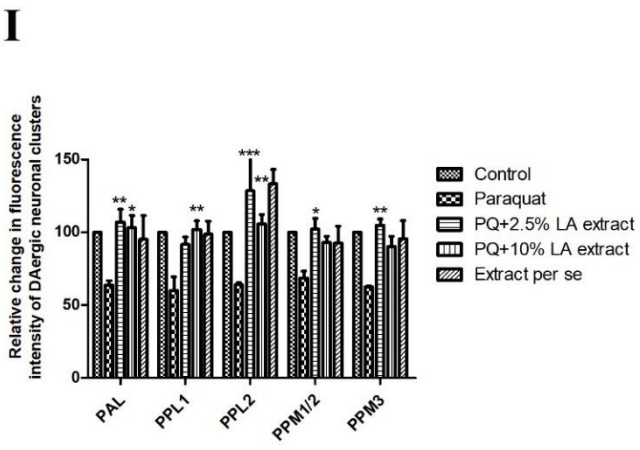
3.6.6 *Leea asiatica* (LA) intervention rescues "DAergic neuronal dysfunction" by replenishing reduced tyrosine hydroxylase (TH) protein synthesis levels in the TP-PD brain

In order to get insight into the neuroprotective efficacy of LA at the level of DAergic neurons during TP, LA co-feeding to the experimental groups was investigated. Co-feeding with LA during the TP replenishes the diminished levels of TH protein synthesis (**Figure 3.18J**). The cluster-wise depletion of FI was found to be rescued during the TP-PD brain. In the PAL, PPL1, PPL2, PPM1/2, and PPM3 clusters, the FI of the fly brain(s) DAergic neurons upon LA interventions/co-feeding increased significantly during the TP by about 43.17% and 39.40% (**p<0.01; **p<0.01), 31.56% and 41.94% (*p<0.05; **p<0.01), 64.44% and 41.65% (***p<0.001; **p<0.01), 33.35% and 24.30% (*p<0.05), and 42.21% and 27.69% (**p<0.01; *p<0.05), respectively **(Figure 3.18I)**. Similarly, group-wise merged FI of all the quantifiable DAergic neurons were also investigated. The result illustrates that LA co-feeding in a concentration of R2.5% (10mM PQ + 2.5% LA extract) and R10% (10mM PQ +10% LA extract), up-regulate the TH protein synthesis levels by 30~35% (**p<001) (**Figure 3.18J**). These findings suggest that during the TP-PD brain, LA can reverse the neurodegeneration brought on by 10mM PQ exposure. Further, these results substantiate the significance of LA extract in alleviating the DAergic neurodegeneration induced by PQ during the transition stage mediated fly PD model.


F
20µm
CTR
TD
R2.5
R10
LA


G
Cluster specific DAergic neuronal numbers
30
20
10
0
NS
NS
NS
NS
NS
NS
PAL
PPL1
PPL2
PPM1/2
PPM3
VUM
Control
Paraquat
PQ+2.5% LA extract
PQ+10% LA extract
Extract per se


H
Number of DAergic neurons
100
80
60
40
20
0
NS
Control
Paraquat
PQ+ 2.5% LA extract
PQ+10% LA extract
Extract per se
Treatment Groups


I
Relative change in fluorescence intensity of DAergic neuronal clusters
150
100
50
0
**
*
**
***
**
*
**
PAL
PPL1
PPL2
PPM1/2
PPM3
Control
Paraquat
PQ+2.5% LA extract
PQ+10% LA extract
Extract per se

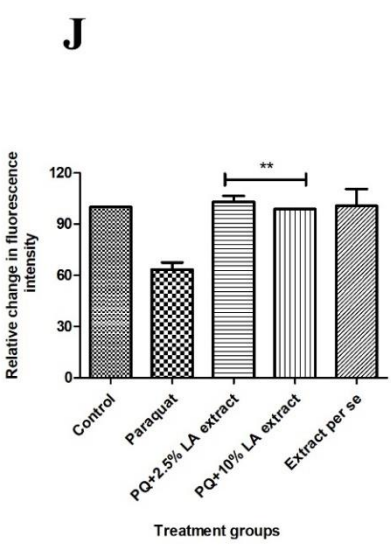

J
Relative change in fluorescence intensity
120
90
60
30
0
**
Control
Paraquat
PQ+2.5% LA extract
PQ+10% LA extract
Extract per se
Treatment groups

Figure 3.18: Characterization of DAergic neurodegeneration in the whole fly brain of *Drosophila* during the TP through anti-TH antibody immunostaining **(F)** reveals that there is no loss in the number of DAergic neurons upon exposure with neurotoxin PQ (10mM) alone and, among all the studied groups **(G&H)**. However, feeding the *Drosophila* during the TP with PQ (10mM) leads to "neuronal dysfunction" characterized by quantification of DAergic neuronal FI that is directly proportional to the amount of TH protein synthesis by ~30-35 % which could be significantly rescued upon co-feeding with LA **(I&J)**. The reduction in the FI has been referred to as "neuronal dysfunction" by Navarro et al., (2014); Ayajuddin et al., (2023). The significance was drawn by analyzing a minimum of three to five brain(s) using One-way ANOVA followed by "Newman-Keuls Multiple Comparison Test" and two-way ANOVA followed by "Bonferroni post-test". The significance was presented by ***p<0.001; **p<0.01; *p<0.05; NS-Not Significant when compared to PQ (10mM) treated group. The scale bar of all the images in the panel (A) is 20 μm. Represented images are "merged" Z-stacking images; however, the quantification of DAergic neuronal number and fluorescence intensity is performed in 3D Z-stack images. (CTR- Control; Td- Treated with 10mM Paraquat; R2.5% - 10mM Paraquat+2.5% LA Extract; R10% - 10mM Paraquat+10% LA Extract; *Perse*7.5% - Sucrose + LA extract alone)

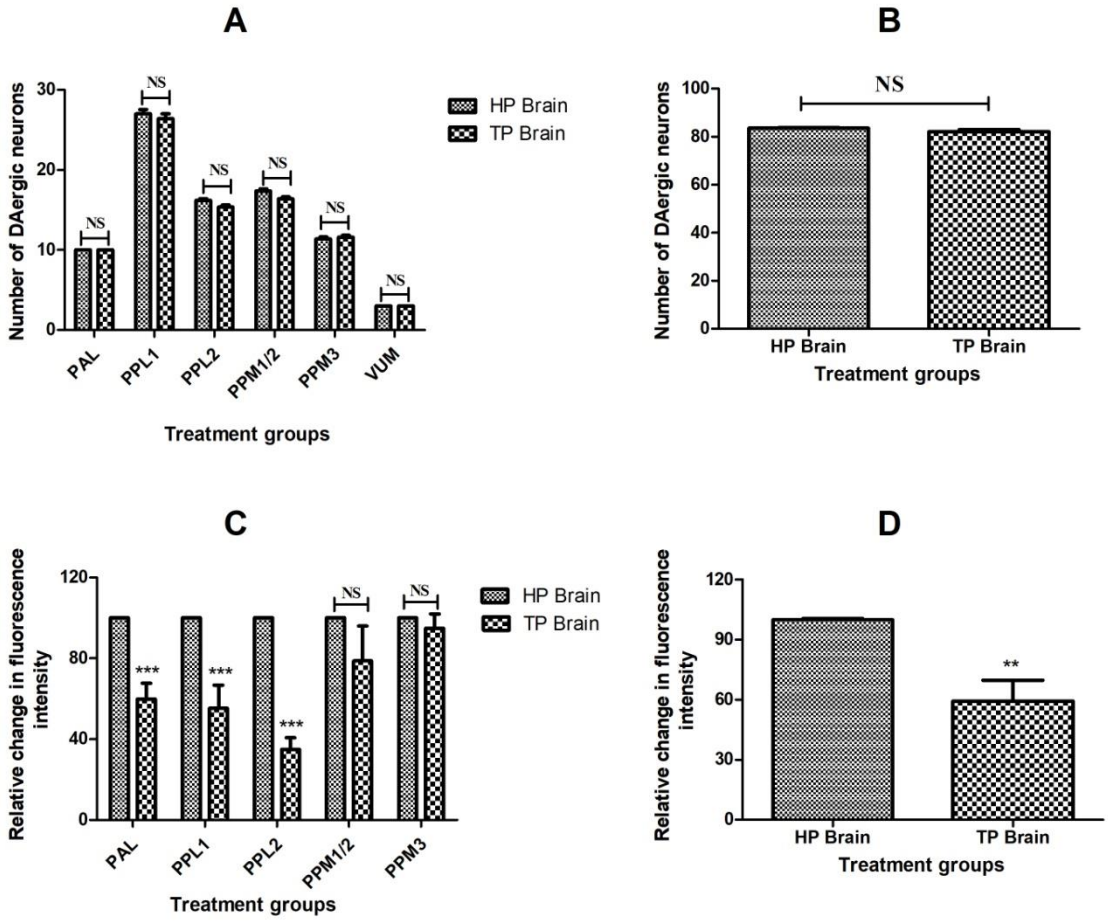


Figure 3.19: Brain-specific aging-associated changes in the DAergic neuronal numbers and relative changes in FI. TH immunostaining of whole-mount *Drosophila* brain reveals that there is no age-dependent change/alteration in the number of quantifiable DAergic neurons among the HP brain and TP brain (**A&B**). However, characterization/quantification of DAergic neuronal FI that is directly proportional to the amount of TH protein synthesis is downregulated by around 30~40% (**p<0.01) in the TP brain when compared to the HP brain (**C&D**). The significance was drawn by

analyzing a minimum of three to five brains through an unpaired t-test. The significance was presented by ***p<0.001; **p<0.01; *p<0.05; NS-Not Significant.

The findings of the current investigation corroborate with the results of the negative geotaxis assay described above in this chapter, which showed that LA corrects the mobility defects during the transition phase of *Drosophila* life span/PD brain.

3.7 Discussion

Epidemiological studies indicate that exposure to environmental toxins is associated with the onset of PD in the later stage of life (Ascherio A, Schwarzschild, 2016). Inevitably, sporadic or idiopathic PD affects 90–95% of populations (Bloem et al., 2021), whereas hereditary factors associated with PD account for only 3-5% of PD in most populations. Studies have reported that exposure of flies to PQ reproduced the characteristic pathological features of PD (i.e., alteration of locomotor behavior, time and concentration-dependent mortality, and progressive deficiency of DAergic neurons) (Ayajuddin et al., 2023; Das, 2022; Phom, 2018; Phom et al., 2014; Jahromi et al., 2013; Zhou, 2011; McCormack et al., 2002). The mortality pattern and mobility impairments of adult *Drosophila* male flies exposed to PQ were shown to be time- and concentration-dependent, according to studies conducted by Phom et al. (2014; 2018). To better understand the pathophysiology of PD and screen for small molecules/nutraceuticals with possible neuroprotective effectiveness, Phom et al. (2014; 2018) findings highlight the relevance and usefulness of using animal models relevant to life phases. This chapter

details my efforts to use the TP fly model of sporadic PD, as described and developed by Phom et al. (2014), in conjunction with therapeutic neuroprotection through the intervention of LA, to reduce the PQ-induced deficit in locomotion, as measured by climbing ability, through the use of a pre-and co-feeding regimen. Many studies have shown that animals exposed to PQ demonstrated impaired locomotion and a significant mortality rate (Chaouhan et al., 2022; Niveditha et al., 2017; Phom et al., 2014; Jahromi et al., 2013). The present study indicates that feeding the TP fly with 10mM PQ alone adversely decreases mobility (~60%), which could be improved upon co-feeding with LA leaf extract (**Figure 3.15 A&B; ***P<0.001**). Feeding the fly with LA extract *per se* showed no mobility defects (**Figure 3.15 A&B**). Results indicate that in both the co-feeding (**Figure 3.15 A&B; ***P<0.001**) and three days pre-feeding regimens (**Figure 3.16 A; ***P<0.001**), LA extract could confer neuroprotection during the transition phase. This observation is congruent with previous studies that revealed protective potential using extracts from *Decalepis hamiltonii* root, *B. glabra*, *Sanguisorba officinalis*, *Bacopa monnieri*, and *Zedoariane rizhoma*, as well as calycosin against PQ-induced locomotor impairment in various animal models (Chaouhan et al., 2022; Srivastav et al., 2018; Jahromi et al., 2013; Park et al., 2012). However, all these studies used young animals belonging to the adult health stage. This is the first report to decipher that LA has a TP-associated therapeutic ability in the PQ-mediated sporadic *Drosophila* model of PD.

The defining pathogenic aspect of PD is the loss of DAergic neurons in the *substantia nigra pars compacta* of the human brain. The loss of DAergic neurons, reduced brain DA level, and progressive age-dependent mobility impairments are exhibited in the fly

models of PD (Ayajuddin et al., 2022; Chaudhuri et al., 2007; Feany and Bender, 2000). The loss of DAergic neurons is the specific pathophysiological feature of PD. As a result, DAergic neurons in the whole fly brain were counted using fluorescence microscopy before DA "neuronal dysfunction" was defined. Upon the assessment of the number of DAergic neurons, "neuronal dysfunction" (if any) was determined by assessing the FI of a secondary antibody that is fluorescently tagged and that specifically binds to the anti-TH primary antibody. The emanating FI can be correlated to the TH protein abundance and synthesis. This was carried out to assess the degree of DAergic neurodegeneration/dysfunction in induced PD circumstances/brain and potential neuroprotection with LA interventions. The result demonstrates that the number of DAergic neurons in the control and induced PD brains did not differ during the transition stages (TP) of adult life of *Drosophila* (**Figure 3.18 G&H**). This observation is in line with earlier conclusive findings from other investigations (Ayajuddin et al., 2023, 2022; Das, 2022; Navarro et al., 2014; Menzies et al., 2005; Meulener et al., 2005; Pesah et al., 2005). These results make sense in the context of the "dying back" phenomenon, which argues that neurodegeneration begins at the axonal terminus (Wong et al., 2019). This further explains why tried-and-true L-DOPA supplementation therapy has failed, as chronic L-DOPA supplementation will result in irregular DA receptor and signaling activation, dyskinesia, and toxicity from plasma L-DOPA due to irregular uptake (due to axonal degeneration) by DAergic neuronal terminals (Nakmodde et al., 2023).

Recently, our laboratory has demonstrated that there is no diminution in DAergic neuronal number during the HP and TP of the adult life of *Drosophila* but there is a substantial shift/change in the level of TH protein synthesis in a mitochondrial complex I

inhibition and PQ mediated fly model of PD (Ayajuddin et al., 2022, 2023; Das, 2022). Here, it is important to underline that the loss of DAergic neuronal cell bodies (loss in number of DAergic neurons) in the fly PD models has been the subject of controversy in the field of *Drosophila* neurobiology. Feany and Bender (2000) were the initial investigators who demonstrated that the loss of DAergic neurons occurs at an adult-onset in the *Drosophila* model of PD. Then, numerous investigators exploited that model to investigate and illustrated the varying level of DAergic cell death in various DAergic clusters (Rai and Roy, 2022; Maitra et al., 2021; Bordet et al., 2021; Sur et al., 2018; Song et al., 2017; Barone et al., 2011; Chen and Feany, 2010; 2005; Trinh et al., 2008, Cooper et al., 2006; Pesah *et al.,* 2005 Auluck et al., 2002). Using the same flies, Auluck and Bonini (2002), Auluck et al. (2002), and Yang et al. (2003) observed a 50% loss of DAergic neurons. Similar to that, in flies with loss-of-function mutations in PD-associated genes like *PARKIN*, and *PINK1*, just two to four neurons from a specific DAergic neuronal cluster (PPM1/2 or PPL1) were reported to be deteriorated/degenerated (Kim et al., 2012; Trinh et al., 2010, 2008; Cha et al., 2005; Whitworth et al., 2005). On the contrary, Pesah et al. (2004) detected no loss of neurons in the PPM1/2 cluster, suggesting that just a particular DAergic cluster in *PARKIN* mutants may be susceptible to degeneration. Studies using the *PINK1 Drosophila* model of PD have revealed significant discrepancies, ranging from a discrete loss of two to four neurons in the PPL1 clusters in a null mutant (Park et al., 2006) to a significant reduction in neurons in several DAergic clusters in RNAi knockdown flies (Wang et al., 2006; Yang et al., 2006). In addition to genetic models, toxicity-induced PD models, viz., PQ-based models, intrigued the narrative by providing information on varying levels of DAergic neuronal degeneration. In a study concluded by Maitra et al. (2021, 2019) and Chaouhan et al.

(2022) showed that exposure to 5 mM PQ for 12-48 hours causes a significant loss of DAergic neurons in the PPM and PPL1 clusters in the *Drosophila* PD brain. Another independent investigation by Shukla et al. (2014) demonstrated the cluster-wise selective loss of DAergic neurons occurred in flies exposed to 10mM, and 20mM PQ for 24 hours. Additionally, over-expression of *HSP70* inhibits the cluster-wise selective loss of DAergic neurons caused by PQ in *HSP70*-expressed flies for 12 and 24 hours, respectively (Shukla et al., 2014). Similarly, in accordance with Song et al. (2017), a mutant fly with reduced *Aux* expression exhibits alteration in the number of neurons in the PPM1/2 cluster that may be mediated through α-synuclein toxicity. Additionally, flies with reduced *Aux* expression are susceptible to PQ, indicating that genetic and environmental factors may both have a bearing on the DAergic neurodegeneration in the late HP of PD brain (ThirtyThree days old flies) (Song et al., 2017). Similarly, Loss of Catsup [*Drosophila* ortholog of the mammalian zinc transporter SLC39A7 (ZIP7)] function has been found to delay the death of DAergic neurons after paraquat exposure (Chaudhuri et al., 2007). In another independent study, upon treatment with PQ for 24 hours, DAergic neuronal clusters (PAL, PPL1, PPM2, and PPM3) exhibited statistically significant neuron loss, whereas, at 48 hours, the affected clusters continued to deteriorate, while neurodegeneration was also noted in the PPM1 and PPL2 DAergic neuronal clusters (Inamdar et al., 2012).

In PQ induced fly PD model, specific loss of DAergic neurons was found with varying concentrations of the toxin (Chaouhan et al., 2022; Maitra et al., 2019, 2021; Shukla et al., 2014; Inamdar et al., 2012; Chaudhuri et al., 2007; Wang et al., 2007) or no change in the number of neurons (Navarro et al., 2014; Ayajuddin et al., 2023; Das, 2023). As a

result, it can be argued that there is a discrepancy/contradiction between studies conducted in the past and those conducted in the current scenario on the loss of DAergic neurons in the *Drosophila* model of PD. This issue had previously undergone thorough investigation in a number of fly models of PD (both genetic and sporadic), and it was found that there is no structural loss of DAergic neurons but merely a decreased GFP (GFP reporter) / TH protein production that was correlated to decreased in FI of neurons (Chaurasia et al., 2024; Ayajuddin et al., 2022; 2023; Das, 2022; Navarro et al. 2014). In this study, flies treated with 10mM PQ alone resulted in a significant decrease in the FI in all the studied DAergic neuronal clusters. This reduction in the FI significantly reversed when LA was co-fed to the flies during the adult TP of the *Drosophila* PD brain **(Figure 3.18 I)**. These results were further assessed where, the total FI of all the DAergic neurons in the fly brain(s) of the various experimental groups were quantified **(Figure 3.18 J)**. These groups were analyzed separately and produced similar results. Because the fluorescence of the neuronal cell body strongly correlates with the rate at which the rate-limiting enzyme TH is synthesized, decreased levels of FI reflect reduced levels of TH protein synthesis. According to the current study, in the late-onset PD model, "neuronal dysfunction" is defined as a decrease in TH production without a corresponding loss of the neuronal cell body. The magnitude of the DAergic neurodegeneration provided can be precisely determined by quantifying the TH signals in the PQ-induced fly model and the extent of neuroprotection through LA intervention. These findings confirm that LA restores the reduced TH protein level and corrects mobility impairments during TP of the *Drosophila* PD brain. It was also observed in the current study that there was no loss of DAergic neurons (Cell bodies) with natural aging [(TP brain; **Figure 3.19A&B**)]. These observations were inconsistent with previous conclusive results from other studies where

investigators showed an age-related decline in the number of DAergic neurons in normal healthy flies (Menzies et al., 2005; Neckameyer**,** et al., 2000). The current study also demonstrated brain-specific aging-associated changes in FI. Results conclude that with natural aging, in the TP brain, the levels of tyrosine hydroxylase protein synthesis were downregulated/altered by about 30~40 % (**p<0.01) (**Figure 3.19 D)** when compared with the HP brain.

