STUDIES ON DOPAMINERGIC NEUROPROTECTIVE POTENTIAL OF *LEEA ASIATICA* (L.) LEAF EXTRACT IN *DROSOPHILA* MODEL OF PARKINSON'S DISEASE

by

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on Dopaminergic Neuroprotective Potential of *Leea asiatica* (L.) Leaf Extract in *Drosophila* Model of Parkinson's Disease"** is a record of original research work done by **Mr. Kelevikho Neikha.** He is a registered research scholar bearing **Regd. No. PhD/ZOO/00439** 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 the degree of **Doctor of Philosophy in Zoology** under Nagaland University.

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DECLARATION

I, Mr. Kelevikho Neikha, 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 the 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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(This study was conducted at the Drosophila Neurobiology Laboratory (DNBL), Department of Zoology, Nagaland University (Central), Lumami campus, Nagaland)

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ABBREVIATIONS

α-syn: α-synuclein

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

6-OHDA: 6-hydroxydopamine

AADC: aromatic L- amino acid decarboxylase

aaNAT: Arylalkyl amine N-acetyltransferase

AChE: Acetyl-cholinesterase

AD: Alzheimer's Disease

ALS: Amyotrophic lateral sclerosis

anti-TH: anti-tyrosine hydroxylase

AP-1: Activator protein 1

ATP: Adenosine triphosphate

Aux: Auxilin

BBB: Blood-brain-barrier

BDNF: Brain Derived Neurotrophic Factor

BchE: butyryl-cholinesterase

Bcl-2: B-cell CLL/lymphoma 2

CA: Caffeic acid

cAMP: Cyclic adenosine monophosphate

CAT: Catalase

CMG: Curcumin Monoglucoside

CNS: Central nervous system

COMT: Catechol-o-methyltransferase

CRE: Colin red peel

CS: Canton-S

CSF: Cerebrospinal fluid

CU: Curcumin

DA: Dopamine

DAergic: Dopaminergic

DALYs: Disability-adjusted life years

DAT- Dopamine transporter

DHODH: Dihydroorotate dehydrogenase

DOPAC: 3,4-Dihydroxyphenylacetic acid

dUCH: Drosophila ubiquitin carboxyl-terminal hydrolase

EC: Epicatechin

ECD: Electro chemical detector

EGCG: Epigallocatechin-3-gallate

ELAV: Embryonic lethal abnormal vision

ER: Endoplasmic reticulum

ETC: Electron transport chain

FI: Fluorescence intensity

FTD; Frontotemporal dementia

Ga: Gallic acid

GPx: Glutathione peroxidase

GSH: Glutathione

GST: Glutathione s transferase

HD; Huntington's disease

HP: Health Phase

HPLC: High-performance liquid chromatography

HVA: Homovanillic acid

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

JNK: c-Jun N-terminal Kinase

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

LBs: Lewy bodies

LCIG: Levodopa/carbidopa intestinal gel

LID: L-DOPA-induced dyskinesia

LNs: Lewy neurites

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

L-DOPA: Levodopa

LRRK2: Leucine-rich repeat kinase 2

MAO: 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

NDDs: Neurodegenerative diseases

NF-κB: Nuclear factor-κB

NGS: Normal Goat Serum

NIRF: Near-infrared fluorescence imaging

NMDA: N-methyl-D-aspartate

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

OMIM: Online Mendelian Inheritance in Man

OS: Oxidative stress

OXPHOS: Oxidative phosphorylation

PAL: Protocerebral anterior lateral

PAM: Protocerebral anterior medial

PARK1: Parkinson disease 1, autosomal dominant

PBS: Phosphate-buffered Saline

PBST: PBS containing TX-100

PC: Protein carbonyl

PD: Parkinson’s Disease

PE: Plant extract

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

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

“Dedicated to my Parents

Chapter: 1

Review of Literature

1.1. Introduction

1.1.1. Neurodegenerative diseases (NDDs)

Neurodegenerative diseases (NDDs) are irreversible pathologies that are characterized by progressive loss of synapses and neurons, leading to functional decline. The deterioration of circuitry undermines both cognitive function and physical capabilities in patients, resulting in a cascading decline in independence and overall quality of life. (Wilson et al., 2023; Cheslow et al., 2024). NDDs primarily appear in the elderly population and encompass a range of disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), frontotemporal dementia (FTD), and amyotrophic lateral sclerosis (ALS) (Shusharina et al., 2023; Wilson et al., 2023). Despite advancements in research and pharmacology, there is currently no definitive drug to fully cure NDD, though ongoing studies aim to alleviate symptoms and prolong lifespan. (Pohl and Kong, 2018; Breijyeh and Karaman, 2020). NNDs arise from many factors and complexities; however, it is widely acknowledged that gene-environment interactions and aging processes play pivotal roles. These diseases exhibit distinctive pathological markers, including oxidative stress (OS), impaired proteosomes, dysfunctional mitochondria, and the accumulation of abnormal protein aggregates (Tan et al., 2016; Karvandi et al., 2023). Another contributing factor to neurodegeneration is the incapacity of central nervous system (CNS) neurons to regenerate cells autonomously following death or damage, posing a significant threat to human longevity (Fawcett, 2020; Cooke et al., 2022).

1.1.2. Parkinson’s Disease (PD)

200 years have passed since James William Keys Parkinson first published the ‘Essay on the Shaking Palsy’ in 1817, subsequently termed PD (Parkinson, 2002; Palacios-Sánchez,

et al., 2017). PD is the second most prevalent and the fastest-growing NDDs (de Lau and Breteler, 2006; Phom et al., 2014; Dorsey et al., 2018). The projection for 2019 indicated that PD affected about 8.5 million people globally. According to current estimates, PD caused 5.8 million disability-adjusted life years (DALYs) in 2019, an increase of 81% since 2000, and 3,29,000 deaths, an increase of almost 100% since 2000 (WHO, 2023). The pathology of PD is enigmatic as it involves convoluted molecular pathways. The loss of dopaminergic (DAgeric) neurons in the *substantia nigra pars compacta (SNpc)* and subsequent deregulation of the basal ganglia is one of the primary pathogenic features of PD (Radhakrishnan and Goyal, 2018; Tsalenchuk et al., 2023). These neurons synthesize dopamine (DA), the key neurotransmitter that regulates the body's movement. So, death or impairment of the neurons can lead to a shortage of DA in the basal ganglia, leading to resting tremors, postural instability, rigidity, and bradykinesia which are classic motor symptoms. It is also accompanied by multiple non-motor symptoms, including dementia, constipation, sleep behavior disorder, hyposmia, cognitive decline, and restless leg syndrome, which may antecede more than ten years before motor symptoms (Radhakrishnan and Goyal, 2018; Santiago and Potashkin, 2023; Giri et al., 2024). Another inevitable non-motor disorder among PD patients is sexual dysfunction, which remains underrated and neglected (Koza et al., 2023). A further pathological feature is the development of intraneuronal Lewy bodies (LBs) and Lewy neurites (LNs) caused by the accumulation of misfolded proteins and primarily result in prominent neuronal death of DAergic in *SNpc* (Lewy, 1912; Gibb and Lees, 1988; Goodheart and Blackstone, 2024). The α-synuclein (α-syn) protein, which is extensively tagged with ubiquitin, covers up majority of the LBs. Other proteins include αβ-crystallin and neurofilament, followed by other molecular elements like lipid membranes, mitochondria, and lysosomal structures (Tsalenchuk et al., 2023). The exact physiological functions of α-syn are not

entirely known, but it is presumed to play a role in synaptic vesicle release. In PD, aggregates of α-syn are passed between neurons, facilitating disease spread throughout the brain. Pathological accumulation of α-syn filaments occurs in regions such as the substantia nigra (SN), amygdala, and striatum, leading to the formation of LBs and LNs. In PD, prion-like aggregates of α-syn localize primarily in the brain stem and SN within the mesencephalon, contributing to neuronal dysfunction and death (Abdelmoaty et al., 2023). The etiology of PD is still obscure, but the strongest known risk factor is age. Approximately 1% of those over 60 years of age and more and an increase of 1% to 3 % of senior citizens over 80 are affected (Driver et al., 2009). Around 85-90% of PD cases are sporadic. However, several genes have been identified that give rise to rare familial forms of PD when harbouring mutations (Tsalenchuk et al., 2023). Genetic forms of PD account for approximately 5 -10% of all cases. The first genetic connection to PD was discovered relatively recently, in 1997, when a mutation in the *SNCA* gene, which encodes the protein α-syn, was identified in families with a high prevalence of the disease (Boger et al., 2023). Some genes increase the risk of PD development, while others are linked with a Mendelian inheritance pattern. To date, approximately 24 genes or loci have been identified and catalogued in the Online Mendelian Inheritance in Man (OMIM) database as being implicated in the development of PD. (Funayama et al., 2023; Trevisan et al., 2024). A complex interplay between gene and environmental factors may also influence the progression of the disease. For instance, it has been demonstrated that pesticides and heavy metals exacerbate the risk of PD by inducing gene variations associated with familial forms of PD, such *as PARK1*, *LRRK2*, and *PINK1*. These genetic alterations contribute to PD-related pathological mechanisms, including mitochondrial dysfunction, OS, and impaired protein degradation (Ball et al., 2019).

1.1.3. Therapeutic strategies and limitations of PD

PD is currently incurable and results in irreversible brain damage along with gradually worsening symptoms. Therapeutic options available for PD patients remain limited, with few alternatives addressing the disease progression. One drug that has revolutionized the management of this crippling illness and remains the gold standard is L-3,4-dihydroxyphenylalanine (L-DOPA), also referred to as levodopa. L-DOPA is an amino acid precursor of DA that can cross the blood-brain barrier (BBB). Once within the brain, it is converted to DA in the presynaptic terminals of DAgeric neurons by the enzyme aromatic L- amino acid decarboxylase (AADC) thereby replenishing depleted DA levels (Fabbri et al., 2018; Alarcón et al., 2023; Ramesh and Arachchige, 2023). It is envisaged that the present pharmacological treatments for PD will mostly focus on symptomatic relief. However, these pharmaceutical treatments only alleviate the related motor symptoms by supplementing with DA, which restores striatal DAgeric tone. Apart from L-DOPA, other therapeutic drugs include:

(a) DA antagonists (DAagonists): Apomorphine, bromocriptine, pramipexole, cabergoline, rotigotine, ropinirole, pergolide, lisuride, and piribedil.