3.8 Conclusion

To better understand the efficacy of therapeutic compounds in PD models, numerous investigators co-treat or pre-treat young animal models for a brief period. They investigate if the prospective therapeutic molecule protects the toxin-mediated DAergic neuronal degeneration by assessing behavioural markers like motor defects, mortality, etc. LA improved the locomotory defects in the TP of the adult life of flies, suggesting its potent therapeutic ability in PD. Hence, I tried to understand how LA mediates TP-specific DAergic neuroprotection in the *Drosophila* PD brain through the quantification of DAergic neuronal number and through quantification of the level of TH protein in DAergic neurons. TH is the rate-limiting enzyme necessary for the synthesis of DA. FI of the secondary antibody targeting the primary anti-TH antibody represents TH abundance in the DAergic neuron.

The results established that LA mediates the transition stage-specific neuroprotection in the *Drosophila* PD brain, suggesting its potential upstream protective function in DA anabolism, as the cause of the elevation of decreased TH production. The effect of LA on DA catabolism and turnover rate will be fascinating to examine because it may shed light on the effectiveness of the transition stage-specific DAergic neuroprotection. As a result,

in Chapter 4, DA catabolism and oxidative turnover to immediate downstream metabolites (DOPAC and HVA) will be assayed using HPLC-ECD (High- performance liquid chromatography with an electrochemical detector) in order to gain more understanding/insight into the neurochemistry underlying "neuronal dysfunction" and LA- mediated transition stage-specific neuroprotection.

Chapter 4

Leea asiatica Mediated Regulation of Brain Dopamine (DA) Metabolism in the *Drosophila* Model of Parkinson's Disease: Implications to its therapeutic efficacy

Chapter 4

4.1. Introduction

The DAergic system has a sizable impact on the neuromodulation of motor control, motivation, reward, cognitive function, and maternal and reproductive behaviors. The neurotransmitter DA interacts with G-protein-coupled receptors and is produced in the peripheral and central nervous systems. An imbalance in DAergic signalling pathway activity may lead to dysfunctions associated with NDD because the maintenance of the DAergic system is crucial for sustaining physiological functioning (Klein et al., 2019). The primary cause of PD is found to be a deterioration in the health of DAergic neurons. Insight suggests that the number of DAergic neurons gradually decreases as people age normally at a rate of roughly 4% (Fearnley and Lees, 1991). However, PD accelerates the decline in DAergic neurons, resulting in a final decline of 70% DAergic neurons and a 40–50% decline in striatal DAergic neurons compared to age-matched controls (Cheng et al., 2010). Thus, in the event of late-onset diseases like PD, the increased DAergic neuronal death may also lead to a progressive decline in DA levels. The amount of catechols, including DA, is lower in postmortem PD brains. However, people who were at high risk of developing PD or had a family history of PD had lower levels of CSF (cerebrospinal fluid) DA (Goldstein et al., 2018; 2011). These results strongly imply that DA dysregulation is not only a contributing factor to the abnormal motor and non-motor behaviour seen in PD, but a reduced level of DA in the early stages can also signal the development of PD. There are multiple ways through which DAergic neurodegeneration contributes to reduced DA levels. According to Masato et al. (2019), under specific circumstances, poor DA metabolism and oxidation can exacerbate the ROS stress and cause the degeneration of DAergic neurons. In mammals, a presynaptic neuron releases

DA into the synapses after processing or packaging it in vesicles. Presynaptic neurons have the capacity to reabsorb extra DA, and MAO can then catabolize the extra DA to synthesize DOPAC. In addition, astrocytes, a specific type of glial cell, can utilize DOPAC for further catabolism into HVA through COMT activity (Winner et al., 2017) (**Figure 4.1A**). Although DA dynamics and signalling in *Drosophila* are virtually identical to those in mammals, however, there are three important metabolic variations (Yamamoto and Seto, 2014). **1**. In mammals, DA metabolism exploits only oxidation and methylation, whereas, in flies, N-acetylation and β-alanylation may also be used in the metabolism of DA. **2**. Additionally, flies lack the genes needed to produce norepinephrine and epinephrine from DA. **3**. In flies, but not in mammals, DA is required for the synthesis of epidermal melanin. Dopamine N-acetyltransferase, also known as arylalkyl amine N-acetyltransferase (aaNAT), and enzymes coded by *pale*, *ebony*, and *tan* are believed to be involved in one of the mechanisms by which DA is metabolized in flies (Wright, 1987). In flies, NBAD (N-β-alanyl dopamine) and NADA (N-acetyl dopamine) are the byproducts of DA breakdown. Through the use of enzymes like Tan, NBAD can be converted back to DA (Yamamoto and Seto, 2014). The fly epidermal cells and the brain cells both undergo the same DA metabolic cycle. From DA to NBAD, and NADA are synthesized in epidermal cells, and thereafter, these metabolites/byproducts are transferred to the cuticle for pigmentation and sclerotization (**Figure 4.1B**). Whereas, in the fly brain, DA is broken down into NBAD and NADA by glial cells, and NBAD is then converted back to DA by DAergic neurons (**Figure 4.1C**).

It's interesting to note that *Drosophila* lacks the orthologs of the COMT and MAO coding genes found in mammals. Yet, in addition to NBAD and NADA, fly brains also contain

DA oxidative products like DOPAC and HVA (Freeman et al., 2012; Wakabayashi-Ito et al., 2011; Chaudhuri et al., 2007; Zhang et al., 2005). According to Yamamoto and Seto (2014), this revelation raises the possibility that the fly brain has metabolic components and pathways that are similar to those in the mammalian system.

DOPAC and HVA are known as endogenous neurotoxins in addition to being natural DA catabolites because DA oxidation to DOPAC-HVA results in the formation of ROS and peroxides (Cao et al., 2021; Zhang et al., 2019). The antioxidant defense system may be able to counteract the ROS and peroxides in DAergic neurons. However, external insults can also disrupt the DA metabolism pathway, increasing catabolism and generating more DOPAC-HVA, which can accelerate the deterioration of DAergic neurons through the creation of ROS and peroxides (Zhang et al., 2019; Winner et al., 2017). This insight emphasizes the DAergic neurons propensity for degradation that results in the PD phenotype.

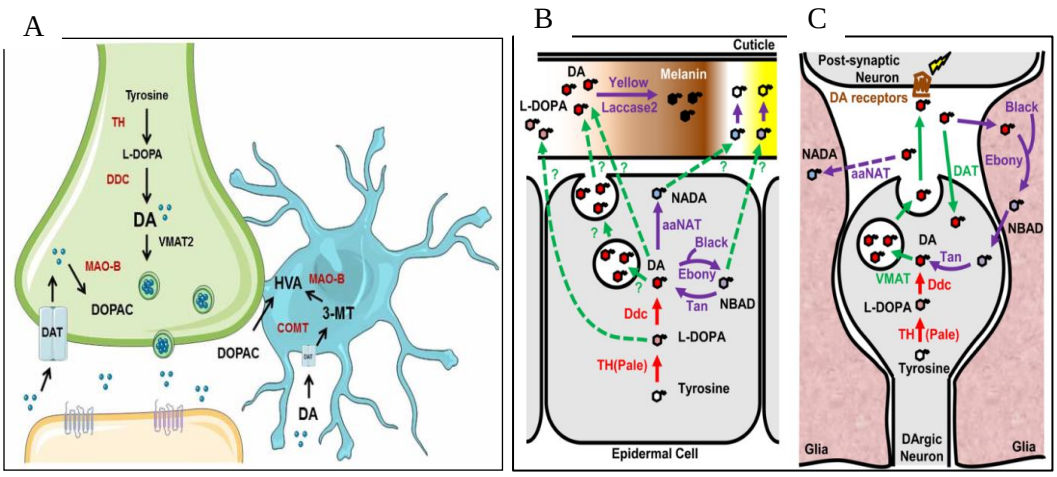


Figure 4.1: Representation of the DA metabolism in mammalian brain, fly epidermal tissue, and fly brain. In a presynaptic neuron (green), DA is synthesized, processed, or packaged into vesicles before being released into the synapses. After being synthesized from tyrosine by TH, L-DOPA is converted to DA by dopa-decarboxylase (DDC). Monoamine oxidase (MAO) can then convert DA to DOPAC, which can subsequently be exported to astrocytes (blue) for further metabolism into HVA by COMT (A; from Winner et al., 2017). On the other hand, insight into DA catabolism in the fly brain and epidermal cell suggests DA is converted to NBAD and NADA by the activity of aaNAT, *Ebony*, *Black*, *Tan* (B, C; from Yamamoto and Seto, 2014). DA is transported to the cuticle for pigmentation, while NBAD and NADA are transported to the cuticle for hardening (B). The fly brain utilizes neuronal and glial cells for DA catabolism. Excess DA in synapses is retaken by glial cells to convert it into NBAD and NADA by aa-NAT, Black, and Ebony activity. NBAD can be transported again to neuronal cells and can be reconverted to DA through TAN activity (C).

According to a study by Stefani et al. (2017), patients with mild PD symptoms had higher levels of CSF DOPAC and HVA. Motor impairment and the rise in DA metabolites are directly connected. Further, as reported in other independent studies, increased DA breakdown to its metabolite(s) was observed in brain tissue of PQ-induced sporadic *Drosophila* and mouse models of PD (Das, 2022; Rudyk et al., 2015; Shukla et al., 2014; Inamdar et al., 2012; Chaudhuri et al., 2007). These models highlight the significance of investigating DA catabolism in addition to the DA pool in the brain to comprehend PD pathophysiology.

The HPLC-ECD is the most trustworthy method for assessing the level of catecholamines in a model system. Catechol-modified proteins can be measured using SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Rees et al., 2007) and the protein pull-down test (Plotegher et al., 2017; Liu et al., 2014). NIRF (Near-infrared fluorescence imaging) scanning is used to detect and quantify O-quinones and other charged proteins that are also present in cells and tissues (Jinsmaa et al., 2018; Burbulla et al., 2017). HPLC-ECD is the best technique for catecholamine measurement because all other techniques are less accurate. Speed and precision in identifying brain-specific catecholamines are two advantages of HPLC-ECD. The technique's versatility is further strengthened by its increased capacity for detecting other related catecholamines (Das, 2022; Ayajuddin et al., 2022; Allen et al., 2017).

It was shown in Chapter 3 that DAergic neuronal dysfunction during the adult transition phase is caused by neurotoxicant PQ. Additionally, leaf extract from LA could prevent neuronal dysfunction in late-onset PD. The reduced production of TH, a rate-limiting enzyme for DA anabolism, has been referred to as “neuronal dysfunction” (Navarro et

al., 2014). Using the HPLC-ECD method, the goal of the current Chapter 4 is to understand how neuronal dysfunction affects the DA pool in the fly brain. Further, as DA catabolism plays a clear role in PD, I also intended to investigate its correlation with late-onset PD and LA extract-mediated DAergic neuroprotection.

4.2. Material and Methods

(Described in Chapter-3, please refer the section/subheading 3.1 to 3.3)

4.2.2. Quantification of DA and its metabolites using HPLC

In order to comprehend the biological importance of DA metabolism in the *Drosophila* model of PD, I, therefore, attempted to quantify the levels of DA and its metabolites (DOPAC and HVA) using the HPLC-ECD equipment (Ayajuddin et al., 2023; Das, 2022; Ayajuddin, 2022). Standard DA and metabolites were quantified to provide a precise retention time and area with which samples were compared in order to quantify catecholamines in the tissue samples. Quantification of DA and metabolites was done following the protocols of Ayajuddin et al., (2021).

Catecholamine quantification

Sterilized 1.5 mL centrifuge tubes (Tarsons, WB, India, catalog number: 500010), Parafilm^{TM} wrapping film (Bemis, WI, USA, catalog number: PM996), Sterilized micro tips (Tarsons, WB, India, catalog number: 521010), Freshwrapp aluminum foil 9–11 μm (Hindalco, Maharashtra, India, catalog number: HV2241), Whatman^{TM} filter paper (GE Healthcare, Buckinghamshire, UK, catalog number: 1001917), Phosphate buffered saline (PBS) pH 7.4 (HiMedia, Maharashtra, India, catalog number: ML023), Dissecting fine

forceps (EMS, PA, USA, catalog number: 78620-4B), Brush (TEYUP, Delhi, India, model number: SR-1013), Delicate task Kim wipers (KIMTECH™, GA, USA, catalog number: 370080), Micropipette i.e., 1,000 µL, 50 µL, 10 µL, 2 µL (Gilson, WI, USA, catalog number: 30040), pH/mV meter (Hanna Instruments, RI, USA, model: HI2211-02), -20°C ES Series refrigerator (Thermo Scientific, MA, USA, model: 50616100444443250), -80°C ultra-low temperature freezer (New Brunswick Innova, Hamburg, Germany, model: U101-86), Stereo zoom microscope (Carl Zeiss, Jena, Germany, model: Stemi 305), Dopamine (DA) (Sigma-Aldrich, St. Louis, MO, USA, catalog number: H8502), 3,4-Dihydroxyphenylacetic acid (DOPAC) (Sigma-Aldrich, St. Louis, MO, USA, catalog number: 11569), Homovanillic acid (HVA) (Sigma-Aldrich, St. Louis, MO, USA, catalog number: 69673), Trichloro Acetic Acid (TCA) (SRL, Maharashtra, India, catalog number: 204842), MDTM mobile phase (Thermo Scientific, Waltham, USA, catalog number: 701332), HPLC grade water (JT Baker, Radnor Township, USA, catalog number: 4218-03), Acetonitrile (JT Baker, Radnor Township, USA, catalog number: 9017-03), Methanol (JT Baker, Radnor Township, USA, catalog number: 9093-68), HPLC-ECD 3000 RS system (Thermo Scientific, MA, USA, model number: Dionex Ultimate 3000), Nanodrop® 2000c Spectrophotometer (Thermo Scientific, Waltham, USA, model number: ND2000CLAPTOP)

Tissue sample

Fly heads were used for brain-specific catecholamine quantification. After 24 hrs of exposure, flies were immediately frozen. For each treatment group of the transition phase, 15 fly heads were decapitated. To avoid the thawing of tissue and degradation of biomolecules, the heads of frozen flies were decapitated on top of an ice tray having a

chilled metal sheet. Dissection equipments were cleaned with 70% ethanol to avoid contamination.

A) Preparation of standard DA, DOPAC, and HVA

Preparation of standards

To prepare the standards, 2 mg of commercially available catecholamines were dissolved in 2 mL of PBS. Different concentrations were achieved through dilution, as shown in **Table 4.1.** For loading the standard, 200 ng/mL of the concentration was used.

Standard	**PBS**	**Concentration**	**Stock Name**
2 mg	2 mL	1000 µg/mL	S
100 µL of **S**	900 µL	100 µg/mL	S1
100 µL of **S1**	900 µL	10 µg/ mL	S2
100 µL of **S2**	900 µL	1000 ng/ mL	S3
200 µL of **S3**	800 µL	200 ng/ mL	S4
150 µL of **S3**	850 µL	150 ng/ mL	S5
100 µL of **S3**	900 µL	100 ng/ mL	S6

Table 4.1: Preparation of multiple concentrations of catecholamine standards

The concentration of catecholamine that is to be loaded on the HPLC system was mixed with 5% TCA (the 5%TCA should be centrifuged at 6000 rpm for 10 minutes at 4°C prior to application to remove any undissolved solute particles) in a 1:1 ratio.

B) Sample preparation

1. In 300 µL of 1x PBS (prepared in HPLC grade water), 15 heads of adult flies were collected.

2. It was then homogenized and subjected to sonication for 20 seconds at intervals of 5 seconds with 30% amplitude (always kept on ice during the process).

3. At 4°C, the samples were centrifuged for 10 minutes at 6000 rpm.

4. The supernatant was collected.

5. 200 µL of the supernatant was removed (the remaining were set aside for protein quantification), and to it, 200 µL of the 5% TCA was added.

6. After that, the supernatant solution underwent two centrifugations at 5000 rpm for 10 minutes each at 4°C.

7. The supernatant was collected for downstream assay.

Precautions and Recommendation

1. The tissues should be homogenized and sonicated on the ice to avoid heat generation and prevent degradation.
2. In order to prevent any degradation of the molecules of tissue extract and standard catecholamines, both solutions should be kept on ice in between procedures.

3. All the reagents were made in HPLC grade or Milli-Q water to prevent any contaminating molecules that could create a false positive peak in the chromatogram.

4. Autoclaved fresh pipette tips were used to prepare serial dilutions of the standard and to transfer tissue extract.

C) Quantification of protein:

Bradford technique was used to measure the protein. 2 mg/mL of BSA was dissolved in PBS to make BSA stock. The 100 µL of stock solution was dissolved in 900 µL of PBS to create a working concentration of 0.2 µg/ µL. According to **Table 4.2**, the serial dilution was carried out.

BSA (µg/mL)	Working solution (µL)	PBS (µL)	Bradford (µL)
0.5	2.5	497.5	500
1	5	495	500
1.5	7.5	492.5	500
2	10	490	500
2.5	12.5	487.5	500
3	15	485	500
3.5	17.5	482.5	500

Table 4.2: Preparation of serial dilutions using standard BSA.

The NanoDrop 2000C (Thermo-Scientific, Waltham, USA) was used to read the absorbance at 595 nm after 5 min of room temperature incubation to produce a standard graph. The protein was measured using 3 µL of the pure tissue extract. Therefore, the

µg/mL of total protein concentration that was achieved during the assay was derived from 3 µL of extract which was combined with PBS and Bradford reagent. Thus, to get the real protein amount per µL, the total µg of protein was divided by 3 µL.

D) Setting up the HPLC system:

Solvents

Load the solvent tubing ports of the HPLC-ECD system with the following reagents

1. 100% HPLC grade Methanol

2. 80% Acetonitrile (Prepared in HPLC grade water)

3. 20% Acetonitrile (Prepared in HPLC grade water)

4. MDTM Mobile phase

The following "Preloading instructions" was followed for solvents

Preloading instructions for solvents

The act of setting solvent reagent bottles on the HPLC solvent rack and securing the corresponding tubing to the bottles is known as "preloading of the solvents." The "Preloading Instructions" listed below serve as a manual for handling the solvents and their containers. The instructions are regarding solvent preparation, setting them on the solvent rack, and connecting them to the tubing ports of the HPLC platform.

1. Each reagent bottle was optimally filled (minimum 350 mL in each).

2. To prevent the production of bubbles, the bottle was slanted while being filled.

3. As described in point 2, the mobile phase was poured onto the appropriate reagent container only after being filtered using 0.22-micron filter paper. (Miscibility of the mobile phase's constituents is an issue when passing through the column, due to minute-level coagulation of the mobile phase's organic components. Even ready-made mobile phases may contain undissolved salt residues and suspended particles. Filtering using a 0.22-micron nylon membrane ensured that such residues which could otherwise clog the C18 column of the HPLC-ECD system, are isolated).

4. Prior to being connected to the HPLC system, all the reagent bottles were sonicated in a bath sonicator for 15 minutes at room temperature using a 40 kHz ultrasonic frequency.

System/ Column cleaning

Tissue debris from the prior HPLC experiment may be present inside the columns and electrodes of the detectors. In addition, to prevent the growth of any fungi, the HPLC platform's components, such as the column, ECD, and tubing, were loaded and stored in 100% methanol. In order to remove any leftover tissue debris and methanol, it is crucial to clean the system with the flow of the mobile phase. Additionally, the cleaning makes sure that there are no air bubbles present when the HPLC platform was kept idle for a long time between experiments. The system and column were cleaned using the procedures below:

1. The system was cleaned by purging (Putting each solvent port into a high flow rate from the pump to outside the system while the purge valve was kept open) from all the ports for 5 minutes at a time to get rid of any trapped air bubbles.

2. The purge knob was then operated to close the purge valve and direct the flow from the pump to the column. To start cleaning the column after purging, 100% flow with 20% acetonitrile was enabled in the system for 30 minutes at a flow rate of 0.5 mL/min.

3. For a further 30 minutes, a 100% flow of the mobile phase was allowed to pass through the column at the same flow rate, following which the mobile phase may be recycled (Drainage pipe outlet from the column will be wiped with a lint-free tissue soaked in the mobile phase and will be inserted back into the mobile phase container bottle).

Setting up the HPLC parameters

The ideal oxidation potential for catecholamine detection by ECD is within the range of 340 mV. (Yang and Beal, 2011). In our lab, it is discovered that the catecholamines are most effectively identified with the DIONEX ULTIMATE ECD 3000 system, utilizing a reduction and oxidation potential range of -175 mV and 225 mV respectively. The reduction potential inside the HPLC platform creates an identical state for all the catecholamines like that of *in-vivo*. The excitation of all concerned catecholamines inside the HPLC platform is regulated by the optimum oxidation potential, and within the range of this oxidation potential, the concerned catecholamines may be detected.