(b) Inhibitors of MAO-B: Selegiline, rasagiline, and safinamide.

(c) COMT: Entacapone, and tolcapone.

(d) DOPA decarboxylase inhibitors: Carbidopa, and benserazide.

(e)Antimuscarinic medications: Trihexyphenidyl, benztropine, orphenadrine, procyclidine, and biperiden.

(f) N-methyl-D-aspartate (NMDA) antagonist: Amantadine [**Figure 1**: Based on the pathophysiology of PD, the possible therapies are listed along with their associated

adverse effects, (Nakmode et al., 2023)]. L-DOPA significantly improved PD symptoms, quality of life, and normalizing life expectancy, even countering late-onset PD symptoms (Jankovic, 2002; Tambasco et al., 2018; Nakmode et al., 2023). However, L-DOPA's short half-life (90–120 minutes) limits its use by causing blood level variations that modify clinical symptoms in later stages. This leads to the 'wearing-off phenomenon,' in which PD symptoms resurface 3 to 4 hours after a treatment when the benefits of L-DOPA wear off. Even long-term treatment may lead to considerable toxicity by inducing motor abnormalities and spontaneous movements, known as L-DOPA-induced dyskinesia (LID) and it shows up as DA-resistant motor and non-motor neurotoxicity (Rascol et al., 2003). Within 2 years of initiating L-dopa therapy, up to 50% of patients may experience modest motor fluctuations, after 9 years it rises to up to 70% of patients (Fabbri et al., 2018).

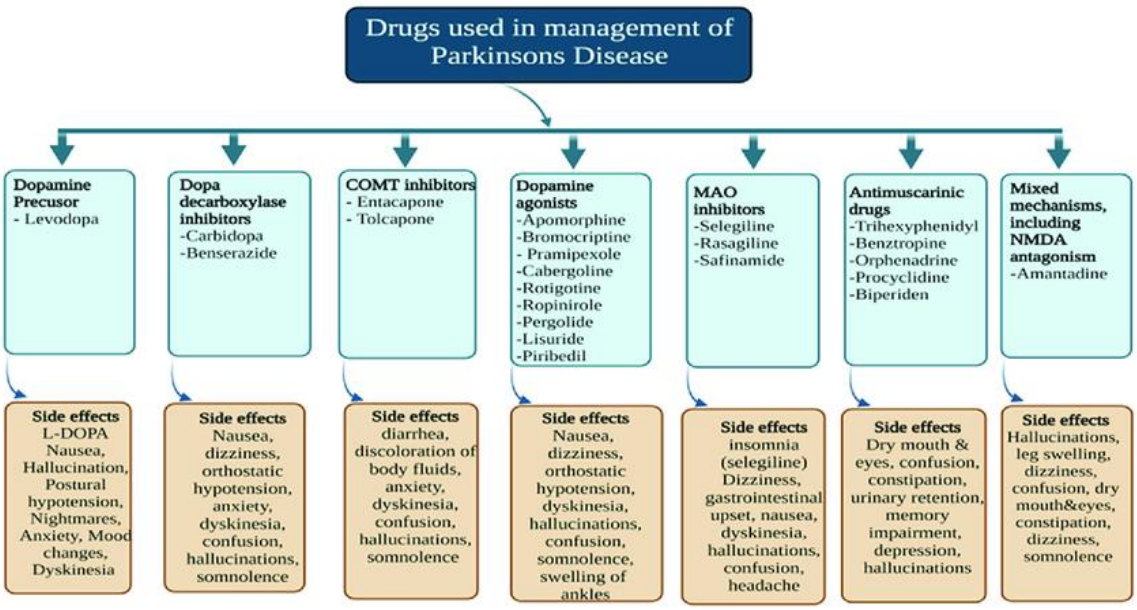


Figure1.1: Various drugs used in PD management (Nakmode et al., 2023)

Hickey and Stacy (2011) have also suggested that L-DOPA-mediated interventions exhibit ambiguity as they progress through an "On-Off" phase. In the "On" phase, patients with PD respond to treatment and their symptoms are under control; in the "Off" phase, patients do not respond to treatment, and their symptoms, which include stiffness, tremors, and slow movement, reappear. The patients also become less susceptible to DAergic drugs as their illness worsens and need higher dosages of DA supplements (Chou et al., 2018). L-DOPA-induced toxicity is hypothesized to arise from pulsatile activation of DA receptors. Under normal physiological conditions, DA release is regulated by both phasic (fast-acting, responding briefly to stimuli) and tonic (slow-adapting, maintaining action potentials at a continuous frequency) receptor firing. The tonic firing of DA neurons is the primary mechanism that is essential for sustaining DA levels and ensuring continuous receptor stimulation (Grace et al., 1984; Papa et al., 1994). Throughout the early stages of PD, a sufficient number of DAergic neuronal terminals maintain the propagation of proper tonic firing, which permits the storage and absorption of DA and L-DOPA from plasma. However, PD-related DAergic neuron terminal degeneration leads to an imbalance in internal DA storage/transport as well as decreased tonic firing. As a result, the short half-life of plasma L-DOPA makes DAergic neurons dependent on it, and variations in tonic firing also led to an imbalance in the intake of plasma L-DOPA levels. These fluctuations in plasma L-DOPA led to corresponding changes in L-DOPA and DA levels, altering receptor exposure to DA, which can trigger the reappearance of PD symptoms (Nakmode et al., 2023). As PD advances, the gradual loss of DAergic neurons disrupts the regulation of neuronal DA and reduces the responsiveness to symptomatic pharmacological treatments. Consequently, symptomatic treatment is inadequate to halt the onset and progression of PD. Ergo, decarboxylase inhibitors, like carbidopa or benserazide, are added to current formulations of L-DOPA to boost the drug's

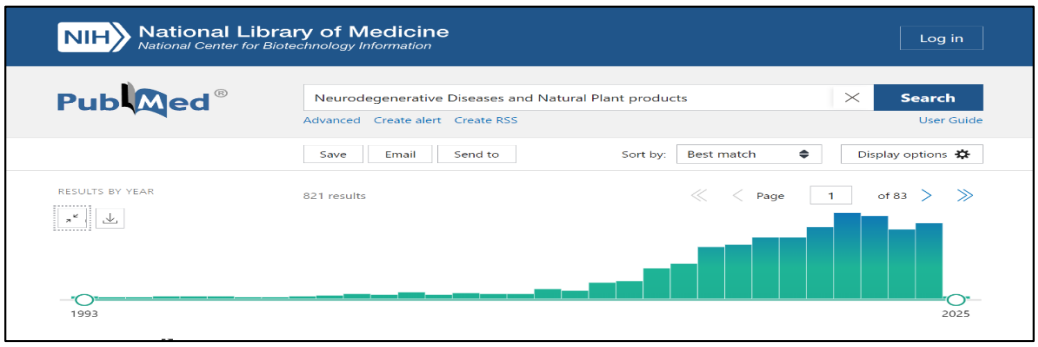
bioavailability by preventing the peripheral metabolism of DA (Muller et al., 2015). To reduce the risks associated with taking large doses of a single medication, it is advised to co-administer L-DOPA with other drugs (Dhanawat et al., 2020). So, adjunctive treatment options for L-DOPA include MAO-B inhibitors, and COMT inhibitors which have shown to increase the levels of DA (Oertel et al., 2016; Regensburger et al., 2023). Another subset of drugs is DA agonists, such as ropinirole and pramipexole, which have been described as safe and effective when used alone or in combination with L-DOPA (Kulisevsky et al., 2010; Binde et al., 2020). Although these treatments have made a substantial improvement in the management of PD symptoms, researchers are now reassessing the design of clinical trials due to the declining effectiveness of oral medications, limited therapeutic options for patients in an advanced stage of the disease, and the persistent failures of clinical trials for disease-modifying agents (Cedarbaum, 2018). As a result, the creation of novel and potent biological treatments for disease modification is becoming the main focus of PD research (Antonini et al., 2020). Several advanced formulations of L-DOPA have been developed to address L-DOPA therapy shortcomings (Armstrong et al., 2020). Continuous duodenal infusion of levodopa/carbidopa intestinal gel (LCIG) (Nilsson et al., 1998; Buongiorno et al., 2015; Thomsen et al., 2024), continuous intra-jejunal infusion of LCIG is a long-term proven and effective treatment in the later stage PD (Olanow et al., 2014; Szatmári et al., 2024). Rotigotine, available as transdermal patches, provides continuous drug delivery helping to manage motor symptoms in individuals (Carbone et al., 2019). Additionally, apomorphine delivered via subcutaneous pumps, administers the drug directly into the small intestine of individuals with PD, resulting in a marked reduction of motor fluctuations by reducing the plasma L-DOPA variability (Armstrong et al., 2020; Henriksen and Staines, 2021).

Therefore, a disease-modifying strategy that can directly address the etiology rather than providing symptomatic alleviation remains a major unmet clinical need in the care of PD to slow or stop the illness's progression and limit mobility. Consequently, it is proposed that the next "cutting edge tools" that might fundamentally alter the etiopathogenesis of PD are disease-modifying medicines, such as immunotherapies, small molecules/compounds, and nanoformulations (Nakmode et al., 2023). Plant products, extracts, and nutraceuticals have gained recognition as potent disease-modifying agents, valued for their accessibility, safety, and multi-target mechanism of action (Lama et al., 2020; Yuan et al., 2016; Modi et al., 2016). In recent years, there has been a growing body of research, using the therapeutic potential of plant products and extracts in studying and managing NDDs, particularly PD (Figure **1.2**). So, exploring natural plant products with appropriate model organisms may lead us to new insights into PD progression and help strategize new therapeutic targets that burden humankind.