The following parameters were set for efficient detection and analysis of catecholamines

Reduction potential	:	-175 mV
Oxidation potential	:	+225 mV
Omni cell	:	+500 mV (for noise reduction)

Gain range	:	1 μA
Data collection rate	:	5 Hz
Detection Filter	:	2.0 (for all cells)
Column temperature	:	Room temperature
Auto sampler temperature	:	4^0C
Flow rate	:	0.5 mL/min

ECD priming

1. The ECD was primed after the mobile phase was switched to recycle mode.

2. To monitor the state of the baseline, the system was kept in acquisition mode for at least 2 hrs after configuring the necessary parameters for ECD.

3. Over time the parallelism of the two lines (Denoting the signal acquisition of ECDRS 1 & ECDRS 2 electrode), representing equilibrium and non-parallelism representing fluctuation was verified. The lines are supposed to be parallel if the system is equilibrated properly.

4. If the drifting was less than 2 nA/hour, the baseline was regarded as stabilized.

E) Standard and sample loading:

20 µL of standards were injected followed by 50 µL of samples for analysis. Standard, DOPAC, DA and HVA showed an optimum peak at 20 µL injection of 200 ng/mL concentrations.

Note: The same PBS was used to prepare samples as well as to dissolve standard metabolites. A minimum of 300 µL of standard and tissue extract was kept in the vial for injection.

F) Analysis

Quantitative analysis was performed using Chromaleon® version 6.8 software provided along with the HPLC system.

1. For analysis, the chromatogram obtained using the ECDRS2 channel was employed.

2. The "Inhibit Integration range" option was applied to the entire solvent-front area of the standard and sample chromatograms, inhibiting noisy or false peak integration to the chromatogram.

3. The sample chromatogram was superimposed over the standard chromatogram.

4. One may determine the peaks of the specific catecholamine present in the sample by comparing it with the standard chromatogram. Factors such as retention time, and the behaviour of the peaks with respect to other catecholamines are to be considered. To accurately pinpoint the DA, DOPAC and HVA peak in the sample, 10 µL of the

composite standard was mixed and the sample was run again in HPLC. The peaks that spiked according to the detection sequence were identified as the monoamines of interest.

5. The peaks were split into two peaks with the user interface if the peaks were co-eluted i.e., peak shoulders were joined.

6. Software tools such as the automated tool, delimiter tool, peak tool, baseline tool, etc. were used to increase the accuracy of the peak area.

7. After the peak was precisely determined, additional processing for quantitative analysis could be done.

8. In order to quantitate catecholamine levels in tissue extract, the sample catecholamine's peak area was normalized to the standard.

G) Calculation of concentration of catecholamines in the sample with example:

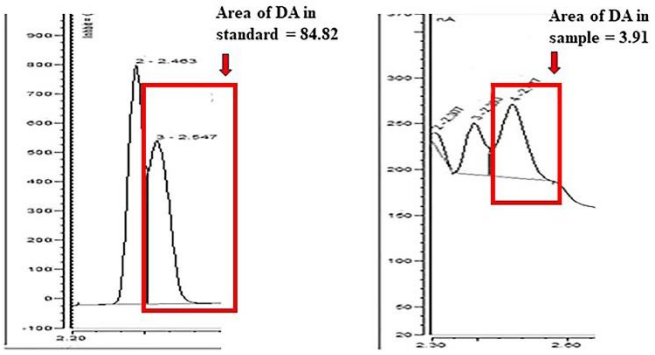


Figure 4.2: Image of a chromatogram showing the area of the standard and sample

i. The concentration of the standard catecholamines: DA (DA_{Std}), DOPAC ($DOPAC_{Std}$) and HVA (HVA_{Std}) used in the HPLC assay was 200 ng/mL each.

ii. Injection volume of all standard catecholamine to the HPLC column was I_{Std} = 20 µL.

iii. Peak area for a catecholamine was obtained from standard and sample chromatograms (**Figure 4.2**)

iv. Area of the peak of the standard catecholamines (DA, DOPAC and HVA) in the chromatogram was

A_{DA_Std} = 84.82, A_{DOPAC_Std} = 90.21 and A_{HVA_Std} = 60.41

v. Injection volume of tissue extract to the column was I_{Samp} = 50 µL.

vi. Area of the peak of catecholamines (DA, DOPAC and HVA) in the tissue sample chromatogram was A_{DA_Samp} = 3.91, A_{DOPAC_Samp} = 3.45 and A_{HVA_Samp} = 9.92.

vii. Suppose, a particular tissue extract from an experimental group that was used for HPLC assay, was quantified beforehand for total protein which was TP_{Samp} = 0.143 µg/ µL.

viii. The following steps were followed for calculating the actual amount of the catecholamines in tissue extract **(Table 4.3).**

Calculation

Calculation Steps	**Metabolites**		
	DA	**DOPAC**	**HVA**
Step I: Concentration of standard catecholamines in 20 µL	DA_{Std} X I_{Std}/1000 i.e. (200 X 20)/1000 = 4 ng	$DOPAC_{Std}$ X I_{Std}/1000 i.e. (200 X 20)/1000 = 4 ng	HVA_{Std} X I_{Std}/1000 i.e. (200 X 20)/1000 = 4 ng

Step II: Concentration of catecholamines in brain tissue extract	(A_{DA_Samp} X 4)/ A_{DA_Std} i.e. (3.91 X 4)/84.82 = 0.18 ng	(A_{DOPAC_Samp} X 4)/ A_{DOPAC_Std} i.e. (3.45 X 4)/90.21 = 0.15 ng	(A_{HVA_Samp} X 4)/ A_{HVA_Std} i.e. (9.92 X 4)/60.41 = 0.65 ng
Step III: Determining the total protein in 50 µL that was injected into column	(TP_{Samp} X I_{Samp}) i.e. (50 X 0.143) = 7.15 µg	(TP_{Samp} X I_{Samp}) i.e. (50 X 0.143) = 7.15 µg	(TP_{Samp} X I_{Samp}) i.e. (5 X 0.143) = 7.15 µg
Step IV: Determining the catecholamine in 1 µg of protein	[0.18/7.15] = 0.025 ng	[0.15/7.15] = 0.021 ng	[0.65/7.15)] = 0.091 ng
Step V: Determining the actual amount of catecholamine per head as injected tissue extract solution had brain tissue extract + TCA in 1:1 ratio	0.025 X 1000/(2 X 15) = 0.83 pg/brain	0.021 X 1000/(2 X 15) = 0.7 pg/brain	0.091 X 1000/(2 X 15) = 3 pg/brain

Table 4.3: Steps for calculation of the amount of catecholamines for single fly brain.

3.2.3. Statistical analysis

Graphs were created using Graph Pad Prism 5.0 software (Graph Pad Inc., San Diego, CA, USA). Statistical analysis was completed, and results were expressed as the mean ± standard error of the mean (SEM). A Two-way ANOVA followed by Bonferroni post-test and one-way ANOVA followed by Newman-Keuls multiple comparison test was carried out to draw significance for catecholamines quantification. P-values < 0.05 were regarded as significant.

4.3. Results

4.3.1. PQ diminishes brain DA level and induces alteration in its metabolites (DOPAC and HVA) levels in the TP-PD brain

Utilizing the standard and sample chromatogram obtained from the HPLC-ECD unit I measured the concentration of brain DA and its metabolites (DOPAC and HVA) to understand DA metabolism (**Figure 4.3**).

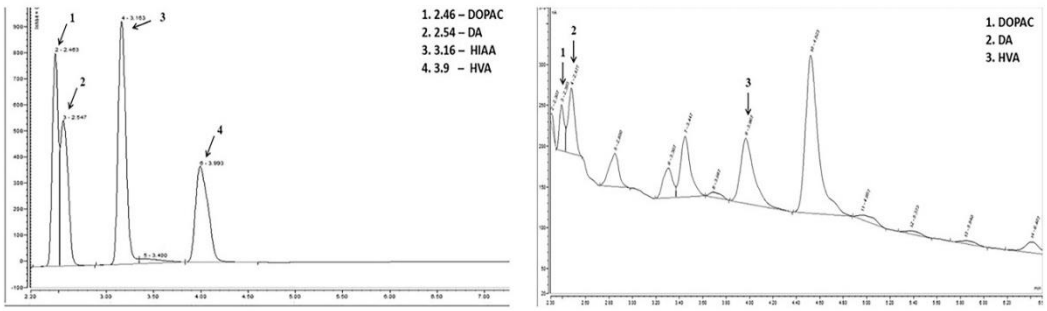


Figure 4.3: Characterization of retention time of standard DOPAC, DA and HVA (A) and brain-specific DA and its metabolites levels (B): Chromatogram of the standard catecholamines gives a particular RT comparing with which the catecholamines in the fly brain sample is analyzed.

The levels of DA in the PD brain were reduced by 44.29% (***$p<0.001$) during TP (**Figure 4.4A**). The FI intensity of DAergic neurons was significantly reduced after PQ treatment, which is due to TH-reduced production and neuronal dysfunction (**Figure**

3.4J). Therefore, decreased TH synthesis, as established in Chapter 3, is directly linked with reduced DA levels. The accumulation of these events may result in motor impairments in the PD model (Das, 2022; Phom et al., 2014). The subsequent metabolite of DA, i.e., DOPAC, was also reduced by 33.77% (***p<0.001) in the TP-PD brain (**Figure 4.4B**). Reduced DA and DOPAC levels have also been reported in the post-mortem brains of PD patients (Goldstein et al., 2011). HVA is the end product of DA metabolism in the fly, where DA and DOPAC can be reduced to HVA via an MAO/COMT equivalent pathway (Yamamoto and Seto, 2014; Meiser et al., 2013). As a result, HVA levels were examined too, and it was discovered that in the TP-PD brain, HVA levels are reduced by 28.24% (**p<0.01) (**Figure 4.4C**). Whereas, when compared to DOPAC and HVA, the DA depletion in the TP-PD brain is substantially greater. This shows that DOPAC and HVA synthesis in the PD brain exceeds DA synthesis during TP. Like the TP, depletion of DA by 32.48% was also observed in the HP PD brain. Further, high DOPAC and HVA synthesis was also postulated in HP PD brain as DOPAC was depleted only by 20%, whereas HVA level increased by 37% (Results from a colleague in lab Neikha K). Higher DA downstream DOPAC and HVA synthesis in the PD brain indicates that these monoamines might play a role in the initiation and progression of early and late-onset PD.

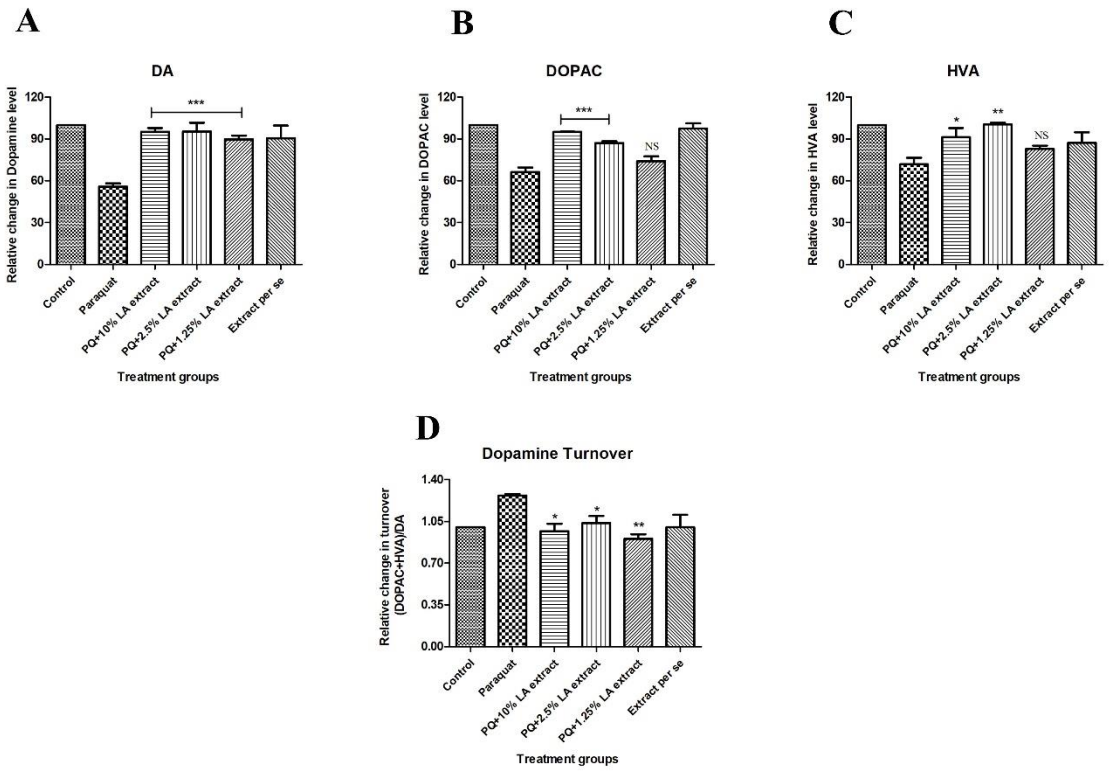


Figure 4.4: Quantification of catecholamine levels in fly brain using HPLC. During the TP, feeding the flies with PQ alone led to a significant reduction in brain DA (A), DOPAC (B), and HVA levels (C). The level of DA depletion is higher compared to downstream metabolite DOPAC and HVA, suggesting an enhanced DA turnover rate (D). LA intervention during the TP rescue diminished DA, DOPAC, and HVA levels, and the intervention was enough to prevent DA turnover (D). Insight suggests that LA ameliorates DA depletion and reduces DA turnover rate during TP. Significance was drawn by analyzing the data of minimum three replicates with one-way ANOVA followed by Tukey post-hoc test. The significance was presented by ***$p<0.001$; **$p<0.01$; *$p<0.05$; NS-Not Significant when compared to PQ (10mM) treated group.

4.3.2. *Leea asiatica* (LA) replenishes diminished DA level and its downstream metabolites DOPAC and HVA levels in the TP-PD brain

The Co-feeding of LA has been shown to help flies recuperate from PD motor impairment during the TP (**Chapter 3**). In the current investigation, LA extract co-feeding could restore (***p<0.001) reduced DA levels in the TP-PD brain (**Figure 4.4A**), suggesting the therapeutic potential of LA extract in late-onset PD. Furthermore, with LA extract intervention, the DA downstream DOPAC (***p<0.001) and HVA (*p<0.5, **p<0.01) levels were also rescued in the TP-PD brain (**Figure 4.4 B, C**). Interestingly, in HP PD, brain neuroprotection by LA extract intervention also involved the rescue of diminished DA level, whereas DOPAC level was inhibited and HVA was further enhanced (Results from a colleague in lab Neikha K). The insights suggest that LA extract-mediated neuroprotection in early and late-onset PD may be through modulating the DA metabolic pathway. To investigate further the implication of LA extract in DA metabolism and neuroprotection in late-onset PD, I looked into the DA turnover rate.

4.3.3. *Leea asiatica* (LA) rescues enhanced levels of DA turnover rate in the adult TP *Drosophila* model of PD

The impact of alterations in DA, DOPAC, and HVA pools in the PD brain and with LA extract interventions were investigated to comprehend DA catabolism. The [(DOPAC+HVA)/DA] formula was used to calculate the DA degradation/turnover ratio to its catabolites. The DA turnover ratio in the TP-PD brain was found to be higher than in the healthy brain (**Figure 4.4D**). It implies that in the TP-PD brain, the depletion of DA level apart from its reduced synthesis, may also be attributed to its degradation into downstream catabolites, viz., DOPAC and HVA. LA extract intervention effectively

mitigates the elevated DA turnover during TP-PD (**Figure 4.4D**). Similarly, in the HP PD brain, it was also observed that LA extract intervention could rescue enhanced DA turnover (Results from a colleague in lab Neikha K). Overall insight implies that the neuroprotective efficacy of LA extract is possible through modulation of DA catabolism, where enhanced DA breakdown to DOPAC and HVA in the PD brain is inhibited. This may prevent endogenous neurotoxicity during early and late-onset PD.

4.4. Discussion

The findings suggested that neurotoxicant PQ treatment promotes DA depletion in the HP and TP brain (**Figure 4.5**). The depletion of the DA pool in the HP brain was accompanied by moderate depletion of DOPAC and a significant increment in HVA levels, resulting in high DA turnover (**Figure 4.5)**. Whereas a high degree of depletion of the DA pool in the TP brain was accompanied by a comparatively lesser depletion of DOPAC and HVA, which also promoted high DA turnover (**Figure 4.4; Figure 4.5**). Similar patterns of changes in monoamine pools were also reported in the PQ -mediated early and late-onset PD model of *Drosophila* developed in our lab (Das, 2022). It has been reported that PQ-mediated stress depletes extra nigrostriatal DA in the nucleus accumbens of young mice (6-7 weeks old). When the same PQ-intoxicated mice were further exposed to psychological stress, there was reduced DOPAC, elevated HVA levels, and a rise in DA turnover (Rudyk et al., 2015). The motor and non-motor symptoms of PD patients are exacerbated by psychological stress, which leads to depression. In the mice model, Rudyk et al. (2015) demonstrated that enhanced HVA levels with reduced DA and DOPAC levels and enhanced DA turnover in some extra nigral brain regions are associated with the onset of PD having psychological impairment. Insight from our lab suggested similar changes in the monoamine pool in the HP PD brain

(**Figure 4.5**). On the other hand, the TP-PD brain also showed high DA turnover resulting from relatively lower depletion of DOPAC and HVA pool compared to DA, which in turn suggests higher DA breakdown to its metabolites (**Figure 4.5)**. Therefore, further insight is needed to conclude if such change is associated with the onset of psychological disorder in the current fly PD model.

In one of the studies conducted on adult young flies (2-4 days old) belonging to the CS strain and a particular white eye strain {*y W1118, Df (1)w, y*}, exhibited reduced DA levels and elevated DOPAC levels in the brains upon exposure to 10 mM or 20 mM PQ on filter paper for 24 hours (Inamdar et al., 2012; Chaudhuri et al., 2007). The observations on DOPAC regulation in the HP brain from our lab and that of Inamdar et al. (2012) are different, despite their apparent similarities. Inamdar et al. (2012) indicate that exposure to a neurotoxicant (10mM PQ) similar to that of the present study elevates DOPAC levels in the brains of young adult flies (2-4 days old). However, it was observed in the current study that during HP, the DOPAC level, along with DA, is diminished. Yet a closer observation reveals that DA depletion is relatively higher compared to that of the DOPAC pool in the HP PD brain (**Figure 4.5**). This suggests higher DA oxidative degradation to downstream DOPAC, therefore the postulation of higher DA turnover in HP PD brain, as suggested by Inamdar et al., (2012), corroborates to our insight from the current study. On the other hand, in the TP-PD brain, a higher DA depletion is observed compared to DOPAC (**Figure 4.5**), which, as discussed, preludes to high oxidative turnover of DA to DOPAC. Further insights from the current study suggest that oxidative degradation of DA, majorly contributes to HVA synthesis as the HVA level in the HP PD brain is enhanced, whereas the degree of HVA depletion is relatively lower compared to DA and even DOPAC in the TP-PD brain (**Figure 4.5**). Therefore, it's feasible that the

HVA might be produced from DA predominantly through the DA>3-MT>HVA pathway (**Figure 4.5**). As to why the DOPAC level is depleted like DA, although to a different extent, it may be explained by the fact that DOPAC is the immediate catabolite of DA and, therefore, responds to the changes in the total DA pool. In fact, the DOPAC pool in CSF is used as a valid indicator/marker of DA deficiency in cases of human PD (Goldstein et al., 2018; 2011).

While DA metabolism in the late-onset fly model of PD has not been thoroughly studied, all of the information gathered from this study rewards the possibility that PQ-induced sporadic PD condition elevates DA oxidative breakdown to the downstream catabolites in addition to facilitating DA depletion during TP. Owing to the neurotoxic natures of the DA catabolites and the generation of ROS/peroxides due to the catabolic process, neurodegeneration ensues (Cao et al., 2021; Zhang et al., 2019).