1.1.4. *Drosophila* as a model organism

Drosophila, though a miniature fruit fly continues to inspire researchers and has paved the way in advancing our understanding of a broad range of biological processes, including genetics, development, aging, learning, behavior, neurogenesis, neurodegenerations, and many other areas. (Jennings, 2011; Ram and Chowdhuri, 2014; Tolwinski, 2017). *Drosophila* has many advantages when categorizing it as a model organism: low maintenance, cost-effectiveness, sophisticated genetic toolkit, and fewer ethical restrictions. The genome of *Drosophila* has been fully sequenced, which comprises ~13,600 protein-coding genes that are distributed on four pairs of

(a)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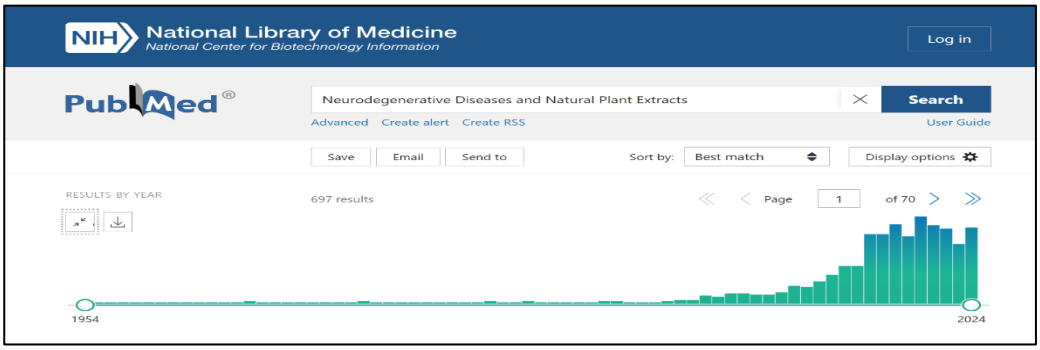
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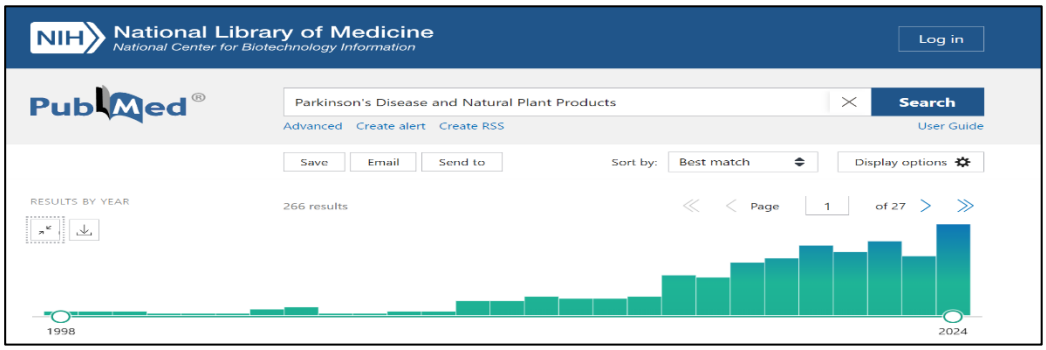
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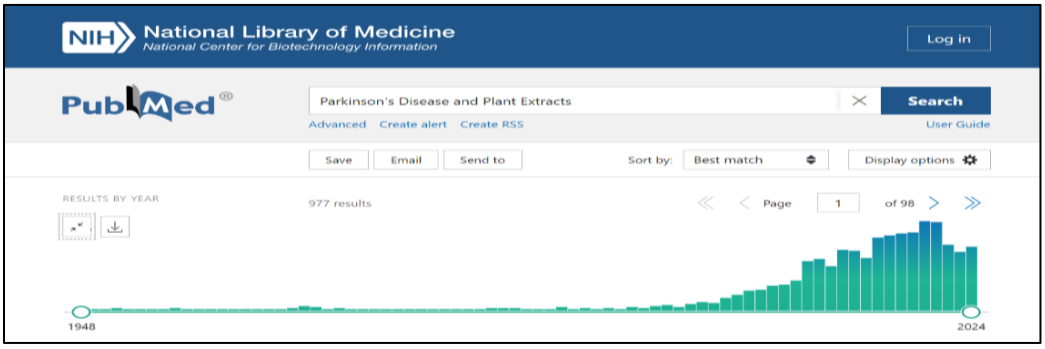
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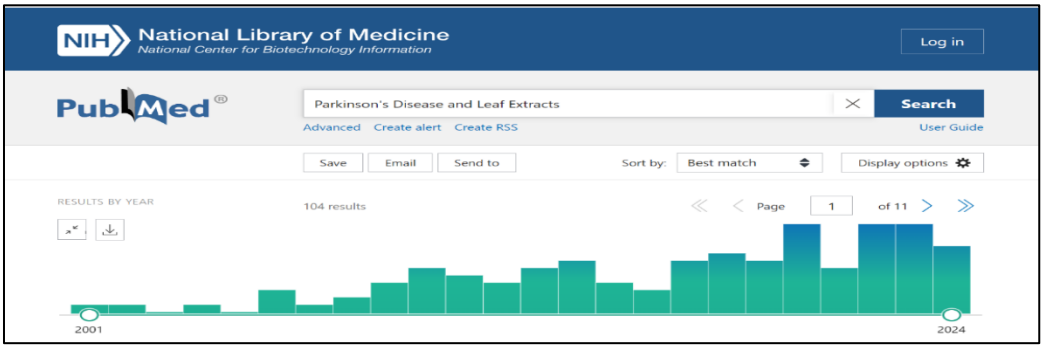
National Library of Medicine
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PubMed
Parkinson's Disease and Leaf Extracts
Search
Advanced Create alert Create RSS
User Guide
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Sort by: Best match
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RESULTS BY YEAR
104 results
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2001
2024

Figure 1.2: Snapshots depict search results over the last few decades in the PubMed network database. The search phrase "Neurodegenerative Diseases and Natural Plant Products" retrieves 821 peer-reviewed articles (**a**), "Neurodegenerative Diseases and Natural Plant Extracts" retrieved 697 peer-reviewed articles (**b**), "Parkinson's Diseases and Natural Plant Products" retrieved 266 peer-reviewed articles (**c**), "Parkinson's Diseases and Natural Plant Extracts" retrieved 977 peer-reviewed articles(**d**), and "Parkinson's Diseases and Leaf Extracts" retrieved 104 peer-reviewed articles(**e**) chromosomes (Adams et al., 2000). The fly has 57.9 % homologues of all human genes and 73.1% of all human disease-related genes have a fly orthologue (Yamamoto et al., 2024). The nucleotide or protein sequence similarity between mammals and flies is about 40% but, in conserved functional domains, it can reach as high as 80–90% (M. Ayodele-Asowata et al., 2024). *Drosophila* is used more than genetic analysis with a purpose, making it one of the valuable organisms to study human diseases (Fischer et al., 2023; M. Ayodele-Asowata et al., 2024). Thus, there have been many investigations of employing environmental toxins in the *Drosophila* model to understand the pathology of PD and to examine and explore the therapeutic interventions of natural plant products/ extracts (**Tables 1.1 and 1.2**).

1.1.5. Neuroprotective efficacy of plant products/nutraceutical/plant extracts/polyphenols in *Drosophila* model of Parkinson's disease (PD)

Even before the advent of modern neuropharmacology, physicians intuitively believed that plant-based medications could alleviate the symptoms of PD. Although Jean-Martin Charcot (1825-93) was not the first to experiment with such treatments, he refined James Parkinson's description of the disorder, and his use of the alkaloid hyoscyamine may represent the first documented anti-parkinsonian drug effect (Kempster and Ma, 2022). This section aims to conduct a literature search to identify publications that discuss the neuroprotective efficacy of plant extracts (PE) or nutraceuticals in neurotoxin-mediated *Drosophila* models of PD using Paraquat (PQ) or Rotenone (ROT). Additionally, it includes studies examining the relationship between PE, *Drosophila* models, and PD. Numerous trials have demonstrated that plant products, nutraceuticals, and plant extracts

are effective in reducing NDDs, including PD (Scalbert et al., 2005). Plant products, nutraceuticals, and extracts have recently attracted much attention from researchers seeking a cure for PD. 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**).

The most promising candidate in developing therapeutic drugs for PD is to explore traditional medicinal plants 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 of Dh root extract. Furthermore, Dh extract reduced neurotoxicity against 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 antioxidants 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 (Chaumontet et al.,1994; Hirano et al., 1995; Datla et al., 2001; Seo et al., 2011). 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 NDDs, like PD (Long et al., 2009; Wang et al., 2010; Cha et al., 2017). Feeding grape extract and grape seed extracts to flies expressing α-syn resulted in 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 by 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 the fly, the impaired climbing ability induced by PQ is 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, increased the life span, and reduced OS, resulting in a reduction in LP and PC content, as well as apoptosis (Phom et al, 2014; Phom, 2018; 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**and**1.2** are potential PD drug therapy sources.

S. No.	Fly	Neurotoxin treatment strategies	Age/Sex	Plant extract intervention strategies	PD phenotypes scored	Remark(s)	Reference(s)
1.	Oregon K	500 µM ROT for 7 days in diet	10 days old, Male	*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 levels	Rescued PD phenotypes	Hosamani and Muralidhara, 2010
2.	Oregon K	20-40 mM PQ for 24 and, 48 hrs on filter paper	8-10 days old, Male	*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
3.	Oregon K	500 µM ROT for 7 days in the diet	9-10 days old, Male	*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
4.	Oregon K	15-20 mM PQ for 24 and 48 hrs on filter paper	2 days old	Dietary feeding of *Decalepis hamiltonii (Dh)* aqueous extract: 0.1, 0.5 % for 14 days in food media	a) Mobility defects b) Increased mortality c) Elevated OS markers *viz.*, Elevated MDA, AchE, CAT, SOD levels, GSH depletion	Rescued PD phenotypes	Jahromi et al., 2013
5.	Oregon K	10mM Paraquat (PQ) for 24 and 48 hrs on filter paper	5, 50 days old, Male	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	Rescued PD phenotypes during the health Phase, but not during	Phom et al., 2014; Phom, 2018

					f) No DAergic neuronal loss	the transition Phase	
6.	Oregon K	500 µM ROT for 7 days in diet	8-10 days old, Male	*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 (NPSH) 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
7.	Oregon K	30mM PQ for 48 hrs on filter paper	9-10 days old	*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
8.	Oregon K	500 µM ROT for 7 days in diet	10 days old, Male	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.*, e) Increased ROS, H_2O_2 levels, GSH depletion	Rescued PD phenotypes	Pandareesh et al., 2016
9.	Oregon K	500 µM ROT for 7 days	9–10 days old, Male	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

10.	Oregon K	5.375 of PQ for 24 hrs, alternatively for 8 days	6 days old, Male and Female	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
11.	Oregon K	5.375- and 20-mM PQ for 24 hrs, alternatively for 8 days	6 days old, Male and Female	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 et al., 2018
12.	Oregon K	500 µM ROT in media for 7 days	8-10 days old, Male	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 Mitochondrial complex impairment	Rescued PD phenotypes	Pramod and Prashanth, 2020
13.	Oregon R	2 hrs starvation, 20 mM PQ for 24 hrs on filter paper	10, 20, 30 days old, Male	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
14.	Oregon R	2 hrs starvation, 20 mM PQ for 24 hrs on filter paper	10, 20, 30 days old, Male	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
15.	Oregon R	4 hrs starvation, 20mM PQ for 48 hrs on filter paper	3 days old, Male	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

16.	Oregon R-P2	20mM PQ for 48 hrs in the diet	3 days old	*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
17.	Oregon R	20 mM PQ for 24 hrs on filter paper	5–7 days old	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
18.	Oregon R	20 mM PQ for 24 hrs on filter paper	5 days old	*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
19.	Canton S	3 hrs starvation, 20mM PQ for 24 and 48 hrs on filter paper	3 days old, Female	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
20.	Canton S	10 mM PQ for16 hrs on filter paper	5 and 20 days old	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

21.	Canton S	10mM PQ for 0-16 hrs on filter paper	20 days old, Male and female	*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
22.	Canton S	200 µg/mL (0.8 mM) PQ for 3 days in diet	1 day old, Female	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 d) Loss of ELAV (embryonic lethal abnormal vision) and α-spectrin proteins for neuronal viability and synaptic stability	Rescued PD phenotypes	de Oliveira Souza et al., 2019
23.	Canton S	500 µM ROT for 7 days in food media	8-10 days old, Male	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
24.	Canton S	1 mM PQ for 5 days on filter paper (Co-treatment) and 10mM PQ for 60 hrs (Pre-treatment)	2 days old, Male and Female	*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.	Canton S	5 mM PQ for 18, 24, and 48 hrs on filter paper	3-5 days old, Male	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	Rescued PD phenotypes	Maitra et al., 2021

					d) DAergic neuronal loss (PPM and PPL1 subset)		
26.	Canton S	6 hrs starvation, 5 mM PQ for 48 hrs on filter paper	5 days old, Male	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
27.	Canton S	0.2mg/ml PQ in food diet	15 days old	*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	de Alencar et al., 2023
28.	*D. melanogaster* (B Line)	12.5 mM PQ on filter paper	Male and Female	*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
29.	*D. melanogaster*	500 µM ROT for 7 days in diet	1-3 days old, Male and Female	*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
30.	*D. melanogaster*	3.5 mM PQ for 4 days in the diet	1- 4 days old, Male	*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

31.	*D. melanogaster*	2 hrs starvation, 20mM PQ on filter paper	24-day-old, Male and Female	*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
32.	*D. melanogaster*	3.5mM PQ for 4 days in the diet	Male	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., 2020
33.	*D. melanogaster*	3mM PQ for 4 days	1-4 day old	*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
34.	*D. melanogaster*	500 µM ROT for 7 days in a cotton-soaked flask	2-8 days old, Male	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
35.	*D. melanogaster*	4 hrs starvation, 5 mM PQ for 12 and 24 hrs on filter paper	3 days old, Male	Mulberry fruits (MF): pre-treatment with 1% MF extracts	a) Mobility defects b) Increased mortality d) Elevated OS markers *viz.*, Increased MDA, ROS, CAT, SOD and AchE levels, GSH depletion	Rescued PD phenotypes	Mahesh et al., 2022
36.	w^{1118}	18mM PQ in diet for 24 Hrs	10 days old, Females	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
37.	w^{1118}	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	1, 20, and 30 days old, Male and female	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

38.	W^{1118}	2 hrs starvation, 20mM PQ on filter paper	20 days old	*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
39.	w^{1118}	250 µM Rotenone (ROT) in media for 7 days	1 day old, Male	*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

Table 1.1: Summary of the environment toxin-mediated *Drosophila* models of PD and nutraceutical/ plant extracts based therapeutic intervention.

S. No.	Fly (Gain of function – GOF; Loss of function – LOF)	Neurotoxin treatment strategies	Age/Sex	Plant extract intervention strategies	PD phenotypes scored	Remark(s)	Reference(s)
1.	*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
2.	*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
3.	*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
4.	*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	Mobility defects	Rescued PD phenotypes	Siddique et al., 2012
5.	*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	Mobility defects	Rescued PD phenotypes	Siddique et al., 2012
6.	*a-synuclein*-(Elav-Gal4 overexpression)	---	21 days old, Male	Acetone extract *Eucalyptus citriodora L*. leaf: 0.25, 0.50	a) Mobility defects Elevated OS markers *viz.*, Elevated LP	Rescued PD phenotypes	Siddique et al., 2013

				and 1.0 µl/ml in diet for 21 days			
7.	*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
8.	*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
9.	*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
10.	*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
11.	*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
12.	*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

13.	*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
14.	*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.*, d) Reduced GSH, Elevated PC, LP, and GST levels	Rescued PD phenotypes	Siddique et al., 2018
15.	*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
16.	*a-synuclein* (*A30P* and *A53T*α-synuclein)	3 hrs starvation, 10 mM PQ for 48 hrs in culture media	5, 25 days old, Male	*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
17.	*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
18.	*Parkin* Knockdown (KD)	3 hrs starvation, 1 mM PQ for 48 hrs on filter paper	3 days old, Female	*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 No DAergic neuronal loss	Rescued PD phenotypes	Ortega-Arellano et al., 2011, 2019

19.	*Parkin* knockdown (KD)	3 hrs starvation, 1 mM PQ for 15 days on filter paper	3 days old, Female	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
20.	*Parkin* (TH-GAL4; UAS-RNAi-parkin Knockdown)	3 hrs starvation, 1,5,20mM PQ for 15 days on filter paper	3 days old, Female	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
21.	*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
22.	*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
23.	*Pink1*^{B9} mutant (LOF)	20mM PQ in food media for 2 days	larvae, 5, 18, 30 days old, Male	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 e) Elevated OS markers *viz.*, Enhanced ROS levels f) Mitochondrial dysfunction *viz.*, Inhibited Complex-I, II, Reduced ATP, mtDNA, and MMP	Rescued PD phenotypes during 3–5 (I), 15–18 (II) but failed during (25-30 III)	Liu et al., 2020

					g) DAergic neuronal loss (PPL1)		
24.	*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
25.	*Pink1*^{B9} (LOF)	PQ	1st instar larvae, 20, 30 days old, Male	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
26.	*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
27.	*Park13* or *DJ 1βDelta93*	6 hrs starvation, 10mM PQ for 24 hrs on filter paper	Larvae, 1 and 35 days old, Male	*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
28.	*park*^{13}	10mM PQ for 24 hrs	7, 15, and 20 days old, Males	*(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 e) Mitochondrial dysfunction *viz.*, Inhibited complex I activity, Reduced MMP	Rescued PD phenotypes	Pradhan et al., 2020
29.	*Park*^{25} (LOF)	---	1 day old	Aqueous extract of *Ashwagandha*-root (ASH-root extract): 0.6% w/v of ASH-	a) Mobility defects b) Increased mortality	Rescued PD phenotypes	Murthy et al., 2023

				root extract from larval stage till the last day of their survival (L^+/A^+) (1 to day 20)			
30.	*DJ-1RNAi*	---	1, 10, and 25 days old	*Triperygium 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
31.	*DJ-1β*$^{\Delta}$ 93, *Oregon R+*	10, 20, and 30 mM PQ in the diet	Male, female	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.*, d) Elevated SOD, CAT activity	Rescued PD phenotypes	Kumar et al., 2017
32.	*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., 2023
33.	*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
34.	Canton-S, *Ddc-GAL4*	3 hrs starvation, 1 mM PQ for 15 days on filter paper	3 days old, Female	1% to 10% glucose for 15 days on filter paper	a) Mobility defects b) Increased mortality	Rescued PD phenotypes	Ortega-Arellano et al., 2011
35.	Canton-S, TH > dj-1-β-RNAi/+ (knocked down *dj-1-β*function)	3 hrs starvation, 1 mM PQ for 10 and 15 days on filter paper	3 days old, Female	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

36.	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	Rose et al., 2016
37.	*(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
38.	Harwich	500 µM ROT for 7 days in diet	1-5 days old, Male and Female	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
39.	Harwich	5mM PQ on filter paper for 3 days	1-4 days old, Male and Female	*Anacardium microcarpum* hydroalcoholic extract (AMHE), methanol (AMMF) and acetate (AMAF) fraction of *A. microcarpum*: 1 and 10 mg/ml for 5 days in standard medium	a) Mobility defects b) Reduced survival c) Elevated OS markers *viz.*, Increased ROS levels	Rescued PD phenotypes	Müller et al., 2017
40.	Harwich	250 µM ROT for 7 days in diet	1-3 days old, Male and Female	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	Rescued PD phenotypes	Farombi et al., 2018

					activity, Inhibited CAT and GST activities		
41.	Harwich	0.44 mg/g of PQ in diet for 7 days	1-5 days old	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
42.	*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
43.	Tyrosine hydroxylase (TH) Gal4/UAS-X (RNAi Knockdown), Canton-S	3 hrs starvation, 1 mM PQ for15 days on filter paper	3 days old, Female	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
44.	UAS-MitoGFP/Cy; Mef2	170 mg/kg body weight PQ for 3 days in diet	1 day old, Female	Diet 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
45.	W1118& 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
46.	esg-Gal4 UAS-GFP	2 hrs starvation, 20mM PQ for 24 hrs on filter paper	25-days old, Male	*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	Rescued PD phenotypes	Han et al., 2021

					e) Reduced autophagy pathway activation		
47.	*Hsp70Ba, Hsp70Aa, CecA1, Amy-p, and Drs (RNAi* knock-down*)*	PQ	3- and 20-days old Male	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
48.	Oregon K, UAS-*sod1*-IR, UAS *cat*-IR, RNAi line	10 and 8mM PQ for 24 hrs on filter paper	5 days old, Male and Female	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)
49.	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
50.	dUCH-knockdown	---	larval stage, 5, 10 days old	Dietary supplementation *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