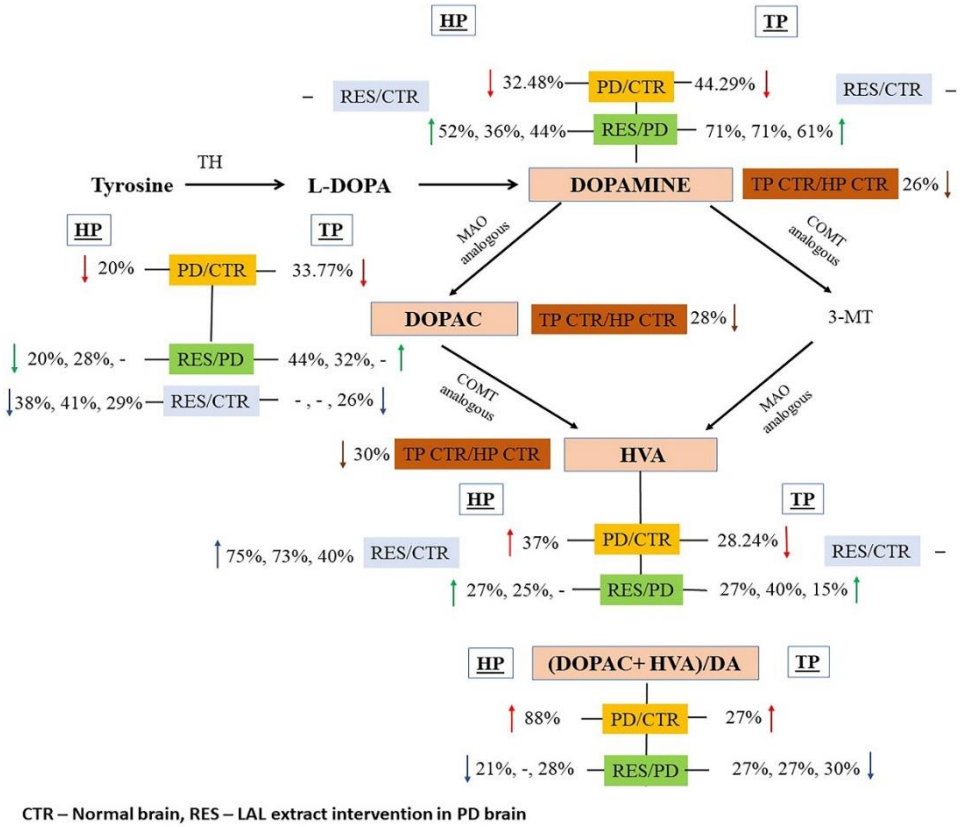


Figure 4.5: Schematic representation of DA metabolism in PD brain and with LA extract interventions: During HP, DA and DOPAC levels are decreased, while HVA level is increased under PQ-mediated PD condition. The relatively higher DA depletion compared to DOPAC and enhanced HVA suggests, higher DA oxidation in the PD condition in the fly brain. LA extract intervention rescued diminished DA levels and altered DA turnover during HP. In the TP-PD brain, DA, DOPAC, and HVA were depleted, although DA depletion was higher compared to DOPAC and HVA. This implied higher DA turnover. LA extract rescued the diminished DA, DOPAC, and HVA levels and further rescued the altered DA turnover. Also, with natural aging, DA, DOPAC, and HVA levels are decreased in healthy TP brains. (Data relating to the HP is adapted from the thesis of a colleague in the lab, Kelevikho Neikha, DNBL)

Insight on the neuroprotective efficacy of LA extract suggests that LA extract effectively rescues diminished DA levels during HP and TP. On the other hand, while LA intervention inhibits the DOPAC level in the HP PD brain, the HVA level is further enhanced, whereas in the TP brain, DOPAC and HVA levels are rescued with LA intervention (**Figure 4.5)**. However, in both age groups, the altered DA turnover is rescued. Previously I discussed that it is possible that oxidative turnover of DA in the PD model is more directed towards HVA synthesis than DOPAC. It is observed with LA intervention that although DOPAC level is differentially modulated between two age groups, upon resuscitation of DA level in HP and TP-PD brain, HVA level is also enhanced and rescued respectively. This suggests that LA extract -mediated regulation of DA metabolism is more focused toward the upstream pathways like DA anabolism rather than downstream pathways like DA catabolism. A high DA synthesis in PD condition may, in turn, trigger high DA metabolite production, although it effectively prevents DA depletion/breakdown mediated onset of PD. Further insights illustrated that healthy aging brain showed/exhibited diminished DA, DOPAC, and HVA levels (**Figure 4.5**), suggesting natural dysregulation of DA metabolic pathways with age. Yet, the ability of LA extract to effectively bypass the natural aging associated DA metabolic dysregulation and promote neuroprotection even during TP suggests possible modulation of DA agonistic pathways.

However, as mentioned before, a higher DA synthesis may also run a risk of higher ROS production and associated OS, more so in the later stages of life. Perhaps LA extract is a highly potent antioxidant that counters these additive ROS generation and associated OS in the PD brain, thereby ensuring neuronal integrity. Therefore, with the speculation in

Chapter 5, I attempted to look into the extent of the antioxidant capabilities of LA extract in late-onset PD.

(Contributions: 1. Animal treatments and climbing assay: Rahul Chaurasia; 2. HPLC experiment and analysis: Dr. Abhik Das and Dr. Mohamad Ayajuddin)

Chapter 5

Oxidative Stress-mediated DAergic Neurodegeneration and its Sequestration by *Leea asiatica* (LA) in the *Drosophila* Model of Parkinson's Disease

Introduction

5.1 Oxidative stress in human diseases

Helmut Sies coined the term "oxidative stress" (OS), which alludes to an imbalance between the generation of free radicals and cellular antioxidant defenses that compromise the biological systems (Watanabe et al., 2024; Sies 1985, 2018, 2020a). In a normal physiological state, the level of ROS is characterized by low to mild levels of oxidants, also referred as “good stress,” involved in the regulation of various biochemical transformations such as carboxylation, hydroxylation, peroxidation, or modulation of signal transduction pathways such as nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) cascade, phosphoinositide-3-kinase, nuclear factor erythroid 2–related factor 2 (Nrf2) (Reddy, 2023; Hajam et al., 2022; Forman and Zhang, 2021). Elevated levels of ROS, which are produced by both exogenous (environmental adversities/radiation, anticancer therapy, smoking, alcohol intake, and certain pharmaceuticals) and endogenous (mitochondria and NADPH oxidases) sources, culminating in a detrimental state known as OS or "bad stress" (Reddy, 2023; Hajam et al., 2022; Forman and Zhang, 2021). Conversely, the endogenous sources are either enzymes that produce ROS outside of the mitochondria or inside the mitochondria (Balogun et al., 2021; Kim et al., 2015). During cellular respiration, the electron transport chain (ETC) first three complexes allow an electron to break free and bind to oxygen, producing superoxide anions ($O_2^{\bullet-}$). Three different kinds of SOD are found in the mitochondrial membrane: zinc superoxide dismutase (Zn-SOD), copper superoxide dismutase (Cu-SOD), and manganese superoxide dismutase (Mn-SOD). Mn-SOD transforms O^{-}_2 into H_2O_2, which is converted to hydroxyl radical by the enzyme aconitase via the Fenton reaction (Reddy, 2023; Hajam et al., 2022; Vásquez-Viva et al., 2000).

Meanwhile, $O_2^{\bullet-}$ is changed into H_2O_2 and oxygen in the intermembrane space by Cu-SOD and Zn-SOD (Chidambaram et al., 2024; Hajam et al., 2022; Okado-Matsumoto et al., 2001). The production of ROS can also occur through the mitochondrial cytochrome catalytic cycle, which includes the cytochrome P450 enzyme and a variety of organic substances, including steroids, lipids, and xenobiotics. It generates a variety of reactive by-products, including H_2O_2 and $O_2^{\bullet-}$ (Hajam et al., 2022). Since MAO and mitochondrial glyceraldehyde-3-phosphate dehydrogenase (mGDPH) are more active, there is also an increase in H_2O_2 synthesis in the mitochondria. According to Pesta and Roden (2017), H_2O_2 induces dysfunction in the Insulin signaling pathway by activating the mitogen-activated protein kinase (MAPK) and C-Jun-N-terminal kinase (JNK) pathways and hinders the uptake of glucose. Endoplasmic reticulum (ER) stress also causes mitochondrial OS by releasing calcium into the mitochondria (Pesta and Roden, 2017). Elevated calcium levels in the mitochondria obstruct electron transport and energy production, which raises ROS production (Pesta and Roden, 2017). Furthermore, pro-inflammatory transcription factors, including NFκB and activator protein-1 (AP-1), are activated by increased ROS production and accumulation in the cell, which in turn activates a variety of pro-inflammatory chemokines/cytokines and adhesion molecules (Rendra et al., 2019). Likewise, a variety of protein complexes in mammals, including succinate dehydrogenase (SDH), dihydroorotate dehydrogenase (DHODH), nicotinamide adenine dinucleotide (NADH)-cytochrome b5 reductase (b5R), and MAOs, also produce ROS (Jamova et al., 2023; Hajam et al., 2022; Zhou et al., 2021; Hey-Mogensen et al., 2014) causes DNA damage. Hastoy et al. (2018) state that ROS can attack nucleic acids, nitrogenous bases, and sugar-phosphate backbone, resulting in DNA breaks linked to premature aging. OS-induced DNA damage elevates the levels of the mutagenic base8-

oxo-2′-deoxyguanosine in the tissue, which serves as a biomarker for OS (Hastoy et al., 2018). Additionally, OS produces a complex mixture of LP products, some of which are principally responsible for DNA alterations and ensuing carcinogenesis, such as malondialdehyde (MDA) and 4-hydroxy-trans-2-nonenal (HNE) (Wei and Yin, 2015). OS also compromises the blood-brain barrier (BBB), aggravating neuronal damage in AD and TBI patients (Wang et al., 2023). Despite the widespread acceptance that their origins are complex, OS is an umbrella term among many chronic diseases. A prolonged higher OS increases the risk of developing several fatal pathological conditions, including diabetes, lung disease (chronic pulmonary obstruction, lung cancer), mental illnesses (depression, schizophrenia, bipolar disorder), cardiovascular disease, aging, and neurodegenerative disorders (AD, PD, ALS) (Jomova et al., 2023; Mangione et al. 2022; Forman and Zhang 2021; Halliwell 2020). Understanding the fundamental processes that underlie the pathophysiology of many clinical diseases caused by OS can aid in creating therapeutic interventions to combat these illnesses and disorders to promote or advance human health.

5.2 Oxidative stress and PD

The etiology of OS-induced pathology encompasses multiple processes, including DA metabolism, mitochondrial failure, neuroinflammation, and environmental variables that produce ROS. ROS is a critical component in the development and progression of PD (Houldsworth, 2024). In the brain of PD patients, DA auto-oxidation or metabolization by MAO results in the production of ROS and DA quinones (Zucca et al., 2014; Segura-Aguilar et al., 2014). DA quinones have been shown to cause inactivation of the DA transporter (DAT) and the TH enzyme (Whitehead et al., 2001; Kuhn et al., 1999), as well as modification of several proteins, such as α-syn, parkin, DJ-1, SOD2, and UCH-

L1, alterations of brain mitochondria, and failure in Complex I activity (Houldsworth, 2024; Blesa et al., 2015; Zhou et al., 2014; Jana et al., 2007). Recent reports demonstrated that PD patients exhibit reduced complex I activity in the SNpc, which leads to an excessive generation of ROS and the degeneration of DAergic neurons (Hajam et al., 2022; Hauser and Hastings, 2013). Moreover, studies also evidenced that point mutations and deletion in the mitochondrial DNA might cause a defect(s) in complex I and encode complex I subunits in patients suffering from PD (Ma et al., 2021; Ellmore et al., 2020; Simon et al., 2010). Reduced complex I activity, increased ROS, and increased DA toxicity through OS were linked to α-syn accumulation in DAergic neurons (Moradi Vastegani et al., 2023; Dryanovski et al., 2013; Xiang et al., 2013; Martin et al., 2006). The reduction of GSH in the SNpc of PD is caused by ROS-induced OS (Oakley et al., 2007). Whereas GSH and glutathione disulfide (GSSG) levels are lower in the brain tissue of PD patients than in the brain tissue of healthy individuals (Pearce et al., 1997; Sian et al., 1994). Elevated OS accelerates the rate of LP by breaking down membrane polyunsaturated lipids and proteins, causing DNA fragmentation in the PD brain (Locatelli et al., 2003). The altered gating features of ion channels linked with increased OS led to PD pathology. K^+ channels are critical in PD and are accountable for neuronal excitability, neurotransmitter release, neuroinflammation, and synaptic transmission. Voltage-dependent K^+ currents in DAergic neurons control repolarizing action potentials and fine-tune pacemaker firing rates, while voltage-gated Na^+ channels modulate pacemaker frequency (Picher-Martel and Dupre, 2018; Braga Neto et al., 2016; Sarva and Shanker, 2014; Chen et al., 2012). Elevated non-enzymatic OS markers (ROS, LP, HP, and PC) are also demonstrated in the PQ-mediated ALSS *Drosophila* model of sporadic PD. Similarly, the enzymatic OS markers, viz., SOD, CAT, and GST, are found to be

elevated in the PQ-mediated ALSS *Drosophila* model of sporadic PD (Phom, 2018; Phom et al., 2014). In the present study, we have also demonstrated the elevated levels of non-enzymatic OS markers like LP and enzymatic OS markers like SOD in the transition stage-specific PQ-mediated *Drosophila* model of sporadic PD (**Figure 5.1 & Figure 5.2**). These pathways eventually lead to the degeneration of DAergic neurons in the PD brain.

5.3 Understanding DAergic neurodegeneration using oxidative stress markers: Insights from human to animal models of PD

5.3.1 Non-enzymatic oxidative stress marker

5.3.1.1 Lipid Peroxidation (LP)

Mammalian cell membrane lipids (glycerophospholipids and phosphatidylinositol) carry saturated, mono-unsaturated (esterified in the sn-1 position), and polyunsaturated fatty acids (esterified in the sn-2 position), which are most sensitive to ROS and hence, are primary targets of peroxidation. LP is caused when ROS interacts with lipids within cell membranes (Su et al., 2019). The major degradation products of LP are 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) (Gaschler and Stockwell, 2017). MDA, a highly reactive and toxic dialdehyde, forms an adduct with primary amines on proteins or DNA. MDA forms an adduct with Deoxy-guanosine on the pyrimidine ring to give pyrimido[1,2a] purine-10(3H), one of the significant products. MDA also forms adducts with deoxyadenosine and deoxycytidine, which, in the absence of intracellular DNA repair mechanisms, would result in mutagenicity and carcinogenicity (Vijayraghavan and Saini, 2023) and is a reliable marker of OS (Gaschler and Stockwell, 2017; Ayala et al., 2014). 4-HNE, the primary by-product of LP, under homeostatic conditions, is a protective signaling molecule; however, at high concentrations, it acts as a cytotoxic molecule inhibiting gene expression (Zhong and Yin, 2015). LP alters

membrane permeability, fluidity, and ion gradients (Gaschler and Stockwell, 2017). In turn, 4-HNE interaction with proteins or lipids creates protein-lipid adducts responsible for protein aggregation, enzyme inactivation, and cytotoxicity (Bilska-Wilkosz et al., 2022; Gaschler and Stockwell, 2017). LP is a well-studied molecular pathway involved in the pathogenesis of PD (Reddy, 2023; Angelova et al., 2021). However, whether LP products are the cause or consequence of PD remains unclear (Ayala et al., 2014). It has been reported that 4-HNE-induced neuronal loss and α-Syn aggregation (Peña-Bautista et al., 2019). Furthermore, it has been reported that oligomeric α-Syn induces LP (Angelova et al., 2015). Angelova et al. (2020) have recently reported that oligomeric α-Syn produces an iron-dependent increase in ROS and LP, which induces α-Syn aggregation in membranes, disrupts calcium flux, and leads to ferroptosis. A meta-analysis reported by Wei et al. (2018) concluded that MDA levels increased in PD patient's blood. Similarly, 4-HNE is also found in the LB of post-mortem PD brain tissues (Castellani et al., 2002). Furthermore, higher levels of MDA, a marker of OS, have been reported in the SNpc of PD patients (Dexter et al., 1989). Sharma et al. (2008) demonstrated a 2-fold increase in the plasma MDA levels of PD patients compared to healthy age-matched controls. Asako Yoritaka et al. (1996) revealed that 58% of the SN neurons contain 4-HNE modified proteins (compared to 8% in control) and an elevated level of MDA; other studies revealed a multi-fold increase in levels of LP products in the PD SN (Munch et al., 1998; Dexter et al., 1994). Fedorova et al. (2019) studies revealed that regardless of the stage of the PD disease (stages 2–4), an average increase in LP by 20% was observed. Elevated levels of MDA were registered in patients with a higher disease severity who were at the advanced stages of the disease (stages 3 and 4) (Fedorova et al., 2019). Whereas, when albino rats were administrated with PQ (10 mg/kg, injection,

twice a week) for three weeks, the level of MDA was enhanced (Fathy et al.,2021). Similarly, a study conducted by Saadullah et al. (2022) and Vegh et al. (2021) demonstrated that exposing Wistar rats to PQ, 10 mg/kg for 40 days and 10 mg/kg 5 times for 20 days enhanced the MDA levels and 4-HNE fluorescence levels in PD brain. Furthermore, Ravi et al. (2018) demonstrated that the levels of MDA were increased twofold in SK-N-SH cells when treated with PQ at a concentration of 14 μM for 24 hours. In *Drosophila*, MDA levels were significantly elevated in PQ-treated PD brains (20 mM PQ for 48 hours) (Srivastav et al., 2018, 2020; Shukla et al., 2014). A study concluded by Maitra et al. (2021) and Chaouhan et al. (2022) showed that exposure to 5 mM PQ for 48 hours causes elevation of LP in the *Drosophila* PD brain. Another independent investigation by Mahesh et al. (2022) demonstrated the enhanced LP upon exposure to PQ (5 mM PQ for 48 hours) in the *Drosophila* PD flies. Similarly, prolonged exposure to PQ (20mM for 24 hours) increased LP levels in the *Drosophila* PD brain. Meanwhile, the inclusion of 0.44 mg/g PQ in the seven-day diet enhanced the LP in Harwich PD flies (Nunes et al., 2019). Similarly, the inclusion of 3.5mM PQ for four days in the diet increased the LP in *Drosophila* (Salim et al., 2021; Soares et al., 2017). Also, a study conducted by Phom, (2018) exhibited elevated MDA levels when ALSS flies were exposed to 10 mM PQ for 24 and 48 hours (Phom, 2018). Since MDA and 4-HNE are well-known biomarkers of LP (Tsikas, 2017), the results above suggest that elevated OS leads to increased LP in the PD brain.

5.3.2 Enzymatic oxidative stress markers

5.3.2.1 Superoxide dismutase (SOD)

Enzymatic antioxidants constitute an endogenous antioxidant system and serve both main and secondary defensive roles. SOD is the primary line of defense that prevents the formation of ROS or neutralizes ROS (Chidambaram et al., 2024). O^{2-} is catalyzed by SOD to dismutate into O_2 and H_2O_2. Humans have been shown to have three distinct, well-separated SOD isoenzymes (Nozik-Grayck et al., 2005; Zelko et al., 2002). A study on the PD brain showed an increased SOD-like activity most prominent in the SN and basal nucleus (Marttila et al., 1998). Saggu et al. (1989) revealed that the activity of the SOD enzyme in the SN of PD patients was higher than in the control group. A clinical study reported elevated plasma levels of SOD in PD patients when compared to healthy controls (Sharma et al., 2008). Post-mortem investigations in the PD brain revealed increased activity of encephalic SOD (Taylor et al., 2012). Furthermore, elevated levels of SOD activity were positively correlated with a blood survey of PD patients (de Farias et al., 2016). In contrast, PQ-treated *Drosophila* PD brains (20 mM PQ for 48 hours) showed a 2.2-fold elevation of SOD activity levels (Srivastav et al., 2018). Another independent investigation by Mahesh et al. (2022) demonstrated the enhanced SOD activity upon exposure to PQ (5 mM PQ for 48 hours) in the *Drosophila* PD flies. Similarly, Phom, (2018) found that ALSS flies treated with 10 mM PQ for 24 hours have higher SOD levels (Phom, 2018). Also, prolonged exposure to PQ (20mM for 24 hours) increased the SOD levels in the *Drosophila* PD brain (Oloyede et al., 2020; Niveditha and Shivanandappa, 2018). Conversely, the administration of 5.375 of PQ alternatively for eight days resulted in enhanced SOD levels in the PD flies (Niveditha et al., 2017). The rise in SOD activities might represent a protective response to elevated levels of free radicals in PQ-mediated PD flies (Srivastav et al., 2018). Contrary to that, Chaouhan et

al. (2022) reported that exposure to 5 mM PQ for 48 hours causes reduced SOD in the *Drosophila* PD brain. Similarly, Shukla et al. (2014) reported that PQ-mediated toxicity (20 mM for 24 hours) resulted in a significant decrease in SOD activity by ~ 50% in the brain of the PD flies. Whereas, when albino rats received PQ injection (10 mg/kg, twice a week) for three weeks, the level of SOD activity was reduced (Fathy et al., 2021). Similarly, a study conducted by Saadullah et al. (2022) revealed that when Wistar rats were exposed to PQ, 10 mg/kg for 40 days reduced the SOD activity levels in the PD brain. Regardless of the altered results in PD models, several studies showed that SOD activity was higher in the SNpc and erythrocytes of PD patients (Younes-Mhenni et al., 2007; Saggu et al., 1989; Marttila et al., 1988). These findings indicate that PQ induces OS by reducing the antioxidant defense.

5.3.2.2 Electron transport chain enzymes: Mitochondrial Complex I-III (NADH and Cytochrome C reductase)

Mitochondrial complex I is the initial enzyme in the ETC, responsible for transferring protons from the mitochondrial matrix to the intermembrane space. This process creates an electrochemical gradient that is used to generate ATP. The inhibition of complex I activity hampers the efficient synthesis of ATP, leading to an increased formation of ROS in PD. The suppression of complex I leads to an elevation in generating ROS. Consequently, this increase in ROS suppresses complex I activity, creating a harmful cycle in DAergic neurons. Over time, this cycle results in excessive OS and depletion of ATP, finally leading to DAergic neurodegeneration in the nigrostriatal pathway (Tretter et al., 2004). The decrease in complex I activity in the SNpc of patients with sporadic PD has been extensively documented (Hattingen et al., 2009; Hattori et al., 1991; Schapira et al., 1990). Furthermore, mitochondrial complex I deficiency has been demonstrated in

various brain regions, fibroblasts, blood platelets, skeletal muscle, and lymphocytes of PD patients (Parker et al., 2008; Blandini et al., 1998; Haas et al., 1995; Blin et al., 1994; Mytilineou et al., 1994; Krige et al., 1992; Yoshino et al., 1992; Mizuno et al., 1989). There has been evidence of a decrease in the metabolic activity and protein level of mitochondrial complex I or NADH dehydrogenase in the SNpc and frontal cortex of post-mortem investigation in PD patients (Parker et al., 2008; Schapira et al., 1990). Furthermore, an investigation has shown a reduced complex I in several brain regions among individuals with PD (Flønes et al., 2018). Thus, complex I is regarded as a crucial location for the production of ROS. The use of neurotoxins like PQ established the association between mitochondrial dysfunction and PD. Mitochondrial complex I facilitates the transport of a pair of electrons from NADPH to ubiquinone, resulting in the oxidation of NADPH to $NADP^+$. When PQ dication (PQ^{2+}) enters cells, it goes through redox cycling. During this process, it interrupts the oxidation of NADPH by accepting electrons. This forms PQ mono-cation radical ($PQ^{\bullet+}$), facilitated by NADPH-cytochrome P450 reductase. Ultimately, this inhibits the activity of mitochondrial complex I (Fussell et al., 2011; Fukushima et al., 1993). Fukushima et al. (1995) showed that the brain treated with 500 μM PQ exhibited reduced complex I activity and an increase in LP. According to Srivastav et al. (2018), PQ (20 mM for 48 hours) reduced the amount of ATP in *Drosophila* flies. Choi et al. (2008) proposed that PQ-induced DAergic neuronal death does not need inhibition of complex I. The present study demonstrated that complex-I-III activity was reduced in TP-PD brains.

5.3.2.3 Mitochondria Complex II (Succinate dehydrogenase)

Succinate dehydrogenase (SDH)/ Complex-II, encoded by nuclear DNA (Hattori et al., 1991), is a central component of the oxidative phosphorylation (OXPHOS) system in mitochondria and connects the Krebs cycle to the electron transport chain that is responsible for ATP production (Goetzman et al., 2023; Bezawork-Geleta et al., 2017). Dysfunction or inhibition of the SDH can trigger mitochondrial impairment and disruption in ATP generation. SDH activity is influenced by malate, fumarate, citrate, and oxaloacetate concentrations (Gutman et al., 1971). SDH inhibition prompts the accumulation of fumarate in a matrix of mitochondria, leading to reduced ATP production (Van Vranken et al., 2014). SDH influences the Krebs cycle and contributes to ROS generation (Ralph et al., 2011). The activity of SDH/Complex II was modestly reduced in both platelets and lymphocytes in PD compared to age-matched controls (Haas et al., 1995; Yoshino et al., 1992). Compared to age-sex-matched controls, Complex II activity was also diminished by 20% in PD (Haas et al., 1995). In post-mortem investigations, individual DAergic neurons from PD patients have been found to have varying degrees of SDH deficiency (~65% Complex II deficiency) (Grunewald et al., 2016). Complex II activity has declined with age in multiple tissues, including the brain, liver, heart, and skin (Bowman et al., 2016). Mutation in the SDHC subunit in transgenic mice causes mitochondrial dysfunction and ROS production (Ishii et al., 2011). Meanwhile, 15 mg/kg PQ intraperitoneally for 3 hours reduced the SDH levels in PD mice (Hosamani et al., 2016). SDH may directly and indirectly regulate both lipid metabolism and excitotoxicity. One of the primary signaling proteins, mTOR, is active in PD, and its activation can block the activity of SDH, which acts as a connection between the OXPHOS process and the Krebs cycle. In addition, fatty acid oxidation plays a role in the

Krebs cycle via interacting with the SDH complex. Thus, when the mTOR is active, it inhibits the SDH, reducing fatty acid oxidation and ultimately leading to lipid accumulation. SDH can regulate superoxide generated by complexes I and III of the ETC (Dröse et al., 2011). In *Drosophila*, the activity of SDH is enhanced by the inhibition of mTOR by rapamycin. In contrast, Chronic PQ-mediated toxicity (20mM and 40mM PQ for 24 and 48 hours) in young *Drosophila* PD brains exhibited reduced SDH/Complex-II activity (Hosamani and Muralidhara, 2010). The investigation by Rao et al. (2016) demonstrated the reduced SDH activity upon exposure to ROT (500 µM ROT for seven days) in the *Drosophila* PD flies. Similarly, exposure to 500 µM ROT for seven days in the diet reduced the SDH levels in the *Drosophila* PD brain (Girish and Muralidhara, 2012; Manjunath and Muralidhara, 2015). When flies were fed with PQ (20 mM for 48 hours), they demonstrated reduced SDH activity (Liu et al., 2019). The age-associated decline in complex II activity may contribute to the accumulation of mitochondrial DNA (mtDNA) mutations, OS, and changes in the expression of genes involved in mitochondrial biogenesis (Van Houten et al., 2006). The present study exhibited reduced complex-II enzyme activity in the TP-PD brains. These findings indicate that the decline in Complex II activity is associated with a decrease in ATP production and an increase in the production of ROS, which can lead to OS and cellular dysfunction through damage to proteins, lipids, and DNA in PD brain (Goetzman et al., 2023; Vanova et al., 2020).

5.4 Aging and oxidative stress interaction

OS is a crucial factor in the aging process. It was first proposed by Harman in 1956 as the "free radical theory of aging," which posited that OS might trigger various forms of age-related damage, resulting in cellular aging and senescence (McCord and Luceri et al.,

2017; Fridovich, 1969; Harman, 1956). OS can cause cellular senescence through various mechanisms, including impairing mitochondrial function, causing DNA damage, shortening telomeres, promoting lipid peroxidation, triggering chronic inflammation, modifying proteins through oxidation, and affecting the expression of oncogenes and tumor suppressor genes such as *PTEN*, *RB1*, *NF1*, and *INPP4A* (Davalli et al., 2016; Yang et al., 2024). The "rate of living" hypothesis emphasizes that accelerated aging results from an increase in ROS due to a quicker respiratory rate (Sohal, 1976). Alternatively, according to "Damage-based" theories, which have been put forth by Kirkwood and Austad (2000), Kirkwood (2005b), Longo et al. (2005), and Valavanidis et al. (2015), aging is caused by the accumulation of irreparably damaged proteins, enzymes, membrane lipids, nuclear, and mitochondrial DNA. Under OS, the pro-apoptotic protein p66Shc contributes to the generation of ROS in mitochondria, which in turn causes mitochondrial damage and eventual cell death/apoptosis (Galimov, 2010). Elevated ROS levels activate p66Shc, a crucial protein in the ROS-aging pathway, leading to increased ROS generation and apoptosis while maintaining a gradual aging process (Ray et al., 2012). Furthermore, it has been observed that OS temporarily increases telomerase activity. However, telomerase activity declines as OS continues, resulting in telomere damage and shortening. This, in turn, contributes to aging and age-related diseases (Assavanopakun et al., 2022; Pineda-Pampliega et al., 2020; Blackburn et al., 2015; Ludlow et al., 2014; Cattan et al., 2008; Jiang et al., 2008; Von Zglinicki, 2002). SOD activity increased with age in all brain regions except in the hippocampus of 2-year-old mice (Hussain et al., 1995). Arking et al. (2000) found that in a long-lived (La) fly strain, increasing SOD1 and SOD2 expression was linked to a longer lifetime and resistance to OS, whereas decreasing their expression was linked to early onset of senescence (Arking,

2001; Arking et al., 2000a; 2000b). Rodriguez-Martinez et al. (1997) found that MDA content increased in tissue and plasma samples with age. The frontal cortex, parietal cortex, hippocampus, thalamus, and putamen are some of the brain areas that exhibit MDA protein adduction higher in older individuals (Jové et al., 2020). Mitochondrial complex I activity gradually diminishes within SNpc DAergic neurons with age (Venkateshappa et al., 2012). Also, due to its well-established function in metabolism and OS, Complex II has emerged as an essential player in aging. Studies have shown that complex II activity decreases with age in the liver, heart, and brain (Goetzman et al., 2023; Bowman et al., 2016; Cocco et al., 2005; Kumaran et al., 2004). According to Van Houten et al. (2006), a possible cause of an aging-related decrease in complex II activity is due to the accumulation of mutations in the mtDNA and reduced ATP production, which may, therefore, ultimately lead to OS and cellular dysfunction by damaging proteins, lipids, and DNA (Vanova et al., 2020). In line with numerous investigations (Evans et al., 2004; Reeves et al., 2002; Cardozo-Pelaez et al., 2000), the vulnerability of brain areas responsible for motor activities to OS increases with age. According to Borges et al. (2002), OS hinders TH, the enzyme responsible for DA production. About a third of clinically healthy elderly individuals have mild to moderate decreases in DAergic neuron density (5%-10% each decade) in their post-mortem SNpc (Buchman et al., 2012; Fearnley and Lees, 1991). As the oxidative balance of DAergic neurons continues to decline with aging, the high baseline levels of OS in aged SNpc DAergic neurons are believed to make them more susceptible to oxidative assault. Therefore, OS is still a significant contributor to aging and aging-associated disease.

5.5 *Leea asiatica's* (LA) role in sequestering oxidative stress

Studies have shown the presence of terpenoids, phenolic, flavonoid compounds, triterpenes, and glycosides in methanolic, ethyl acetate, and petroleum ether extract of LA leaves (Kil et al., 2019; Nair et al., 2014; Sen et al., 2013). Sen et al. (2012) have demonstrated the antioxidant efficacy of LA by sequestering OS in Indian adult earthworms (*Pheretima posthuma*) model Helminth infections exhibiting significant anthelminthic activity by scavenging free radicals, nitric oxide radical, and inhibition of LP as quantified through the ferric thiocyanate, diphenyl-picrylhydrazyl (DPPH˙) method and in Wister rats (Sen et al., 2012). The nephroprotective efficacy in cisplatin-induced toxicity in albino mice and Wistar rats have been exhibited through methanol, ethyl acetate, and petroleum ether extract of LA by lowering free radical-mediated OS. The antioxidant activity of LA was quantified by measuring scavenged DPPH˙ responsible for LP inhibition, superoxide anion radical (O_2^{let-}), hydroxyl radical (OH˙), nitric oxide radical (NO˙), H_2O_2, reducing power ability, metal chelating ability. The methanol extract prevents OS-induced damage by scavenging O_2^{let}, OH˙, NO˙, and H_2O_2 (Sen et al., 2013). Sen et al. (2014) demonstrated the hepatoprotective efficacy of LA in acetaminophen-induced hepatotoxicity in Wistar rats by reducing OS. The methanol extract of LA showed dose-dependent increase in antioxidant activity by significantly increasing levels of endogenous antioxidant enzymes such as SOD, CAT, GPx, GSH and by lowering the level of serum SGOT (serum glutamic oxaloacetate transaminase), SGPT (serum glutamic pyruvic transaminase), ALP (alkaline phosphatase), total bilirubin, total cholesterol and triglyceride in acetaminophen-induced hepatotoxicity in Wistar rats (Sen et al., 2014). Increased levels of GSH in serum & liver combined with enhanced activity of antioxidant enzyme SOD, CAT suggests that the LA has multiple ways to impart protection via

manipulating the anti-oxidant defense mechanism. Nair et al, (2014) have observed LA methanolic extract mediated potent anti-inflammatory and wound healing activity on male albino Wistar rats. The efficiency of wound healing was impressive, although not on par with standard wound healing compounds like 10% boric acid solution. Also, rats that were induced inflammation in the paw by carrageenan injection (*Carageenan induced paw oedema* in rats by Winter et al., 1962*)*, when fed water with LA and aspirin, showed marked anti-inflammatory activity, adding to the efficiency as par with marketed drugs. Nair et al. (2014) also addressed the extract's dermal and oral toxicity *in vivo* in a dose-dependent manner. They found no side effects/toxicity caused by the LA. Another major study investigated the potential application of ZnO NRs as an effective anticancer drug (Zinc oxide nanorods obtained via a green synthesis approach utilizing the leaf extract of LA as an effective anticancer drug). The anticancer activity of ZnO NRs was quantified through MTT (3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, XTT ((2,3-bis (2-methoxy-4-nitro-5- sulfophenyl) -5- [(phenylamino) carbonyl]-2H-tetrazolium hydroxide)) assay, NRU (Neutral Red uptake: Mitochondrial lactate dehydrogenase and lysosomic cytotoxic assessments), and LDH assay (Lactase dehydrogenase release: for apoptotic and necrosis cell death), concluding that the cytotoxicity properties of ZnO NRs are time and concentration-dependent (Ali et al., 2021). The authors further demonstrated that the ZnO NRs cause no mortality and toxicity in zebrafish embryos, which established ZnO NRs as a safe potential drug for human screening. Further, when ZnO NRs were exposed against the breast cancer cell line (MCF-7), it resulted in necrosis and apoptosis through OS-mediated ROS production, which can cause DNA damage leading to apoptosis in the breast cancer cell line (MCF-7), concluding its practical anticancer efficacy (Ali et al., 2021).

All these studies reported above demonstrated that LA can sequester OS-mediated dysfunction by scavenging free radicals and metal chelating activity that is as efficient as standard drugs. These antioxidant and anti-inflammatory properties of LA may be primarily responsible for the demonstrated anthelminthic, nephroprotective, hepatoprotective, and anticancer efficacy, which may contribute to imparting therapeutic cures to many other diseases that have not been scientifically validated yet treated by ethnic communities in India.

In this chapter, I attempted to understand LA's antioxidant efficacy by estimating brain-specific enzymatic and non-enzymatic OS markers, mitochondrial dysfunction under induced PD conditions, and LA-mediated rescue of the DAergic system.

5.6 Materials and Methods

5.6.1 Described in Chapter-3, please refer the section/subheading 3.1 to 3.3)

5.6.2 Chemicals

Bovine Serum Albumin (BSA; Sigma Aldrich St. Louis, USA, catalog number: A-2153), Quercetin (fluka, Sigma Aldrich St. Louis, USA, catalog number: 200595), Ethylenediamine tetra acetic acid (EDTA) (SRL, Maharashtra, India catalog number: 054959), Sodium dodecyl sulphate (SDS) (SRL, Maharashtra, India catalog number:1948101), Glacial acetic acid (SRL, Maharashtra, India catalog number:129168), Trichloroacetic acid (SRL, Maharashtra, India catalog number:204842), Mannitol (SRL, Maharashtra, India, catalog number: 134889), Sucrose MB grade (SRL, Maharashtra, India, catalog number: 1944115), HEPES (SRL, Maharashtra, India, catalog number: 84023), TEMED (Sigma Aldrich St. Louis, USA, catalog number: T9281), Tris HCL

(SRL, Maharashtra, India, catalog number: 2049170), Tris (SRL, Maharashtra, India, catalog number: 2049170), Bradford dye (Bio-Rad, CA, USA, catalog number: 5000006), Na_2HPO_4 (SRL, Maharashtra, India, catalog number: 1949147), NaH_2PO_4(SRL, Maharashtra, India, catalog number:1941144), Dimethylformamide (DMF) (SRL, Maharashtra, India, catalog number: 042825), Sodium Succinate dibasic hexahydrate (Sigma-Aldrich, MA, USA, catalog number: S2378), Iodonitrotetrazolium reagent (INT) (SRL, Maharashtra, India, catalog number: 94915), Ethyl acetate (SRL, Maharashtra, India, catalog number: 89362), Phosphate-buffered saline (PBS, HiMedia, India, catalog number: ML-023), Potassium dihydrogen phosphate (KH_2PO_4) (MERCK, Darmstadt, Germany catalog number: 6.175460.5001730), Dipotassium hydrogen orthophosphate (K_2HPO_4) (MERCK, Darmstadt, Germany catalog number:1.93630.0521), Nicotinamide adenine dinucleotide hydrogen (NADH) (SRL, Maharashtra, India catalog number: 44018), Potassium ferricyanide (KCN) (HiMedia, Maharashtra, India, catalog number: GRM627), Cytochrome C (SRL, Maharashtra, India, catalog number: 34015), Motor and pestle (Argos technologies, IL, USA, model number: WW-44468-25), NanoDrop 2000c (Thermo Scientific, MA, USA, catalog number: ND2000CLAPTOP).

5.6.3 Extraction and preparation of whole-head protein lysate for quantification of Lipid peroxidation (LP)

100 heads were homogenized with a pestle motor mixer (Argos technologies) in 175µl 0.1M Phosphate buffered saline (PBS) (pH 7.4). It was then centrifuged at 5000rpm for 10mins @ 4˚C. The supernatant was again centrifuged under the same conditions. The resulting clear supernatant was stored at -80˚C/used for biochemical assay.

5.6.3.1 Estimation of protein

The Protein concentrations of the tissue homogenates were determined by the modified version of the method initially described by Bradford (1976) using Bio-Rad protein assay dye reagent concentrate. The Bradford assay is a colorimetric assay for protein determination based on absorbance shift in the dye Coomassie brilliant blue-G250. Coomassie brilliant blue, which is red in unbound form, on binding to protein changes to stable blue form with absorbance shift from 465nm to 595nm. Since the increase of absorbance at 595nm is proportional to the amount of bound dye and thus to the amount of protein present in the sample. This can be used as a measure of the protein concentration of the unknown sample. Bovine serum albumin was used as the standard prepared in the concentration range of 0.5µgP to 3.5µgP (Table 5.1). The measurement was performed using NanoDrop 2000 (Thermo Scientific).

5.6.4 Extraction and preparation of whole-head protein lysate for quantification of Superoxide dismutase (SOD)

100 heads were homogenized with a pestle motor mixer (Argos technologies) in 175µl 0.1M Phosphate buffered saline (PBS) (pH 7.4). It was then centrifuged at 5000rpm for 10mins @ 4˚C. The supernatant was again centrifuged under the same conditions. The resulting clear supernatant was stored at -80˚C/used for biochemical assay.

5.6.4.1 Estimation of protein

The Protein concentrations of the tissue homogenates were determined by the modified version of the method initially described by Bradford (1976) using Bio-Rad protein assay dye reagent concentrate. The Bradford assay is a colorimetric assay for protein determination based on absorbance shift in the dye Coomassie brilliant blue-G250.

Coomassie brilliant blue, which is red in unbound form, on binding to protein changes to stable blue form with absorbance shift from 465nm to 595nm. Since the increase of absorbance at 595nm is proportional to the amount of bound dye and thus to the amount of protein present in the sample. This can be used as a measure of the protein concentration of the unknown sample. Bovine serum albumin was used as the standard prepared in the concentration range of 0.5µgP to 3.5µgP (Table 5.1). The measurement was performed using NanoDrop 2000 (Thermo Scientific).

5.6.5 Extraction and preparation of mitochondrial lysate from fly head

To extract the mitochondria from the fly head, a mitochondrial extraction buffer was prepared following the protocols established by Moreadith and Fiskum, (1984), Trounce et al. (1996), and Ayajuddin et al. (2022) with minor modifications.