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

1.1.6. Toxin models of PD

Most cases of PD are thought to be sporadic and influenced by environmental variables. The individuals with a higher risk of developing PD are the ones, living in rural areas exposed to pesticides, consume well water, and long-term work in mining (Elbaz et al., 2009; Ritz et al., 2009). Exploring the model organism has contributed to understanding the etiology, pathophysiology, and molecular interactions facilitating the disease progression (Pingale and Gupta, 2020; Dovonou et al., 2023). The neurotoxicants that are regularly used to induce PD in animal models are the 1-methyl-1,2,3,6-tetrahydropiridine (MPTP), 6-hydroxydopamine (6-OHDA), agricultural pesticides, 1,1′-dimethyl-4,4′-bipyridinium dichloride PQ, and Rot. These toxins specifically damage the catecholaminergic system and can mimic PD in animal models. Even though there are variations in their modes of action, all these neurotoxins eventually contribute to the degeneration of DAergic neurons in the *SNpc*, which is evident by the presence of LB structure (Accumulation of α-syn protein, ubiquitin, and gliosis), reduction of DA and its metabolites (DOPAC- 3,4-Dihydroxyphenylacetic acid and HVA- Homovanillic acid), mitochondrial dysfunction, OS (ROS generation), neuroinflammation, impaired UPS (ubiquitin proteasomal system) and autophagy. Neurotoxin models have emerged as the pivotal tool in exploration to test therapeutic intervention strategies intended to mitigate PD symptoms. These animal models can stimulate the neuronal degeneration characteristics of PD, providing a controlled environment for the evaluation of disease mechanisms and potential treatments. Some of the extensively used neurotoxin-mediated animal models of PD will be discussed further.

1.1.6.1. Paraquat (PQ)

PQ is a herbicide that suppresses weeds and grass growth (Richardson et al., 2005). Since its discovery, PQ has been utilized as a neurotoxin to induce PD in model organisms because of

its molecular structure similarities to MPP^+, the active metabolite of MPTP. PQ is analogous to MPP^+, which is unable to pass the BBB but reaches the brain through an amino acid transporter called systemic l-amino acid transporter. In the neuron, it is absorbed 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 GSH and thioredoxin which increases OS and damage the lipids, proteins, DNA, and RNA structure (Niso-Santano et al., 2010). It is also carried 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 levels, mitochondrial dysfunction, reduced mitochondrial membrane potential ($\Delta\Psi m$), endoplasmic reticulum (ER) stress, alteration in DA catabolism, inactivation of TH, and decrease in the neurotrophic factor BDNF, ultimately manifesting the death of DAergic neurons in the *SNpc* (Phom et al., 2014; Das, 2022; See et al., 2022, 2023). 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 us with a larger picture to develop new therapeutic targets shortly.

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 (Phom et al., 2014; Soares et al., 2017, Das, 2022). 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). Whereas, 7- to 10-day-old adult 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). When five days old CS flies starved for 6 hrs 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 two 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 before 4 hours of starvation, they 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 to 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 (Niveditha et al., 2018). PQ has been utilized to induce PD in *Drosophila* model (Phom et al., 2014) and demonstrated impaired climbing, reduced dose, and time-dependent survival, 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 and change in the brain-specific molecular targets (Phom et al., 2014; Das, 2022). 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.

1.1.6.2. Rotenone (ROT)

The first use of ROT was done in 1985 when Heikkila et al. (1985) demonstrated in female Sprague-Dawley rats that when injected with 2µg/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 demonstrated to induce Parkinsonian features in model organisms (Cannon et al., 2009). ROT crosses the BBB as it is a lipophilic compound and does not depend on DAT (dopamine transporter) 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 (Cannon et al., 2009; Bisbal and Sanchez, 2019; Ayajuddin et al., 2022). *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 *Drosophila* model could be used to study the neuro-pathophysiological mechanism of PD as it reproduced 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, it 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) reported that 250 μM of ROT exposure to 3 days old wild-type Harwich flies for 7 days exhibited a defect in negative geotaxis, and reduced acetylcholine levels, OS increasing hydrogen peroxide levels, decrease in catalase, GST, and thiol levels in the treated files (kolet 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 (Pandareesh et al., 2016; Siima et al., 2020;). Recent study on ROT-mediated *Drosophila* PD model demonstrated that when the fly is exposed to ROT it 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 TH 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). Overall, ROT-mediated PD models exhibit all the pathological features of PD making it an attractive model of PD.

1.1.7. *Leea asiatica* (L.) (LA)

Figure 1.3: *Leea asiatica* (L.)

1.1.7.1. Systematic position:

Kingdom	:	Plantae
Division	:	Tracheophyta
Class	:	Mangoliopsida
Order	:	Vitales
Family	:	Vitaceae
Genus	:	*Leea*
Species	:	*asiatica* (L.)
Plant part used	**:**	Leaves

1.1.7.2. Vernacular name:

State/District/Regional language		Local names/ Regional names	Reference
Madhya Pradesh	Jhabua District	Nanli Danhi	Wagh and Jain, 2013
	(Shahdol District)	Nanli Danhi	Bharti, 2015
	Harda District	Hatikan	Jeetendra et al., 2014
	Dindori district	Hansiadhapar	Pandey, 2007
Hindi		Nanli Kumala, Murkhur	Singh, 2015; Singh et al., 2000
Sanskrit		Kakjangha, Jeera vali	Sankara Rao, 2019
Malayalam		Nalugu,	Sankara Rao, 2019
Kannada		Vataal mara	Sankara Rao, 2019
Bengali		Banchalita	Sen et al., 2011
Manipuri		Koknal	Sen et al., 2011
Andamanese		Koya kaccha	Prasad, et al. 2008
Gujarat (Vasavi)		Nanidhini	Chauhan et al., 2018
Maharashtra (Palghar)		Dinda, Gharbanda	Jadhav et al., 2015
Assam (Barpeta district)		Aiha bon	Das, 2016
Assam, Golaghat District		Kukur Thengia	Barukial and Sarmah, 2011
Karnataka, Uttara Kannada district		Bili Nedtige	Bhat et al., 2012

Table 1.3: Some of the vernacular names used by people of India

1.1.7.3. Description of the plant:

LA is a perennial shrub or small tree from the family Vitaceae (grape family). It is characterized by soft wooded, upright, and hirsute texture. It is identified by different names by the various tribes of India (**Table 1.3**). It is widely distributed in tropical and subtropical regions {India (Poonch, Jammu, Sikkim, Darjeeling, Himachal Pradesh, Uttarakhand, Bihar, West Bengal, Arunachal Pradesh, Assam, Nagaland, Manipur, Mizoram, Meghalaya, Odisha, Madhya Pradesh, Rajasthan, Maharashtra, Karnataka, Andhra Pradesh, Tamil Nadu, Kerala); China (Yunnan); Nepal; Bhutan; Bangladesh; Andaman Isl.; Myanmar (Kachin, Mon, Sagaing, Taninthayi); Thailand; Laos; Cambodia; Vietnam}. *L. asiatica* belongs to the genus *Leea* a

well-known genus for being used extensively as folk medicine possessing various beneficial biological and antioxidant properties (Hossain et al., 2021).

1.1.7.4. *Leea asiatica* (L.) (LA) in ethnomedicinal culture and pharmacological preclinical studies

LA is used as non-ethnomedicine and ethnomedicine by many native people of India. It has a wide range of purposes from washing hair to treating various ailments like bone fracture (Bhandary et al., 1995), guinea worms, snake-bite, etc. (Nair et al., 2014). In Gujarat, some tribal people have it as a vegetable by cooking the inflorescence with oil and spices (Chauhan et al., 2018). The whole plant can be utilized, and the method of use varies among native people of India. LA can be used singly or mixed with other plants as polyherbal treatment which is seen in the tribe of Hazaribag district of Bihar where the root of *L. asiatica* is made into paste along with the bark of *Boswellia serrata* to treat snake- bite (Nair et al., 2014). A detailed description of the ethnomedicinal use of LA has been given in **Table 1.4.**

Ethnicities of India	**Plant section employed**	**Processing, if any**	**Method of delivery**	**Therapeutic care/ Health care**	**References**
Siddis of Uttara Kannada district, Karnataka	Whole plant	The whole plant is ground into a paste	Paste is applied over the fractured area, as a thick layer, supported with bamboo sticks	Healing Bone fractures	Bhandary et al., 1995
Tribes of Hazaribag, Bihar	Root of the Plant	The root and bark of *Boswellia serrata are* made into a paste	Paste applied on the site of the snake bite	Snake Bite	Nair et al., 2014; Singh et al., 2002
Tribes of Uttar Pradesh,	Root of the Plant	Root with the bark of *Boswellia serrata*	Pounded and taken orally	An antidote to snake bite	Singh et al., 2002
North Andaman, India	Root	Roots are pounded into a paste	Applied externally on the wound	Boils and blisters	Prasad et al., 2008

West and South district of Tripura	The root of the plant	Pounded to make a paste	Consumed orally	Anthelminthic	Sen et al., 2011
	Leaves	The juice of the leaves is extracted	Consumed orally	Liver disorder	
	whole plant	The whole plant is boiled and dissolved to make a concentrated liquor	Consumed orally	Heart Disorder	
	Leaves	Soaked in the water overnight Drenched in water overnight	Water is consumed the next morning by the patients	Diabetes	
Chiru Tribe of Manipur	Leaves	Extracted leaves juice mixed with water	Solution is applied externally to the hair	Washing of Hair	Nair et al., 2014
Tribes of Madhya Pradesh	Bulbs	Bulb Paste with water	Paste is applied to the joints	Joint disease	Wagh and Jain, 2014
Shahdol district of Madhya Pradesh	Roots	The powdered roots are mixed with mustard oil	Orally consumed on the prepared mixture, twice a day	Arthritis	Bharti, 2015
Harda district of Madhya Pradesh	Flowers	Flowers paste	Paste is applied on wounds	Wounds	Jeetendra and Sudip, 2014
Local community living around the Manas National Park, Barpeta District, Assam, North East India	Roots	Not specified	Not specified	Against fever, ringworm and jaundice	Das, 2016
Golaghat district, Assam	Roots	Not specified	Not specified	Ringworm	Barukial and Sarmah, 2011

Uttara Kannada district, Karnataka	Roots	Roots grounded in washed rice water	Applied on the burned area	Burn	Bhat et al., 2012

Table 1.4: Highlights the ethnomedicinal use of LA among different tribal communities of the Indian subcontinent.

Ethnically, LA has been shown to have many health benefits, yet various scientific validations still need to be performed. Studies have demonstrated that the leaves of LA possess various flavonoid, phenolic compounds, glycosides, and triterpenes in ethyl acetate, methanolic, and petroleum ether extract (Sen et al., 2013; Nair et al., 2014). Sen et al., 2012 reported the antioxidant significance of LA against anthelmintic activity in Indian adult earthworms (*Pheretima posthuman*). The efficacy of antioxidants towards oxidative stress was evaluated through various methods, including 2, 2-diphenyl-1-picryl-hydrazyl (DPPH˙) radical scavenging, nitric oxide radical scavenging, LP assay, and the ferric thiocyanate method. In another study, using methanol, ethyl acetate, and petroleum ether extract of LA the nephroprotective effect in cisplatin-induced toxicity in rats was also evaluated by measuring scavenged DPPH˙ responsible for LP inhibition, superoxide anion radical (O_2^{let-}), hydroxyl radical (OH˙), nitric oxide radical (NO˙), hydrogen peroxide (H_2O_2), reducing power ability, metal chelating ability, and total antioxidant activity. By scavenging O_2^{let-}, OH˙, NO˙, and H2O2, the methanol extract inhibits damages caused by OS (Sen et al., 2013). The methanol leaf extract of LA showed hepatoprotective efficacy by normalizing the level of serum glutamic oxaloacetate transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, total cholesterol, total triglycerides when inducing liver toxicity through acetaminophen. The level of an endogenous antioxidant such as SOD, CAT, GPx, and reduced GSH was also seen to be increased (Sen et al., 2014). According to another study, LA leaf extract possesses strong anti-inflammatory and wound-healing qualities. When adult Wistar albino rats were fed with leaf extract in water, which is

similar to aspirin, the paw oedema that was generated with carrageenan exhibited substantial results. The extract has demonstrated a notable impact on wound healing, albeit one that is not as great as the typical 10% boric acid solution. Additionally, they tested acute oral and acute cutaneous toxicity on rats and New Zealand White rabbits, respectively. According to their findings, neither an oral toxicity test lesion nor an inflammatory or allergic reaction was observed in the dermis (Nair S. et al., 2014). Another significant study explored the potential use of zinc oxide nanorods (ZnO NRs) synthesized through a green method using LA leaf extract, as a potent anticancer agent. ZnO NRs anticancer activity was evaluated 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) assay, and LDH (Lactase dehydrogenase release: for apoptotic and necrosis cell death) assays, and it was found that the cytotoxic properties of ZnO NRs was found to depend on both time and concentration. Also, the introduction of ZnO NRs to the MCF-7 breast cancer cell line caused necrosis and apoptosis due to the formation of ROS, which can damage DNA and trigger apoptosis in MCF-7 breast cancer cells. (Ali et al., 2021). A summary of all the research on the preclinical and scientific validation of LA under conditions of numerous diseases is provided in the **Table.1.5**.

Ethnomedicinal use	**Type of extract**	**Mechanism of action**	**Animal Model**	**Reference**
Anthelmintic (Treating worm infection)	Methanol, Ethyl acetate & Petroleum ether fraction of Leaf extract	Unknown/Anti-oxidant activity	*Pheretema posthuma*	Sen et al., 2011
Nephroprotective (Treating Kidney disorder)		Anti-oxidant, Radical scavenging activity	Wistar rat	Sen et al., 2013
Hepato-protective (Treating liver disorder)		Anti-oxidant activity owing to increased activity of antioxidant enzymes	Wistar rat	Sen et al., 2014
Wound healing Anti-irritant (Treating cuts and snake bites)	Methanol extract of leaf	Anti-inflammatory	Wistar rat	Nair et al., 2014

Anticancer Activity	Zinc oxide nanorods (ZnO NRs) obtained *via a* green synthesis approach utilizing *L. asiatica* extract	Anti-oxidant activity, Apoptotic and Necrosis pathways	Breast cancer cell line (MCF-7); Zebrafish embryos	Ali et al., 2021

Table 1.5: Preclinical and scientific validation of *L. asiatica* as a therapeutic agent in the treatment of various diseases.

1.1.8. Conclusion

The quest for neuroprotective agents in the battle against PD has intensified in recent years, with natural products emerging as potential allies in mitigating the disease's progression. The idea of using plant extracts is intriguing and can have potential therapeutic properties that could be harnessed in modern biomedical research. LA, a plant traditionally used by tribal people of India, has been thrust into the spotlight as a promising candidate. It is revered for its medicinal properties, sparking interest in its potential role in neuroprotection. Some of the preliminary studies that have been discussed above suggested that leaf extract contains bioactive compounds capable of modulating OS and inflammatory responses, a key contributor to neurodegeneration. By employing *Drosophila* as a model organism, the study can effectively analyze the molecular and cellular pathways involved in PD, providing insights that are translatable to human conditions. This approach not only underscores the versatility and relevance of *Drosophila* in neuroscience but also highlights the potential of natural compounds in developing novel neuroprotective strategies. Understanding PD mechanism is crucial for identifying interventions that can halt or reverse neuronal damage, thereby preserving neurological function. By leveraging the *Drosophila* model, I aim to decipher the molecular interactions facilitated by LA, exploring its capacity to protect against DAergic neuronal loss and improve motor function outcomes. In the current investigation, the following approaches

were evaluated to screen LA leaf extract for possible DAergic neuroprotective effects in the *Drosophila* model of PD:

CHAPTER II:
Understanding *Leea asiatica's* (LA)Dopaminergic (DAergic) Neuroprotective Efficacy in the Health Phase *Drosophila* Model of Parkinson's Disease

CHAPTER III:

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

CHAPTER IV:

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

Chapter: 2

Understanding *Leea asiatica's* (LA) Dopaminergic (DAergic) Neuroprotective Efficacy in the Health Phase *Drosophila* Model of Parkinson's Disease

2.1 Introduction

In *Drosophila,* the dopaminergic (DAergic) system is involved in a variety of processes, including locomotion (Pendleton et al., 2002; Ayajuddin et al.,2023), courtship behavior (Liu et al., 2008; Neckameyer, 1998; Koza et al., 2021; 2023), sleep and arousal (Andretic et al., 2005; Foltenyi et al., 2007; Kume et al., 2005), 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). Furthermore, the regulation of light perception, circadian entrainment, and social spacing in flies is influenced by the DAergic system (Kasture et al., 2018). Deficiency of tyrosine hydroxylase (TH) directly links to a reduction in the level of dopamine (DA) so, these flies lack a desire for sucrose, have hypoactivity, longer sleep duration, decreased alertness, defective olfactory, deterrence learning, and mobility defects that tend to get worse with age (Riemensperger et al., 2011; Cichewicz et al., 2017). In *Drosophila*, the gene *ple* is responsible for the expression of TH, which is a rate-limiting enzyme that converts tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) (precursor to DA). The loss of this TH gene is lethal for embryonic development (Neckameyer and White 1993). Riemensperger et al., 2011 showed that it is possible to counteract TH-null lethality by restoring TH expression specific to hypoderm rather than CNS-specific TH expression. Overexpression of TH in DAegric neurons of *Drosophila* enhanced male-male courtship, implying that high levels of DA level act as a catalyst for male-male courtship (Liu et al, 2008). Similarly, the loss of the proto-oncogene *Myc* in DAgeric neurons induces male-male courtship. (Pan et al., 2022). Whereas, flies with TH-deficiency, DA levels can be increased by feeding with L-DOPA and carbidopa (Cichewicz et al., 2017). TH-deficient flies with impaired learning, hypoactivity, and sugar preference are restored by feeding them with L-DOPA. Similarly in the TH-deficient mice model, L-DOPA therapy can

improve the symptoms related to hypoactivity and suppress appetite (Zhou and Palmiter, 1995; Kasture et al., 2018). Complete deletion of TH activity causes heart failure in mice and, most likely, also in humans (Zhou et al., 1995; Kobayashi et al., 1995). Early reduction in the TH activity, followed by a decrease in TH protein levels is thought to critically influence DA deficiency and phenotypic manifestation in PD, DOPA-responsive dystonia, and/ or infantile parkinsonism in mammals (Blanchard-Fillion et al., 2001; Nagatsu et al., 1990; Ng et al., 2015). This investigation highlights the potential therapeutic targets in this pathway that may alleviate symptoms or alter disease progression. It also highlights the importance and relevance of animal models in studying human NDDs like PD. The *Drosophila* models of PD recapitulate the characteristic pathophysiological features of human PD; such as locomotor defects, DAergic neurodegeneration, and reduced brain DA (Feany and Bender, 2000; Chaudhuri et al., 2007; Shukla et al. 2014; Chaouhan et al., 2022; Ayajuddin et al., 2023). The misexpression of human α-Synuclein in the CNS 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 lately started employing the same animal model to study the effects of mutations or over-expression of genes involved in PD. The PQ-mediated fly 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. (Chaudhuri et al. 2007; Lawal et al., 2010; Shukla et al., 2014; Song et al., 2017; Maitra

et al., 2019, 2021; Chaouhan et al., 2022). The compact nature of the *Drosophila* brain offers advantages for studying NDDs. Comprising neurons and glial cells, its functions mirror those found in vertebrates (Nitta and Sugie, 2022). *Drosophila* approximately has 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 (Budnik and White, 1988; Nässel and Elekes, 1992; Friggi-Grelin et al., 2003; Whitworth et al., 2006; Navarro et al., 2014; Ayajuddin et al., 2023; Koza et al., 2023).