The preparation of buffers requires (I) Buffer A: 50 mM HEPES stock was prepared by dissolving 0.1192 g of HEPES in 10 mL Milli Q water, and pH was adjusted to 7.4. Then, 1.82 g of mannitol, 1.195 g of sucrose, and 10 µL of 0.5 M EDTA were added. The final volume was adjusted to 50 mL with Milli Q water. (II) Buffer B: 4.275 g of sucrose was dissolved in 25 mL of Milli Q water, and 100 µL of 1 M Tris (1.211 g of Tris in 10 mL of Milli Q water and adjusted the pH to 7.4) was added. The final volume was adjusted to 50 mL.

5.6.5.1 Mitochondria lysate extraction procedure

The head from the body of the fly was dissected. 500 heads were taken in an Eppendorf tube and homogenized in 500 µL of extraction Buffer B. Proper care was taken so that the sample was not heated while homogenizing. After crushing, the pestle was rinsed with another 500 µL of the same buffer, making a total of 1 mL. The sample was centrifuged

at 5000 rpm for 10 min at 4℃. The supernatant was collected and centrifuged at 8000 rpm for 10 min at 4℃. The supernatant was collected for cytosolic fraction while the pellet was dissolved in 1 mL of mitochondrial Buffer A. It was then centrifuged at 9800 rpm for 10 min at 4℃. The pellet thus obtained was again dissolved in the same mitochondrial Buffer A. For 500 heads, 125 µL of Buffer A was used for dissolving the mitochondrial protein, and quantification was done using NanoDrop 2000 (Thermo Scientific).

5.6.5.2 Quantification/estimation of mitochondrial protein

Quantification of protein was done using the Bradford method. BSA stock was prepared by dissolving 2 mg/mL PBS for normal protein quantification. A working concentration of 0.2 µg/µL was prepared by dissolving the 100 µL of stock solution in 900 µL PBS. For mitochondrial protein quantification, BSA stock was prepared in extraction Buffer A for the mitochondrial fraction and Buffer B for the cytosolic fraction. A working concentration of 0.2 µg/µL was prepared by dissolving the 100 µL of stock solution in 900 µL PBS. A serial dilution of 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 µg/mL standard BSA solution was prepared by diluting the BSA working solution in PBS and 500 µL Bradford dye (Table 1). After 5 min of incubation at room temperature (RT), the absorbance was read at 595 nm using NanoDrop 2000C (Thermo Scientific). Mitochondrial samples were quantified using the same standard graph.

BSA (µg/mL)	Working solution (µL)	PBS (µL)	Bradford (µL)
0.5	2.5	497.5	500
1	5	495	500
1.5	7.5	492.5	500
2	10	490	500
2.5	12.5	487.5	500
3	15	485	500
3.5	17.5	482.5	500

Table 5.1: Preparation of serial dilutions using standard BSA.

5.7 Biochemical Assays

5.7.1 Assessment of Lipid peroxidation (LP) levels

Lipid peroxidation was assessed by estimating MDA by the thiobarbituric acid (TBA) reaction method. MDA, a product of lipid peroxidation, reacts with TBA to form a 1:2 adduct (MDA-TBA2) measured spectrophotometrically at 532nm. 200µgP test samples were allowed to react in 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% TBA, 0.2 ml of 8% SDS (w/v), and vortex, followed by 100°C incubation in a water bath for 30 minutes. The mixture was cool down to room temperature, and optical density absorbance was measured at 532nm using NanoDrop 2000 (Thermo Scientific) (Phom, 2018; Ohakawa et al., 1979)

5.7.2 Assessment of Superoxide dismutase (SOD) enzyme activity

Superoxide dismutase activity was measured indirectly by monitoring the inhibition of quercetin auto-oxidation. Quercetin is oxidized by O2- produced by TEMED, which is effectively inhibited by SOD in the sample. The rate of inhibition of Quercetin oxidation is monitored at 406nm. 100µgP of the test sample was mixed with phosphate buffer

(0.016 M, pH 7.8, containing TEMED- 0.8mM and EDTA-0.08 mM) followed by the addition of quercetin (1.5mg/10 ml DiMethyl Formamide). The rate of its auto-oxidation was monitored at 406 nm for 1 minute with 10-second intervals using NanoDrop 2000 (Thermo Scientific). 50% inhibition of quercetin oxidation in the test sample is defined as one unit of the enzyme and activity expressed as units/mg protein (Phom, 2018; Kostyuk and Potapovich, 1989).

5.7.3 Assessment of Complex I-III (NADH and Cytochrome C reductase) enzyme activity

Mitochondrial NADH-Cytochrome C reductase (complex I-III) was assayed using the modified protocol of Navarro et al. (2004) and Ayajuddin et al. (2022). To determine the complex I-III activity, 60 µg of the isolated mitochondria were mixed with phosphate buffer (0.1 M, pH 7.4). Then, NADH (0.2 mM) and KCN (1 mM) were added and mixed for 10 sec. The reaction was initiated by adding cytochrome C (0.1 mM), and the absorbance was recorded at 550 mm for 5 min. The total reaction volume was 1 mL. The activity was expressed as nmol cytochrome C reduced/min/mg protein (MEC = 19.6 $mM^{-1}cm^{-1}$)

5.7.4 Assessment of Complex II-IV (Succinate dehydrogenase (SDH)) enzyme activity

The succinate dehydrogenase activity was determined by reducing p-iodonitrotetrazolium violet (INT) (Girish and Muralidhara, 2012; Pennington, 1961). To determine the succinate dehydrogenase activity (Complex I-III), 60 µg of the isolated mitochondria protein were mixed with INT (p-iodonitrotetrazolium violet) reagent and incubated at 37℃ for 25 to 30 minutes. The reaction was put to an end by adding 10% TCA. After

that, ethyl acetate was added, and the upper colour phase was taken and measured at 490 nm. The SDH activity was expressed as optical density (OD)/mg protein.

5.8 Statistical analysis

Graphs were created using Graph Pad Prism 5.0 software (Graph Pad Inc., San Diego, CA, USA). Statistical analysis was completed, and results were expressed as the mean ± standard error of the mean (SEM). A Two-way ANOVA followed by Bonferroni post-test and one-way ANOVA followed by Newman-Keuls multiple comparison test was carried out to draw significance for DAergic neuronal number and TH-protein synthesis quantification. P-values < 0.05 were regarded as significant.

5.9 Results

5.9.1 *Leea asiatica* (LA) mitigates PQ-mediated Lipid peroxidation (LP) levels in the TP-PD brain

LP, a potential biomarker of PD, and other numerous NDDs could be facilitated by metabolic failure in antioxidant mechanisms, leading to the activation of phospholipases and promotion of signalling cascades that tenet neuronal cell death (Angelova et al., 2020; Phom, 2018; Hosamani and Muralidhara, 2010,2009). In the present study, the TP-PD brain LP levels were upregulated by 60% (***p<0.001) (**Figure 5.1**). LA significantly attenuated the LP upregulation (***p<0.001) **(Figure 5.1)** when co-administered in the TP-PD brain. The observations suggest that the LA has effective free radical sequestering properties. LA *per se* studies confer no toxic effect on the TP-healthy brain **(Figure 5.1)**. Further investigation revealed that MDA levels were upregulated with natural aging (***p<0.001), higher in TP compared to HP (**Figure 5.5A**).

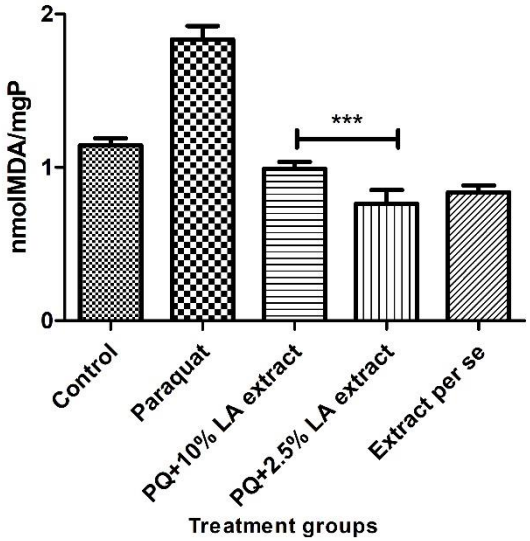


Figure 5.1. Measurement of Malondialdehyde (MDA) levels in the TP-PD brain. Feeding the *Drosophila* TP with PQ (10mM) upregulates the MDA levels by 60%. Upon co-feeding with *Leea asiatica* (LA), enhanced levels of MDA were significantly reduced in the TP-PD brain, suggesting the effective modulatory action of LA against PQ-mediated LP in the fly model of PD. (CTR- Control; Td- Treated with 10mM Paraquat; R10% - 10mM Paraquat+10% LA Extract; R2.5% - 10mM Paraquat+2.5% LA Extract; *Perse* 7.5% - Sucrose + *7.5%* LA extract alone). The significance was drawn using One-way ANOVA followed by the Newman-Keuls Multiple Comparison Test (***p<0.001; **p<0.01; *p<0.05; NS-Not Significant) when compared to the PQ (10mM) treated group.

5.9.2 *Leea asiatica* (LA) abates the PQ-mediated superoxide dismutase (SOD) levels in the TP-PD brain

SOD, a redox equilibrium regulator, serves as a first line of defense against ROS and also performs detoxification as the primary physiological function in the cell. SOD is expressed throughout the central nervous system (CNS), both intracellularly and extracellularly, in the neurons and glial cells (Chidambaram et al., 2024; Phom, 2018; Hosamani and Muralidhara, 2012, 2009). In the present study, the TP-PD brain SOD

levels were upregulated by 54% (***p<0.001) **(Figure 5.2)**. LA significantly reduced the SOD upregulation (***p<0.001) **(Figure 5.2)** when co-administered in the TP-PD brain. The observations suggest that the LA has effective free radical sequestering properties by modulating the OS. LA *per se* studies confer no toxic effect on the TP-healthy brain **(Figure 5.2)**. The investigation also revealed that SOD levels/activity was upregulated with aging (***p<0.001) higher in TP compared to HP (**Figure 5.5B**).

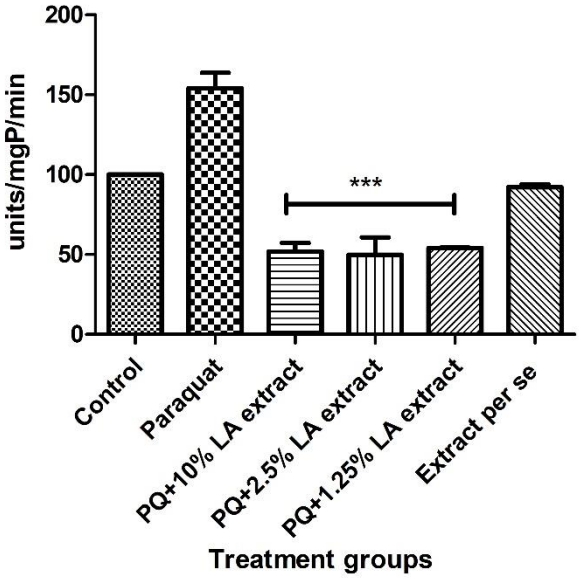


Figure 5.2: Measurement of SOD levels in the TP-PD brain. Feeding the *Drosophila* during TP with PQ (10mM) upregulates the SOD enzyme activity by 54%. Upon co-feeding with *Leea asiatica* (LA), the enhanced/increased activity of SOD was significantly diminished in the TP-PD brain, suggesting that LA has potent anti-oxidative activity against PQ-induced SOD in the fly model of PD. (CTR- Control; Td- Treated with 10mM Paraquat; R10% - 10mM Paraquat+10% LA Extract; R2.5% - 10mM Paraquat+2.5% LA Extract; R1.25% - 10mM Paraquat+1.25% LA Extract; *Perse* 7.5% - Sucrose + *7.5%* LA extract alone). The significance was drawn using One-way ANOVA followed by the Newman-Keuls Multiple Comparison Test (***p<0.001; **p<0.01; *p<0.05; NS-Not Significant) when compared to the PQ (10mM) treated group.

5.9.3 *Leea asiatica* (LA) rescues the inhibited PQ-mediated Complex I-III enzyme activity in the TP-PD brain

Mitochondrial complex I-III/ NADH-Cytochrome C reductase enzyme activity is significantly reduced in the SN, skeletal muscles, and platelets of PD patients (Bose and Beal, 2016). The present study inhibited the TP-PD brain NADH-Cytochrome C reductase activity by 70% (***p<0.001) **(Figure 5.3)**. LA significantly upregulates the inhibited NADH-Cytochrome C reductase activity (***p<0.001) **(Figure 5.3)** when co-administered in the TP-PD brain. The observations suggest that the LA has effective free radical sequestering properties by modulating mitochondrial dysfunction. LA *per se* studies exhibited no detrimental/inhibition effects on the TP healthy brain **(Figure 5.3)**. It was also observed that complex-I-III activities were reduced with aging in the TP brain by 40% (**p<0.01) compared to HP (**Figure 5.5C**).

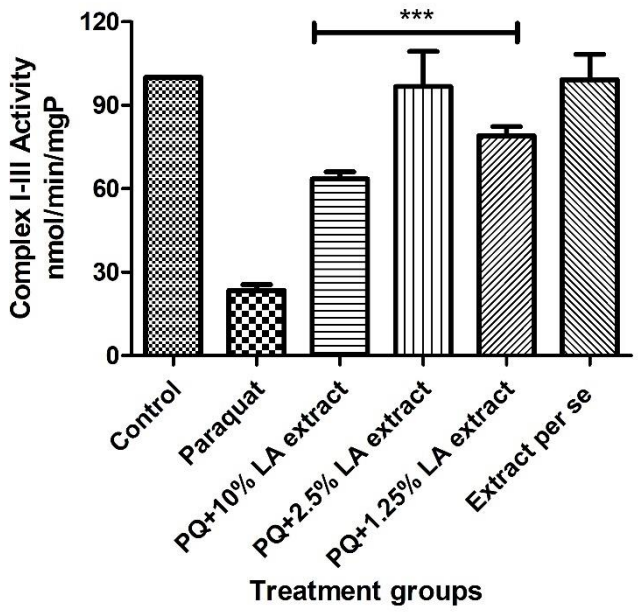


Figure 5.3: The mitochondrial complex I-III activity was assayed to explore the inhibition of NADH- Cytochrome C reductase in PQ-treated flies. Feeding the

Drosophila during the TP with PQ (10mM) inhibits the NADH- Cytochrome C reductase enzyme activity by 70%. This reduction in NADH- Cytochrome C reductase activity indicates the impairment of complex I-III of the electron transport chain. Upon co-feeding with *Leea asiatica* (LA), the NADH- Cytochrome C reductase enzyme activity was significantly upregulated in TP-PD brain (CTR- Control; Td- Treated with 10mM Paraquat; R10% - 10mM Paraquat+10% LA Extract; R2.5% - 10mM Paraquat+2.5% LA Extract; R1.25% - 10mM Paraquat+1.25% LA Extract; *Perse* 7.5% - Sucrose + *7.5%* LA extract alone). The significance was drawn using One-way ANOVA followed by the Newman-Keuls Multiple Comparison Test (***p<0.001; **p<0.01; *p<0.05; NS-Not Significant) when compared to the PQ (10mM) treated group.

5.9.4 *Leea asiatica* (LA) rescues the inhibited PQ-mediated Succinate dehydrogenase (SDH) enzyme activity in TP-PD brain

SDH connects the tricarboxylic cycle to the electron transport chain. Therefore, dysfunction of the SDH could impair mitochondrial activity and ATP generation, exceed lipid synthesis, and induction of excitotoxicity, leading to NDD like PD, AD, and HD (Jodeiri Farshbaf et al., 2016b; Schwall et al., 2012). The present study inhibited the TP-PD brain succinate dehydrogenase activity by 54% (***p<0.001) **(Figure 5.4)**. LA significantly upregulates the inhibited succinate dehydrogenase activity (***p<0.001; **p<0.01) **(Figure 5.4)** when co-administered in the TP-PD brain. The observations suggest that the LA has effective free radical sequestering properties by modulating mitochondrial dysfunction. LA *per se* studies showed inhibition in the TP control brain-specific succinate dehydrogenase activity (**p<0.01) **(Figure 5.4)**. Further investigation

revealed that complex-II-IV activities were downregulated with aging (***p<0.001), lower in TP compared to HP (**Figure 5.5D**).

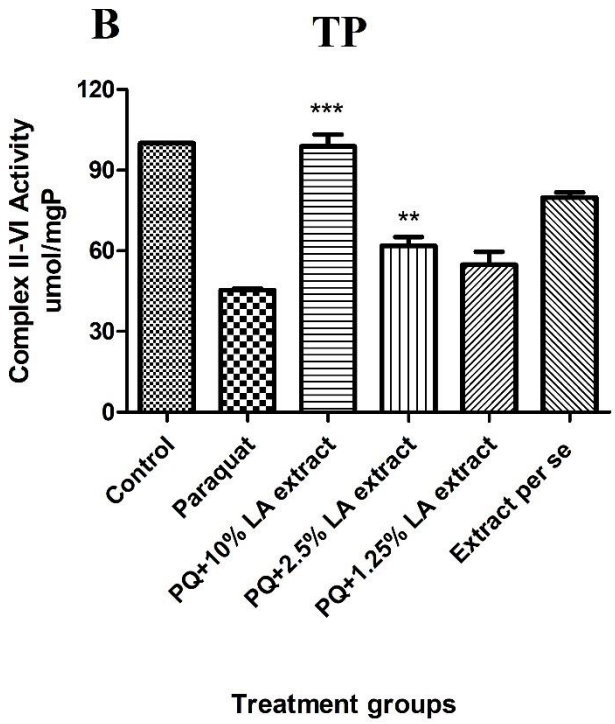


Figure 5.4: The mitochondrial complex II-IV activity was assayed to explore the inhibition of SDH activity in PQ-treated flies. Feeding the *Drosophila* during the TP with PQ (10mM) inhibits the succinate dehydrogenase enzyme activity by 54%. This reduction in SDH activity indicates the impairment of complex II-IV of the electron transport chain. Upon co-feeding with *Leea asiatica* (LA), the SDH enzyme activity was significantly upregulated in TP-PD brain (CTR- Control; Td- Treated with 10mM Paraquat; R10% - 10mM Paraquat+10% LA Extract; R2.5% - 10mM Paraquat+2.5% LA Extract; R1.25% - 10mM Paraquat+1.25% LA Extract; *Perse* 7.5% - Sucrose + *7.5%* LA extract alone). The significance was drawn using One-way ANOVA followed by the Newman-Keuls

Multiple Comparison Test (***p<0.001; **p<0.01; *p<0.05; NS-Not Significant) when compared to the PQ (10mM) treated group.