In this chapter, I attempted to characterize the DAergic neurodegeneration in the health phase (HP) of *Drosophila* model of sporadic PD and therapeutic neuroprotection under *Leea asiatica* (LA) intervention through the quantification of DAergic neuronal number and, through quantification of the level of tyrosine 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.

2.2. Materials and Methods

2.2.1. Procedure for preparing an aqueous extract from the leaves of *Leea asiatica* (LA)

Fresh leaves of LA were harvested and washed with tap water to remove any dirt or impurities still attached to the leaves. The leaves were air-dried in the shade until they became brittle. After drying, the leaves were pulverized using mortar and pestle into fine powder. To prepare aqueous leaf extract, 20 grams of powdered leaves were mixed with 200 ml autoclaved double distilled water in a conical flask.

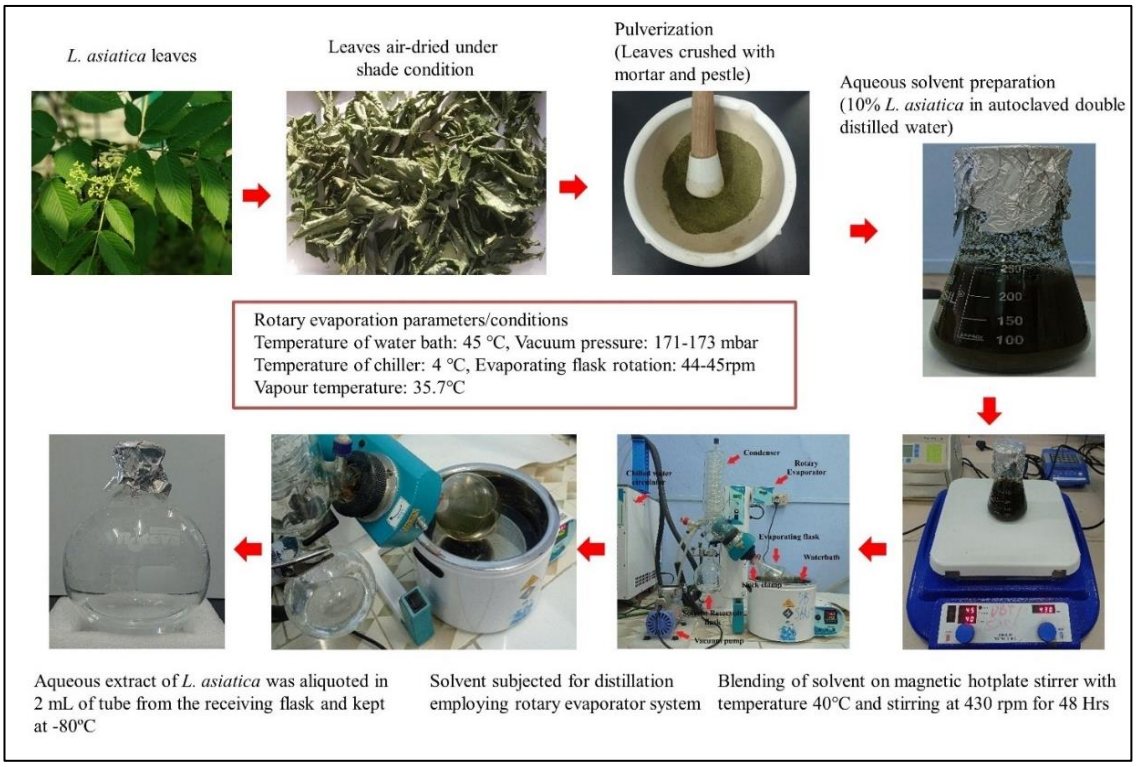


Figure 2.1. Steps involved in preparation of aqueous leaf extract of LA

The flask was then covered with aluminum foil to avoid further evaporation. The solvent was blended on a magnetic hotplate stirrer at 430 rpm at a temperature of 40 ^{o}C for 48 hours. Then the solvent was distilled by using a rotary evaporator system. The temperature of the water bath was 45 ^{o}C and the evaporating flask rotation of 44-45rpm. The final distilled aqueous extract was aliquoted in a 2ml Eppendorf tubes from the receiving flask and kept at -80 ^{o}C. The aqueous extract of LA had a concentration of 10%.

2.2.2. Preparation of multiple concentrations of *Leea asiatica* (LA)

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 LA.

2.2.3. *Drosophila* model of sporadic PD and therapeutic intervention

The Paraquat (PQ) mediated early onset sporadic PD model of *Drosophila* was employed in the present study (Phom et al., 2014), and the therapeutic intervention of LA through co-feeding and pre-feeding. The present study also uses the PQ-induced PD model to understand DAergic neurodegeneration and LA-mediated neuroprotection.

2.2.4. *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

humidity of 60%, 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 (Luckinbill et al., 1984; Phom et al., 2014).

2.2.5. 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, after which the adults were removed. After 10-12 days the flies that eclosed were lightly anaesthetised for separating 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 4-5 days representing the health phase (HP) of the adult life span were used to model PD (Phom et al., 2014).

2.2.6. 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.

2.2.7. 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 No. 1. 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).

2.3. PQ and *Leea asiatica* (LA) feeding regime for negative geotaxis assay during health phase (HP) fly model of 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. 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.

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 an aqueous extract of LA (10%, 7.5%, 5%, 2.5%, and 1.25%) for the co-feeding group. The preparation of aqueous extract LA with PQ for co-feeding was achieved by dissolving 10 mM PQ in 1 ml 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%, dissolving 150 µl of R10% with 150 µl 10mM PQ for R5%, dissolving 75 µl of R10% with 225 µl of 10mM

PQ for R2.5% and by dissolving 50 µl of R10% with 250 µl of 10mM PQ for R1.25% 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 (R10%). 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, 48, and 72 hours of co-feeding with PQ during the health phase flies (HP) (**Figure 2.2 A**).

B) Pre-feeding regime

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 group 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% and, by dissolving 150 µl of R10% extract along with 150 µl of 5% sucrose for R5% and, by dissolving 75 µl of R10% extract along with 225 µl of 5% sucrose for R2.5% 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

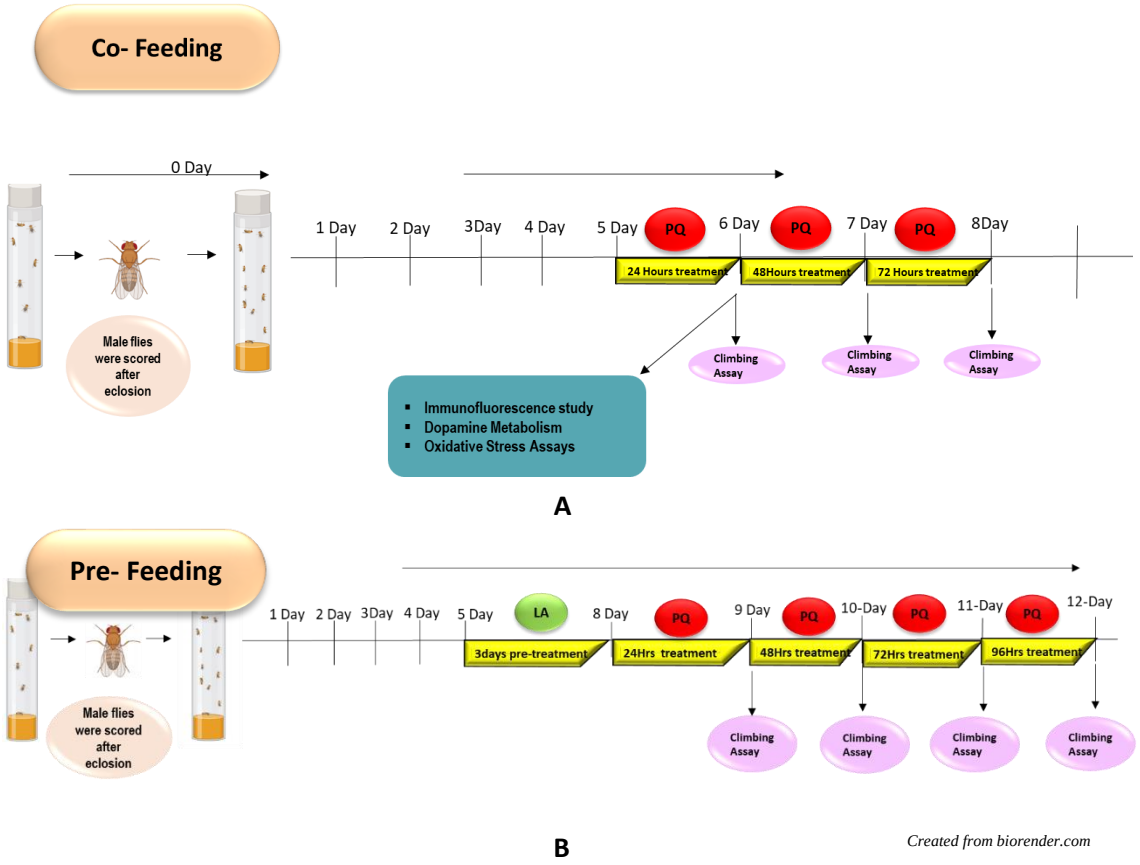


Figure 2.2: Experimental workflow and *Drosophila* feeding regimens: (**A**) **Co-feeding**: Flies were fed with PQ alone or PQ with different concentrations of aqueous extract of LA for 24, 48, and 72 hours in the *Drosophila* model. Control flies were fed with 5% sucrose only, while the *per se* group was fed with LA extract only. The negative geotaxis assay (NGA) was performed after 24, 48, and 72 hours of exposure to PQ in the fly model, respectively. (**B**) **Pre-feeding regimen**: Flies were pre-fed with an aqueous extract of LA for 3 days. The control and the group treated with PQ alone remained at 5% sucrose during this period. The flies were then exposed to PQ for 24, 48, 72 and 96 hours. Control and *per se* group remained in 5% sucrose. The negative geotaxis assay (NGA) was performed after 24, 48, 72, and 96 hours of exposure to PQ in the fly model.

of aqueous extract of LA (R10%). 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, 48, 72 and 96 hours of feeding with PQ (**Figure 2.2 B**). For the experimental group in the HP, a minimum of 100 flies were exposed to quantify LA-mediated neuroprotective efficacy.