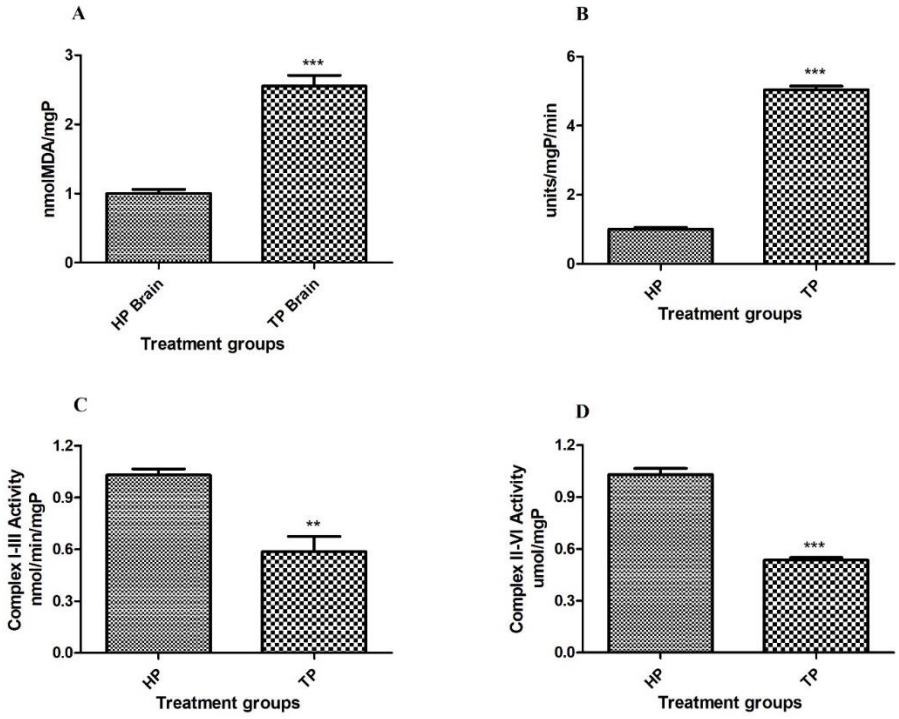


Figure 5.5: Brain-specific aging-associated changes in the level of enzymatic (SOD), non-enzymatic (LP), and mitochondria complexes (I-III, II-IV) markers of OS involved in PD. Natural aging in the brain alters/elevates the MDA (A) and SOD levels (B). However, with aging, Complex-I-III and Complex-II-IV activities were inhibited in the TP brain (C&D). Significance was drawn by analyzing the data of a minimum of three replicates with an unpaired t-test. (*p<0.05; **p<0.01; ***p<0.001; NS: Not significant)

5.10 Discussion

Cellular aging and the onset of age-related diseases are both underpinned by OS (Yang et al., 2024; Kurtishi et al., 2019). Cell senescence increases as a result of stress during aging. Multiple processes, such as telomere shortening, progressive DNA damage, altered metabolic activity, and elevated ROS generation from the altered function of proteins and lipids, are responsible for mediating this occurrence/processes (Luceri et al., 2018; Kuilman et al., 2010). In addition to damaging DNA and impairing mitochondrial function, OS activates the p53 gene, which codes for a protein that regulates the cell cycle and, consequently, activates prooxidant genes. In response, defense mechanisms in the cell become excessively active, such as upregulated expression of antioxidant enzymes viz., SOD, CAT, GST, and reduced GSH (Ren and Zhang, 2017; Zarse et al., 2012), indicating a complex relationship between OS and neurodegeneration. Studies performed on the post-mortem brains of PD patients have implicated the role of OS-mediated pathogenesis linked to the death of DAergic neurons in PD (Zeevalk et al., 2008; Nakabeppu et al., 2007; Bosco et al., 2006). Investigation of two synthetic SOD/CAT mimetics protecting against PQ-induced DAergic cell death in both the rat DAergic cell line and primary mesencephalic cultures *in vitro* and adult mice *in vivo* has also been reported (Peng et al., 2005). Post-mortem studies have shown an increased SOD activity in PD brains (de Farias et al., 2016), where SOD activity was significantly associated with the late PD stage. In the present study, the PQ mediated-TP-PD brain SOD levels were upregulated by 54% (***p<0.001) **(Figure 5.2)**, which is significantly reduced (***p<0.001) when co-administered with LA in the TP-PD brain. The observations suggest that the LA has free radical sequestering properties which modulate the OS. LA *per se* did not influence the SOD levels when compared with the control brain **(Figure**

5.2). The investigation also revealed that SOD activity was higher in TP (compared to HP) suggesting increased SOD activity with age (***p<0.001) (**Figure 5.5B**).

Similarly, studies on post-mortem SNpc of PD brains have shown elevated LP compared with other brain regions and with control tissue (Dexter et al., 1989). MDA levels are also significantly higher in PD patients than in controls, suggesting that high plasma lipid peroxidation rates might contribute as a risk factor for PD (Sanyal et al., 2009). The current study demonstrated that PQ mediated-TP-PD brain LP levels were upregulated by 60% (***p<0.001) **(Figure 5.1)** when compared with TP-healthy brains. LA significantly attenuated the LP upregulation (***p<0.001) when co-administered in the TP-PD brain. These observations suggest that the LA has effective free radical sequestering properties. Meanwhile, LA *per se* studies have demonstrated no adverse effect compared to the control TP brain **(Figure 5.1)**. Further investigation revealed that MDA levels were upregulated with aging (***p<0.001), higher in TP compared to HP (**Figure 5.5A**).

It has been demonstrated that PQ mediates mitochondrial dysfunction by inhibiting ETC complex(s), leading to insufficient generation of energy/ATP and subsequent generation of ROS, resulting in the loss of DAergic neurons (Chen et al., 2019; Shrivastav et al., 2018). The mitochondrial dysfunction was reported to elevate the deleted mitochondrial DNA and reduce metabolic activity and protein level of NADH dehydrogenase in the SNpc and frontal cortex of PD patients (Parker et al., 2008; Bender et al., 2006; Schapira et al., 1990). Meanwhile, the reduced level of complex-I activity (65%) was demonstrated in the SN neurons of PD patients. Studies have also elucidated the reduced complex-I activity throughout the brain of PD patients and the misassembly of complex-I in the ETC (Flønes et al., 2018; Keeney et al., 2006). Since mitochondrial complex-I is the ETC's

first enzyme, it is considered the critical site of ROS generation. In PQ-mediated mitochondrial dysfunction, complex I transfers two electrons from NADPH to ubiquinone, oxidating NADPH to $NADP^+$. Upon entry into the neuron, PQ undergoes redox cycling, where it disrupts the oxidation of NADPH by accepting electrons to form PQ mono-cation radical ($PQ^{\bullet+}$) through NADPH-cytochrome P450 reductase, ultimately inhibiting mitochondrial complex I activity (Fussell et al., 2011; Fukushima et al., 1993). It was reported that the level of ATP was reduced in the fly upon PQ exposure (20mM for 48 hours) (Srivastav et al., 2018). The present study exhibited, inhibited TP-PD brain NADH-Cytochrome C reductase activity by 70% (***p<0.001). LA significantly upregulates the inhibited NADH- Cytochrome C reductase activity (***p<0.001) when co-administered in the TP-PD brain. The observations suggest that the LA has effective free radical sequestering properties by modulating mitochondrial dysfunction. LA *per se* studies exhibited no detrimental effects on the TP healthy brain-specific NADH-Cytochrome C reductase activity. It was also observed that complex-I-III activities were reduced with aging in the TP brain by 40% (**p<0.01) compared to HP (**Figure 5.5C**).

Complex II/ SDH or succinate: ubiquinone oxidoreductase (SQR), is encoded by nuclear DNA (Hattori et al., 1991). The inhibition of the mitochondrial complex II can accelerate neurodegeneration through various mechanisms such as lipid droplets (LD) accumulation, ROS generation, ATP depletion, and excitotoxicity process. Most complexes are encoded by mtDNA, while complex II is the only part of the ETC encoded by the nuclear genome. Mutation or SDH inhibition leads to the accumulation of malate and fumarate in the mitochondria (Van Vranken et al., 2014). For ATP generation, a low concentration of fumarate is critical (Rottenberg and Gutman, 1977), so a high level of fumarate in the matrix can decrease ATP production. SDH influences the Krebs cycle and

contributes to ROS generation (Ralph et al., 2011). Defects in complex II lead to neuronal injuries, and in many NDDs, abnormalities of SDH activity have been reported. In post-mortem studies, varying degrees of complex II deficiency (65%) have been found in individual SN neurons from PD patients (Grunewald et al., 2016). The present study demonstrated that SDH activity was inhibited to 54% in the TP-PD brain (compared to control) (***p<0.001) and LA significantly rescues its SDH activity (***p<0.001; **p<0.01) (compared to that of the TP-PD brain). The observations suggest that the LA has effective free radical sequestering properties by modulating mitochondrial dysfunction. LA *per se* studies demonstrated inhibition in the TP control brain-specific SDH activity (**p<0.01). Further investigation revealed that complex-II-IV activities were downregulated with aging (***p<0.001), lower in TP compared to HP (**Figure 5.5D**). Most previous studies evidence could candidate SDH as an effective target for therapeutic interventions in PD and aging. SDH overexpression or manipulation of its activity could control lipid overloading in NDDs. Moreover, by increasing the protein level of SDH, excitotoxicity and NMDA-dependent signaling can be controlled. Identifying and characterizing the SDH protein provides critical new insights into the mechanisms by which SDH protects neurons from lipid overloading and ROS generating, and it can be a therapeutic candidate to ameliorate PD progression. With regard to the present evidence, the sequestration of OS during the TP-PD could be one of the potential underlying mechanisms for the observed DAergic neuroprotective efficacy of the LA.

5.11 Conclusion

Studies on several PD models and post-mortem PD brains have revealed that OS is the underpinning mechanism causing the death of DAergic neurons. The present investigation revealed that PQ induces OS in TP-PD by elevating the MDA and SOD

levels and inhibiting the complex-I-III and complex-II-IV enzyme activity. The present study also reveals that brain OS levels enhance with advancing age. Feeding LA to the TP-PD flies sequesters the OS that was evident from the reduced levels of MDA and SOD. Similarly, inhibited mitochondrial complexes in the PD brain were rescued/restored upon feeding with LA. The present results suggest that the sequestration of OS during the TP-PD is one of the possible underlying mechanisms for the observed DAergic neuroprotective efficacy of the LA.

Summary

Summary

Parkinson's disease (PD) is a multifactorial disease characterized by progressive and selective degeneration of DAergic neurons (Meissner et al., 2011; Miranda et al., 2022). PD affects more than 1% of the population above 65 years of age and is predicted to double by 2030 (Hoyert et al., 2012; Aarsland et al., 2021; WHO, 2023). There is no cure for PD except the pharmacological interventions to alleviate the motor symptoms via reinstating/replenishing striatal DA tone using dopa mimetic drugs, such as DA precursor *viz.*, L-DOPA, DA agonists *viz.*, apomorphine, bromocriptine, and inhibitors of MAO-B *viz.*, selegiline, rasagiline, and COMT *viz.*, entacapone, tolcapone, and decarboxylase enzymes *viz.*, carbidopa, benserazide. However, L-DOPA brought a revolution in the field of PD management and established as the gold standard treatment by significantly improving PD symptoms, quality of life, and normalizing life expectancy, even countering late-onset PD symptoms (Tambasco et al., 2018; Nakmode et al., 2023). Nevertheless, the administration of L-DOPA has limitations due to its half-life in the blood (about 90-120 minutes), which causes fluctuations in blood levels that result in changes in clinical symptoms in the advanced stage, manifesting as the wearing-off phenomenon, which is the re-emergence of PD symptoms as the effect of L-DOPA diminishes near the end of the dose interval, usually 3 to 4 hours after the dose. Even long-term treatment may lead to considerable toxicity by inducing motor abnormalities and spontaneous movements, known as L-DOPA-induced dyskinesia (LID) (Rascol et al., 2003). Therefore, it is suggested that disease-modifying therapeutics, including small molecules/nutraceuticals, plant products/extracts, and nano-formulations, repurposing of the drugs, are the next "cutting edge tools" that might change the etiopathogenesis of PD at its core (Nakmode et

al., 2023). Plant products/extracts/nutraceuticals have become effective disease-modifying agents because of their affordability, safety, and multi-target mode of action, and present a promising avenue for alternative therapy for PD (Lama et al., 2020).

As PD is a late-onset disease, it is essential to understand the disease progression and therapeutic effectiveness at a later stage (the age equivalent to disease onset in humans) of model organisms. Though aging is the leading risk factor for developing idiopathic PD; most studies exploring the modulatory effect of prospective or potential therapeutic compounds are restricted to young animal models. Consequently, studies in young animal model(s) may not accurately reflect human PD, limiting translational outcomes. Here lies the importance of the ALSS *Drosophila* model of PD (Phom et al., 2014; 2018; Ayajuddin et al., 2022).

Review of the literature reveals that *Leea asiatica* (LA) has anthelmintic (Sen et al., 2011, 2012) and antioxidant-related nephroprotective (Sen et al., 2013), hepatoprotective activities (Sen et al., 2014), and anticancer activity (Ali et al., 2021).

Thus, in Chapter 2, I described an optimized procedure for generating aqueous extract from the leaves of LA. By employing the ALSS fly PD model developed in our laboratory, I decided to understand the neuroprotective efficacy of LA extract during the *Drosophila* adult transition phase (TP corresponds to the adult young life stage of *Drosophila* with 10% mortality) (Chapter 3). Results reveal that PQ induces mobility defects in the TP-PD flies and LA intervention rescues the same under the pre- and co-treatment regime. These results suggest LA aqueous extract has the potency to probe further for critical PD phenotypes in the ALSS *Drosophila* PD model.

Further, I tried to understand LA's DAergic neuroprotective efficacy in the adult TP *Drosophila* model of PD. Anti-TH immunostaining of the *Drosophila* whole brain indicates that PQ does not cause a loss in the number of DAergic neurons but diminishes TH protein synthesis levels (Levels of TH protein synthesis in DAergic neurons is directly proportional to fluorescence intensity (FI) of secondary antibodies which targets the primary antibody anti-tyrosine hydroxylase (anti-TH)) in the TP-PD brain. LA's intervention rescues "DAergic neuronal dysfunction" by replenishing reduced tyrosine hydroxylase (TH) protein synthesis levels in the TP-PD brain. These findings suggest that LA exhibits neuroprotective efficacy in the TP-PD brain.

Subsequently, in Chapter 4, I made an effort to understand how "neuronal dysfunction" affects the DA metabolism in the fly brain as DA catabolism plays a clear role in PD. The result reveals that PQ diminishes brain DA levels and induces alteration in its metabolites (DOPAC and HVA) levels leading to elevated DA turnover in the TP-PD brain. However, LA intervention improved the DA and its metabolites (DOPAC, HVA) levels leading to reduced DA turnover in the TP of the PD flies. These results illustrate that one of the possible mechanisms of DA replenishment is through the upregulation of TH. Overall insight implies that the neuroprotective efficacy of LA extract is possible through modulation of DA catabolism, where enhanced DA breakdown to DOPAC and HVA in the PD brain is inhibited/rescued. This may prevent endogenous neurotoxicity in late-onset PD.

The genesis and course of numerous human neurodegenerative diseases (NDDs) are greatly influenced by oxidative stress (OS) driven changes of enzymes and structural proteins. The accumulation of free radicals (ROS) and consequent neurodegeneration in

specific brain regions have been proposed as the underlying factors in NDDs such as PD (Jellinger, 2010). In the brain of PD patients, DA auto-oxidation by MAO produces ROS and DA quinones (Zucca et al., 2014; Segura-Aguilar et al., 2014; Ayajuddin et al., 2022). DA quinones have been shown to cause inactivation of the DAT and TH enzyme, as well as alterations of the brain mitochondria, and failure in Complex I and II activity (Blesa et al., 2015; Houldsworth, 2024). Reports also demonstrated that PD patients exhibit reduced complex I and II activity in the SNpc, which leads to an excessive generation of ROS and the degeneration of DAergic neurons (Hauser and Hastings, 2013). Furthermore, higher levels of MDA have been reported in the SNpc of PD patients (Dexter et al., 1989). Similarly, PD brain also exhibited an increased SOD activity in the SN and basal nucleus of PD patients (Marttila et al., 1998). Consequently, in the current study (Chapter 5), PQ induces elevated LP, and SOD levels and inhibited complex-I-III and II-IV enzyme activity leading to enhanced OS in the TP-PD brain. Results also indicate that brain OS levels enhance with advancing age. Feeding LA to the TP-PD flies sequesters the OS that was evident from the reduced levels of MDA and SOD. Similarly, inhibited mitochondrial complexes in the PD brain were rescued upon feeding with LA. The present insights suggest that the sequestration of OS in the TP-PD is one of the possible underlying mechanisms for the observed DAergic neuroprotective efficacy of the LA.

The present study illustrates that LA leaf aqueous extract rescues mobility defects, and DAergic neuronal dysfunction, and corrects altered brain DA metabolism. Collectively, these results suggest the neuroprotective efficacy of the LA. This study provides an opportunity to explore the underlying genetic and molecular mechanisms involved

through further studies. The findings derived from this study will aid in the development of more effective therapeutic strategies for late-onset disorders such as PD.

Supplementary Information

ANNEXURE I

Chapter 3: Understanding *Leea asiatica's* Dopaminergic (DAergic) Neuroprotective Efficacy in the Adult Transition Stage *Drosophila* Model of Parkinson's Disease

I. Figure: 3.18G (Cluster-wise DAergic neuronal number in the whole fly brain of control and experimental groups)

TP																									
Neuronal cluster	Control					Paraquat				PQ+2.5% LA extract					PQ+10% LA extract					Extract per se					
PAL	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
PPL1	27	28	27	25	25	25	26	26	27	25	28	28	25	24	25	24	26	27	27	28	27	28	28	27	27
PPL2	15	15	15	14	15	14	14	15	14	14	15	15	14	16	15	15	16	16	15	15	16	16	17	16	16
PPM1/2	16	17	16	16	17	16	15	15	15	15	17	16	16	15	14	15	15	17	15	16	17	17	18	17	16
PPM3	11	12	11	12	12	11	10	12	12	11	10	11	12	12	10	10	11	11	11	11	12	12	12	12	12
VUM	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

II. Figure: 3.18H (Total DAergic neuronal number in the whole fly brain of control and experimental groups)

TP				
Control	Paraquat	PQ+ 2.5% LA extract	PQ+10% LA extract	Extract per se
82	79	83	77	84
85	78	83	81	84
82	81	80	84	85
80	81	80	81	84
82	78	77	83	83

III. Figure 3.18I (Cluster-wise FI of DAergic neurons in the whole fly brain of control and experimental groups)

TP															
Neuronal cluster	Control			Paraquat		PQ+2.5% LA extract			PQ+10% LA extract			Extract per se			
PAL	100	100	100	65.1744	67.9049	58.2266	98.0739	124.52	98.2091	111.311	111.732	86.4622	80.3316	127.727	77.2194
PPL1	100	100	100	42.192	73.9845	63.6986	98.6503	94.4522	81.4559	97.1957	113.763	94.7471	83.0007	113.745	99.6939
PPL2	100	100	100	65.581	61.6002	64.9243	176.65	110.456	98.33	108.419	93.4982	115.153	149.547	135.158	114.684
PPM1/2	100	100	100	62.038	77.4097	66.6651	88.5714	114.707	102.88	93.4297	85.7606	99.835	73.5362	113.628	90.5556
PPM3	100	100	100	63.5449	61.6235	62.7514	108.22	96.4736	109.858	91.0086	78.2725	101.706	77.5827	119.42	89.8441

IV. Figure 3.18J (Total FI of DAergic neurons in the whole fly brain of control and experimental groups)

TP				
Control	Paraquat	PQ+2.5% LA extract	PQ+10% LA extract	Extract per se
100	56.45505	106.4924	98.59185	87.20287
100	70.41418	106.5957	98.72678	119.9134
100	63.57173	95.93482	98.79131	94.81139

V. Figure 3.19: Brain-specific aging-associated changes in the DAergic neuronal numbers and relative changes in TH protein synthesis levels.

3.19 A.

	HP					TP				
PAL	10	10	10	10	10	10	10	10	10	10
PPL1	28	27	28	27	25	27	28	27	25	25
PPL2	17	16	16	16	16	16	15	15	16	15
PPM1/2	17	18	17	17	18	16	17	16	16	17
PPM3	12	11	12	11	11	11	12	11	12	12
VUM	3	3	3	3	3	3	3	3	3	3

3.19 B.

HP	TP
83	82
84	85
84	82
83	80
84	82

3.19 C.

	HP			TP		
PAL	100	100	100	74.7695	56.3834	48.3839
PPL1	100	100	100	65.9072	67.5522	32.7447
PPL2	100	100	100	46.4547	30.673	27.7147
PPM1/2	100	100	100	97.3156	94.6767	44.4007
PPM3	100	100	100	108.562	90.9187	85.1003

3.19 D.

HP	TP
101	76.9188
99	60.1061
100	41.0766

ANNEXURE II

Chapter 4: *Leea asiatica* Mediated Regulation of Brain Dopamine (DA) Metabolism in the *Drosophila* Model of Parkinson's Disease: Implications to its therapeutic efficacy

I. Figure 4.4A, 4.4B, 4.4C (DA, DOPAC, and HVA amount in the fly brain of control and experimental groups)

	TP					
DA	Control	Paraquat	PQ+10% LA extract	PQ+2.5% LA extract	PQ+1.25% LA extract	Extract per se
	100	51.04	97.79	106.77	86.1	85.26
	100	57.47	97.96	84.72	94.91	108.09
	100	58.62069	89.65517	94.20537	88.50575	78.16092
DOPAC	Control	Paraquat	PQ+10% LA extract	PQ+2.5% LA extract	PQ+1.25% LA extract	Extract per se
	100	72.46	95.24	85.21	79.33	98.07
	100	61.62	94.53	87.1	67.77	103.62
	100	64.61538	95.38462	89.23077	75.38462	90.76923
HVA	Control	Paraquat	PQ+10% LA extract	PQ+2.5% LA extract	PQ+1.25% LA extract	Extract per se
	100	62.48	79.78	101.74	78.99	72.23
	100	77.1	102.59	98.08	81.73	94.42
	100	75.7085	91.49798	101.6194	87.44939	95.1417

II. Figure 4.4D (DA turnover ratio in the fly brain of control and experimental groups)

(DOPAC+HVA)/DA	Control	Paraquat	PQ+10% LA extract	PQ+2.5% LA extract	PQ+1.25% LA extract	Extract per se
	1	1.26	0.84	0.93	0.92	0.9
	1	1.29	1.03	1.13	0.83	0.89
	1	1.25	1.03	1.05	0.96	1.21

ANNEXURE III

Chapter 5: Oxidative Stress-mediated DAergic Neurodegeneration and its Sequestration by *Leea asiatica* (LA) in the *Drosophila* Model of Parkinson's Disease

I. Figure 5.1 (LA mitigates PQ-mediated Lipid peroxidation (MDA) levels in the TP-PD brain)

	TP				
LP(MDA)	Control	Paraquat	PQ+10% LA extract	PQ+2.5% LA extract	Extract per se
	1.0675	1.680982	1.067485	0.607362	0.7607362
	1.2209	1.98773	0.9141104	0.917239	0.9141104
	1.1442	1.834356	0.9907975	0.7623005	0.8374233

II. Figure 5.2 (LA abates the PQ-mediated superoxide dismutase (SOD) levels in the TP-PD brain)

	TP					
SOD	Control	Paraquat	PQ+10% LA extract	PQ+2.5% LA extract	PQ+1.25% LA extract	Extract per se
	100	170.8315	42.68351	30.44179	54.87827	95.18071
	100	137.3333	61.16505	68.93204	53.39806	89.10709
	100	154.0824	51.92428	49.68692	54.13816	92.1439

III. Figure 5.3 (LA rescues the inhibited PQ-mediated Complex I-III enzyme activity in the TP-PD brain)

	TP					
Complex I-III	Control	Paraquat	PQ+10% LA extract	PQ+2.5% LA extract	PQ+1.25% LA extract	Extract per se
	100	19.77401	67.79661	74.85876	73.44633	115.0794
	100	27.11864	59.32203	118.6441	84.74577	83.33334
	100	23.44633	63.55932	96.75141	79.09605	99.20635

IV. Figure 5.4 (LA rescues the inhibited PQ-mediated Succinate dehydrogenase (SDH) enzyme activity in the TP-PD brain)

	TP					
Complex II-IV	Control	Paraquat	PQ+10% LA extract	PQ+2.5% LA extract	PQ+1.25% LA extract	Extract per se
	100	46.08567	94.34466	58.40314	49.99231	77.97263
	100	44.83223	103.277	65.19841	59.65785	81.76938

V. Figure 5.5 (Brain-specific aging-associated changes in the level of enzymatic (SOD), non-enzymatic (LP), and mitochondria complexes (I-III, II-IV) markers of OS involved in PD)

	HP	TP		HP	TP		HP	TP		HP	TP
LP	1	2.401069	**SOD**	1	5.23081	**Complex I-III**	1	0.42857	**Complex II-IV**	1	0.50875
	1.1	2.867816		1.1	4.86592		0.99	0.7284		1.1	0.5606
	0.9	2.381909		0.9	5.02022		1.1	0.60229		0.99	0.53527

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Name & Institutional Address of the Supervisor/Joint Supervisor	Prof. Sarat Chandra Yenisetti Nagaland University
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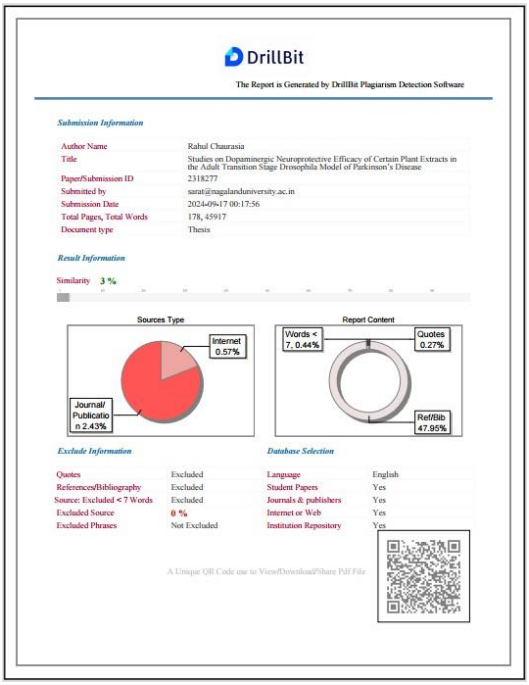

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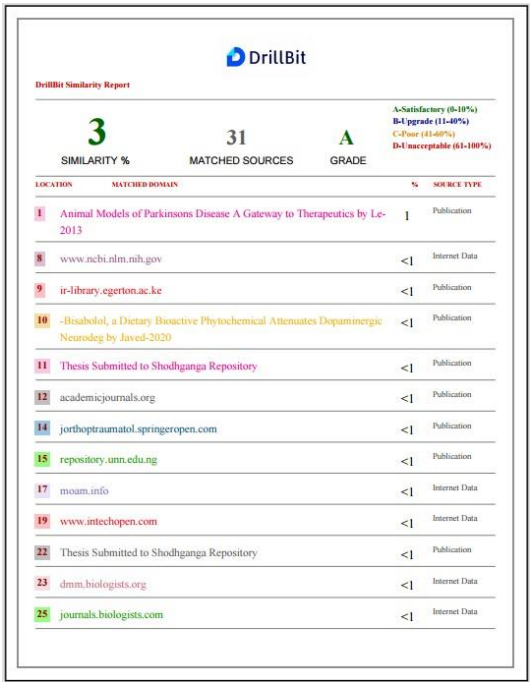

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10	-Bisabolol, a Dietary Bioactive Phytochemical Attenuates Dopaminergic Neurodeg by Javed-2020	<1	Publication
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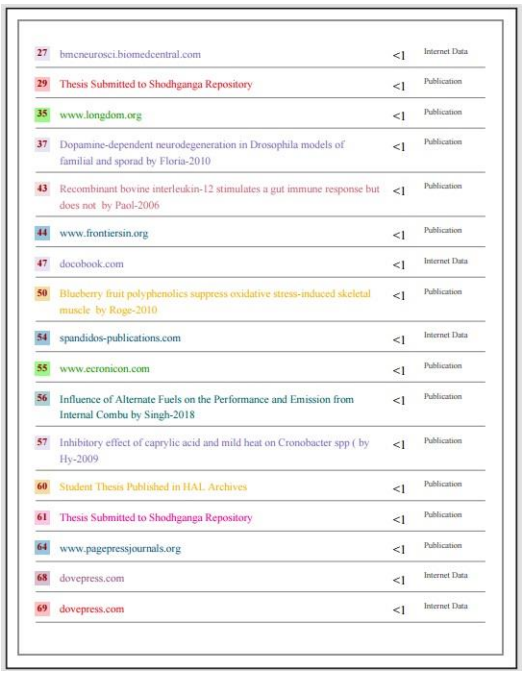

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43	Recombinant bovine interleukin-12 stimulates a gut immune response but does not by Paol-2006	<1	Publication
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CONFERENCES/SEMINARS/SYMPOSIUM/PhD COURSEWORK DOCUMENTS/PUBLICATIONS

International

- **Poster presentation** at the 5th InDRC 2021 (Indian *Drosophila* Research Meeting) hosted by IISER Kolkata and co-organized by the Indian Society for *Drosophila* Researchers from December 13-17, 2021, Kolkata, India, entitled "Fluorescence Microscopy based Sensitive Assay to Decipher Dopaminergic Neuronal Dysfunction in Fly Model of Parkinson's Disease."

- **Poster Presentation** at the 4th International Conference on Nutraceuticals and Chronic Diseases (INCD) hosted by IIT Guwahati from September 23-25, 2019, in Assam, India, entitled "A Novel and Sensitive Method to Screen Dopaminergic Neuroprotective Nutraceutical in *Drosophila* Model of Parkinson's Disease."

National

- **Oral presentation** at the National Conference on Contemporary Excitement in New Biology (CENB) hosted by Nagaland University from October 30-31, 2018, Nagaland, India, entitled "Loss of Neuron or Mere Reduction in Signal Intensity of DA Neurons: A Critical Study to Decipher Neurodegeneration in a Fly Model of Parkinson's Disease."

- **Oral presentation** at the National Seminar on Climate Change and Sustainable
  Development: with Special Focus on North East India hosted by Nagaland University from May 17-18, 2017, Nagaland, India, entitled "A Novel and Sensitive Method to detect Dopaminergic Neurodegeneration in *Drosophila* Model of Parkinson's Disease."

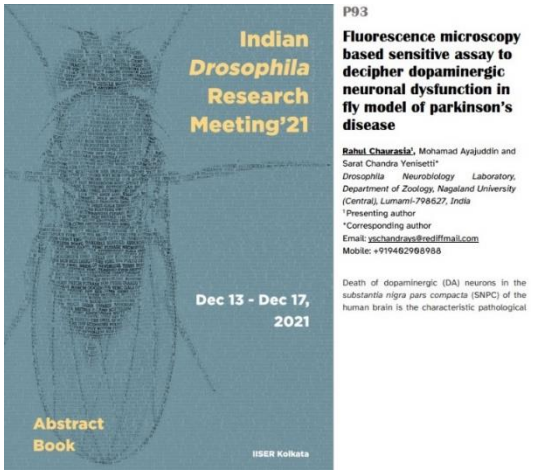
Symposium/Workshops

- **Participated** in an online Symposium on "Recent Advances in Neuro-Techniques" on September 21st, 2021, organized by the Singapore Neuroscience Association (SNA), Department of Anatomy, Singapore.
- **Participated** in the Global Initiative on Academic Network (GIAN) program organized in collaboration with foreign faculty Dr. BB Agarwal, USA, held at the Department of Zoology, Nagaland University from 1-5th October 2019, Nagaland, India.

HONORS/AWARDS

- **Awarded** and availing **Senior Research Fellowship from the Indian Council of Medical Research (ICMR), Government of India, New Delhi, India** from 27th July 2022 to 26th July 2025.
- **Awarded** and availed **DBT U-EXCEL (Unit of Excellence) Senior research fellowship from the Department of Biotechnology, Government of India**, New Delhi, India.
- **Awarded** and availed **DBT U-EXCEL (Unit of Excellence) Junior research fellowship from the Department of Biotechnology, Government of India**, New Delhi, India.
- Awarded and availed a **non-NET fellowship from the University Grant Commission (UGC) through Nagaland University, Nagaland, India**.

CONFERENCES/SEMINARS/SYMPOSIUM



P93

Fluorescence microscopy based sensitive assay to decipher dopaminergic neuronal dysfunction in fly model of parkinson's disease

Rahul Chaurasia[1], Mohamad Ayajuddin and Sarat Chandra Yenisetti*
Drosophila Neurobiology Laboratory, Department of Zoology, Nagaland University (Central), Lumami-798627, India
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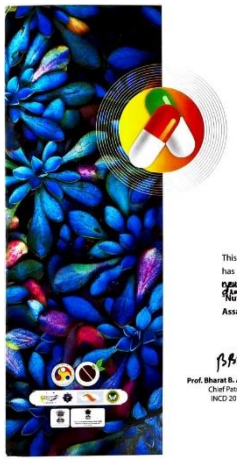
Death of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNPC) of the human brain is the characteristic pathological



Celebrating 25 Glorious Years of IIT GUWAHATI

INTERNATIONAL CONFERENCE ON "NUTRACEUTICALS AND CHRONIC DISEASES" INCD 2019

Certificate

OF APPRECIATION

This is to certify that Rahul Chaurasia
has presented a poster entitled A novel and sensitive method to screen dopaminergic neuroprotective nutraceuticals in drosophila: model of Parkinson's disease
at the Fourth International Conference on 'Nutraceuticals and Chronic Diseases' held at **Indian Institute of Technology Guwahati, Assam, India** from **23rd-25th September, 2019**.

Prof. Bharat B. Aggarwal, Chief Patron, INCD 2019
Prof. Oommen V. Oommen, President, Society for Nutraceuticals and Chronic Diseases
Prof. Ajaikumar B. Kunnumakkara, Organizing Secretary, INCD 2019
Prof. Latha Rangan, Chairperson, INCD 2019

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Contemporary Excitement in New Biology

Certificate of participation

This is to certify that Prof./Dr./Mr./Ms. RAHUL CHAURASIA of NAGALAND UNIVERSITY participated/presented a paper (Key Note Talk/Invited Talk/Presentation Talk/Platform Talk) titled: LOSS OF NEURONS OR MERE REDUCTION IN SIGNAL INTENSITY OF DA NEURONS: A CRITICAL STUDY TO DECIPHER NEURODEGENERATION IN FLY MODEL OF PARKINSON DISEASE in the UGC Sponsored National conference on "Contemporary Excitement in New Biology" organized by the Department of Zoology, Nagaland University, Lumami, Nagaland held during 30 – 31st October 2018.

Prof. M. Indira Devi, Dean, School of Sciences
Prof. Sarat C. Yenisetti, Organising Secretary, CENB-2018
Prof. L. N. Kakati, Convener, CENB-2018
Prof. Bendang Ao, Treasurer, CENB-2018

SINGAPORE NEUROSCIENCE ASSOCIATION

Certificate of Participation

Presented to

RAHUL CHAURASIA

for attending SNA Virtual Symposium

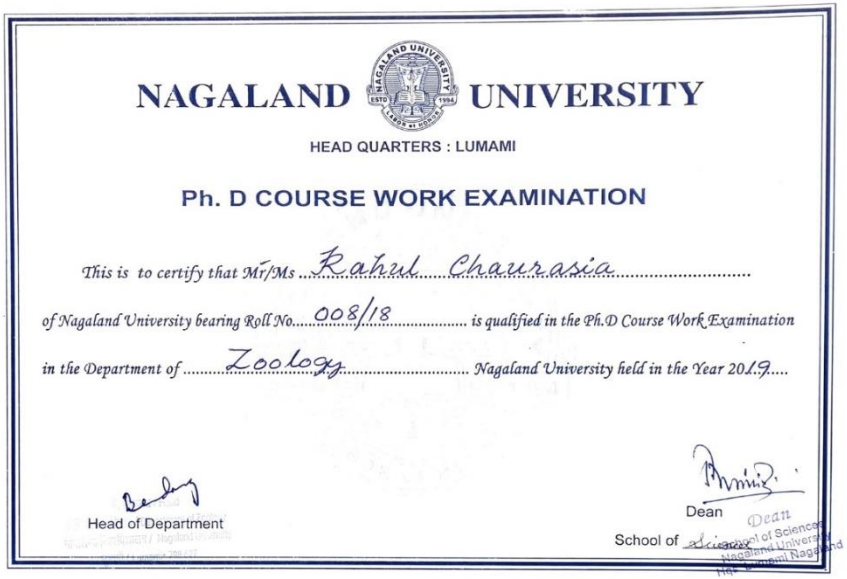
"Recent Advances in Neuro-Techniques"

on September 21st, 2021

Associate Professor S T Dheen
President, Singapore Neuroscience Association
Head, Department of Anatomy
National University of Singapore

NUS National University of Singapore | Department of Anatomy, Yong Loo Lin School of Medicine | Neurobiology Program, Life Sciences Institute | MultiPhoton Microscopy Core Facility

PhD COURSE WORK DOCUMENTS



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Ph. D COURSE WORK EXAMINATION

This is to certify that Mr/Ms Rahul Chaurasia

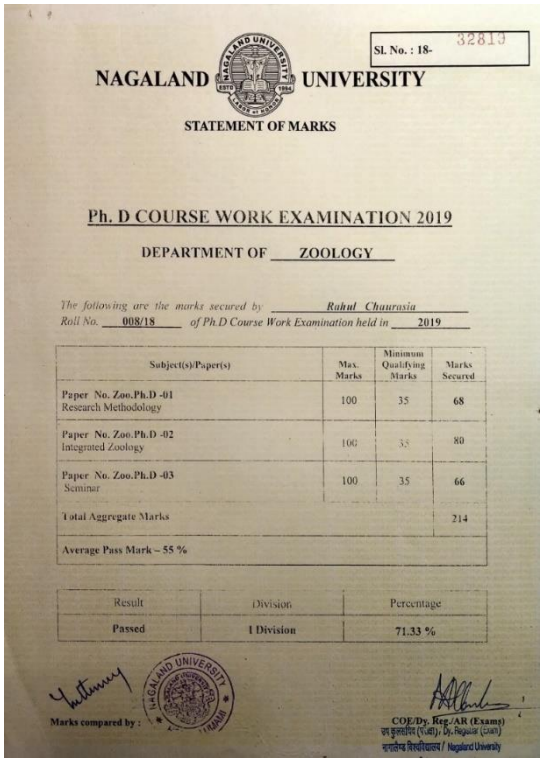
of Nagaland University bearing Roll No. 008/18 is qualified in the Ph.D Course Work Examination

in the Department of Zoology Nagaland University held in the Year 2019.

Head of Department

Dean

School of Science



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STATEMENT OF MARKS

Ph. D COURSE WORK EXAMINATION 2019

DEPARTMENT OF ZOOLOGY

The following are the marks secured by Rahul Chaurasia

Roll No. 008/18 of Ph.D Course Work Examination held in 2019

Subject(s)/Paper(s)	Max. Marks	Minimum Qualifying Marks	Marks Secured
Paper No. Zoo.Ph.D -01 Research Methodology	100	35	68
Paper No. Zoo.Ph.D -02 Integrated Zoology	100	35	80
Paper No. Zoo.Ph.D -03 Seminar	100	35	66
Total Aggregate Marks			214
Average Pass Mark – 55 %			

Result	Division	Percentage
Passed	I Division	71.33 %

Marks compared by :

COE/Dy. Reg./AR (Exams)

PUBLICATIONS

PUBLICATIONS

Research Articles

- **Chaurasia R**, Ayajuddin M, Ratnaparkhi GS, Lingadahalli SS, Yenisetti SC (2024). A Simple Immunofluorescence Method to Characterize Neurodegeneration and Tyrosine Hydroxylase Reduction in Whole Brain of a *Drosophila* Model of Parkinson's Disease. *Bio Protoc.* 14(4): e4937. DOI: 10.21769/BioProtoc.4937.

- Ayajuddin M, **Chaurasia R***, Das A, Modi P, Phom L, Koza Z and Yenisetti S.C. (2023). Fluorescence microscopy-based sensitive method to quantify dopaminergic neurodegeneration in a *Drosophila* model of Parkinson's disease. *Front. Neurosci.* 17:1158858. DOI: 10.3389/fnins.2023.1158858. (*equal contribution)

- Koza Z, Ayajuddin M, Das A, **Chaurasia R***, Phom L and Yenisetti S.C. (2023). Sexual dysfunction precedes motor defects, dopaminergic neuronal degeneration, and impaired dopamine metabolism: Insights from *Drosophila* model of Parkinson's disease. *Front. Neurosci.* 17:1143793. DOI:10.3389/fnins.2023.1143793. (*equal contribution)

- Ayajuddin M, Phom L, Koza Z, Modi P, Das A, **Chaurasia R**, Thepa A, Jamir N, Neikha K and Yenisetti S.C. (2022). Adult Health and Transition Stage-specific Rotenone Mediated *Drosophila* Model of Parkinson's Disease: Impact on Late-onset Neurodegenerative Disease Models. *Front. Mol. Neurosci*. 15:896183. DOI: 10.3389/fnmol.2022.896183.

Research Methodologies

- Das A, **Chaurasia R**, Phom L, Modi P and Yenisetti S.C. (2021). Quantitative Real-Time PCR (qRT-PCR) for Gene Expression Analysis; In Experiments with *Drosophila* for Biology Courses (Eds: Lakhotia SC and Ranganath HA. Indian Academy of Sciences), pp 351-359. ISBN: 978-81-950664-2-1.

- Ayajuddin M, Das A, Phom L, Koza Z, **Chaurasia R**, and Yenisetti S.C. (2021). Quantification of Dopamine and its Metabolites in *Drosophila* Brain Using HPLC; In Experiments with *Drosophila* for Biology Courses (Eds: Lakhotia SC and Ranganath HA. Indian Academy of Sciences), pp 433-440. ISBN: 978-81- 950664-2-1.

Book Chapters

- Ayajuddin M, Das A, Phom L, Modi P, **Chaurasia R**, Koza Z, Thepa A, Jamir N, Singh P.R., Sentinungla, Lal P and Yenisetti S.C. (2018). Parkinson's Disease: Insights from Drosophila model; In Drosophila melanogaster: Model for Recent Advances in Genetics and Therapeutics. [Eds: Perveen FK. Intech, London, UK] pp 157- 192. ISBN: 978-953-51-3854-9. DOI:10.5772/66545.

- Modi P, Mohamad A, Phom L, Koza Z, Das A, **Chaurasia R**, Samadder S, Achumi B, Muralidhara, Pukhrambam RS and Yenisetti SC (2016). Understanding Pathophysiology of Sporadic Parkinson's Disease in *Drosophila* Model: Potential Opportunities and Notable Limitations In Challenges in Parkinson's Disease. [Eds Dorszewska J and Kozubski W. Intech, London, UK] pp 217-244. ISBN: 978-953-51-2464-1. DOI:10.5772/61880.