2.4. Negative geotaxis assay

Taking advantage of the fly's natural tendency to climb toward the light the negative geotaxis assay also called as climbing assay was performed. The assay was employed using a plastic climbing tube where each experiment fly was dropped in the tube using an aspirator and initially left undisturbed to become accustomed to the new environment for 2 minutes. Each tube was fixed on a sponge and the experimental flies were gently tapped to the base of the plastic climbing tube. The distance (measured in centimeters) each fly could climb up in 12 seconds was noted. Each fly was subjected to the experiment three times and data were collected from at least 10 flies in all the experiment sets (**Figure 2.3**) (Phom et al., 2014).

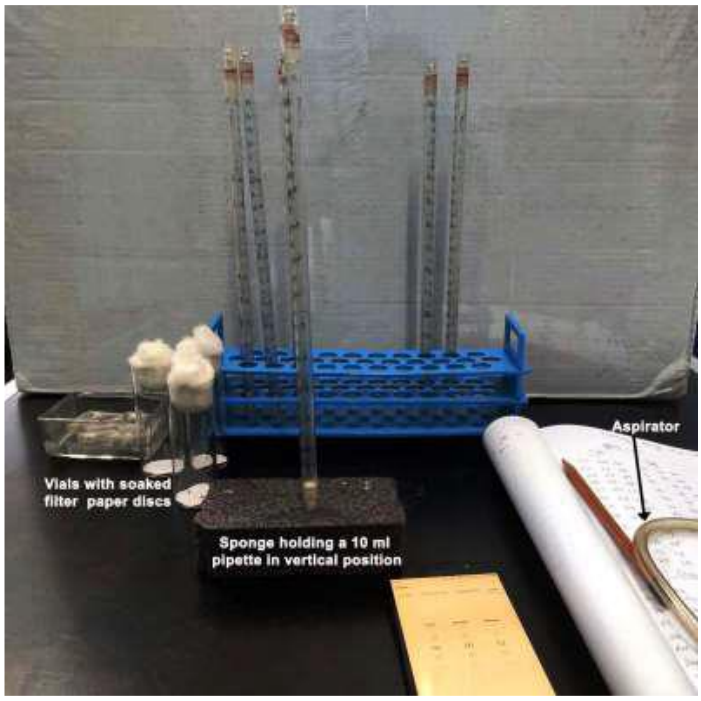


Figure 2.3. Experimental set-up for negative geotaxis assay (adapted from Phom *et al.*, 2021).

2.5. Whole brain immunostaining for Tyrosine Hydroxylase (TH):

2.5.1. TH Immunostaining

1. Sterilized 1.5 mL centrifuge tubes (Tarsons, WB, India, catalog number: 500010)
2. Parafilm™ wrapping film (Bemis, WI, USA, catalog number: PM996)
3. Conical flask (Borosil, Mumbai, India, catalog number: 5100)
4. Magnetic stirrer bar #8 mm × 40 mm (Tarsons, WB, India, catalog number: 4113)
5. SPINNOT™ digital magnetic stirrer hotplate (Tarsons, WB, India, catalog number: 6090)
6. Sterilized micro tips (Tarsons, WB, India, catalog number: 521010)
7. Freshwrapp aluminum foil 9–11 µm (Hindalco, Maharashtra, India, catalog number: HV2241)
8. Glass plate (Suwimut, USA, catalog number: B08FRB2NTM)

9. Fingernail polish (FacesCanada, Mumbai, India, catalog number: CC4403)
10. Glass spacer (Borosil, Mumbai, India, catalog number: 9115S01)
11. Microscopy slides #76 mm × 26 mm (ReliGlas, Haryana, India, catalog number: 7101)
12. Gold-seal coverslips (22 mm^2) (Electron Microscopy Sciences, PA, USA, catalog number: 63765-01)
13. Whatman™ filter paper (GE Healthcare, Buckinghamshire, UK, catalog number: 1001917)
14. Paraformaldehyde (PFA) pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA, catalog number: I58127)
15. Phosphate buffered saline (PBS) pH 7.4 (HiMedia, Maharashtra, India, catalog number: ML023)
16. Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA, catalog number: T8787)
17. Normal goat serum (NGS) (Vector Labs, CA, USA, catalog number: S1000)
18. Rabbit anti-tyrosine hydroxylase (anti-TH) polyclonal primary ab (Millipore, MA, USA, catalog number: Ab152)
19. Goat anti-rabbit IgG H&L (TRITC-labeled) polyclonal secondary ab (Abcam, MA, USA, catalog number: Ab6718)
20. VECTASHIELD® mounting medium (Vector Labs, CA, USA, catalog number: H1000)
21. Fly head capsule handling items e.g., needles #31 G × 6 mm (Tentabe BD, Punjab, India, catalog number: 324902)
22. Dissecting fine forceps (EMS, PA, USA, catalog number: 78620-4B)
23. Brush (TEYUP, Delhi, India, model number: SR-1013)
24. Delicate task Kim wipers (KIMTECH™, GA, USA, catalog number: 370080)

25. Micropipette i.e., 1,000 µL, 50 µL, 10 µL, 2 µL (Gilson, WI, USA, catalog number: 30040)
26. Frost-free refrigerator (Whirlpool, MI, USA, model number: FF26 4S)
27. pH/mV meter (Hanna Instruments, RI, USA, model: HI2211-02)
28. -20°C ES Series refrigerator (Thermo Scientific, MA, USA, model: 50616100444443250)
29. -80°C ultra-low temperature freezer (New Brunswick Innova, Hamburg, Germany, model: U101-86)
30. Stereo zoom microscope (Carl Zeiss, Jena, Germany, model: Stemi 305)
31. Stereo zoom microscope (Leica, Wetzlar, Germany, model: E24)
32. Fume hood (BIOMATRIX, Telangana, India)
33. BOD incubator (Percival, IA, USA, model: DR-36VL)
34. Test tube rotator (Tarsons, Rotospin, , WB, India, catalog number: 3070) and disk for 24 × 1.5 mL tube (Tarsons, WB, India, catalog number: 3071)
35. Axio Imager M2 fluorescence microscope fitted with 100W Mercury lamp (Carl Zeiss, Jena, Germany, catalog number: 430004-9902-000)
36. AxioCam ICm1 monochromatic camera (Carl Zeiss, Jena, Germany, catalog number: 426553-9901-000)
37. ZEN 2012 SP2 blue edition, version 2.0.14283.302 (Carl Zeiss, Jena, Germany)
38. 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 h.

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.

2.5.2. 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 of 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.

2. Circular rotator was used for proper incubation/mixing of primary and secondary antibodies to the brain samples.
3. To prevent brains from being crushed, care was taken by keeping glass spacers while mounting the brain with a cover slip.
4. To prevent the drying of the samples, the edges were carefully sealed with nail polish.
5. 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 the acquisition of *Drosophila* brain Image for quantification of DAergic neurons and fluorescence Intensity (FI) using a fluorescence microscope (Axio Imager 2, Carl Zeiss) with *ZEN 2012 SP2* software illustrates from **Figure 2.4 to Figure 2.14**.

Methods:

1. At a 40x objective lens of a fluorescence microscope, prepared/stained brains were viewed/observed (**Figure 2.4**).

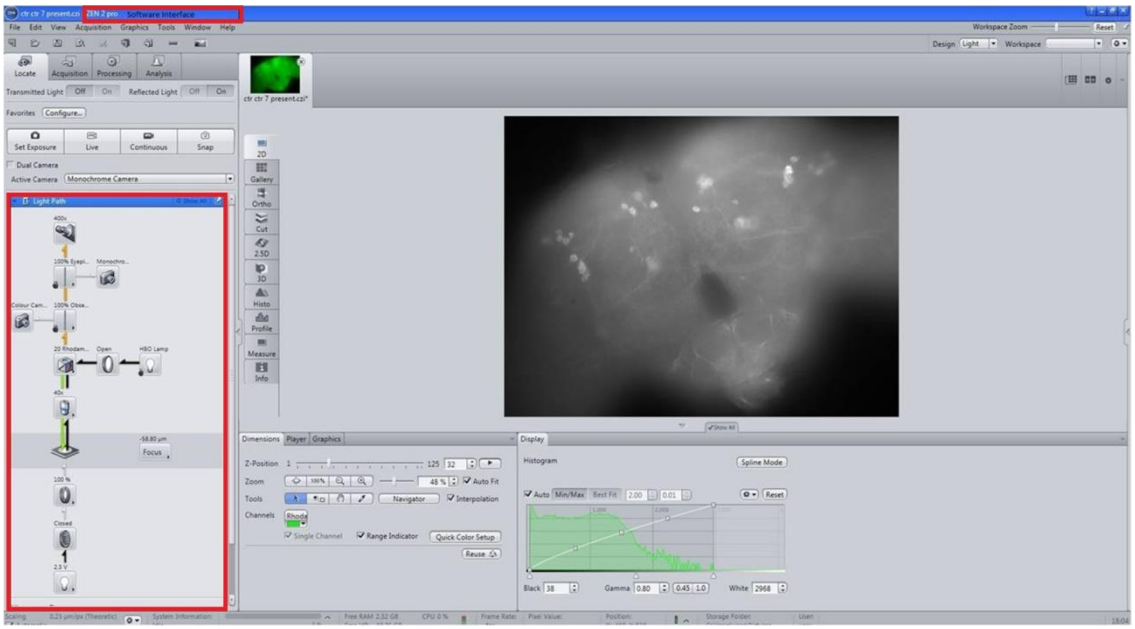


Figure 2.4. Scanning of the whole brain of *Drosophila*. Scan the anti-TH immunostained *Drosophila* brain using Carl Zeiss, Axio Imager M2 (40× objective lens) with ZEN 2012 SP2 software that interactively controls image acquisition, image processing, and analysis of the images.

2. Images were scanned and taken using a monochromatic camera with a Rhodamine fluorescence filter (**Figure 2.5**).

Figure 2.5. 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 2.5**).

4. Then, Z-stack programming was performed with constant interval of 1.08 µm for each image (**Figure 2.6**).

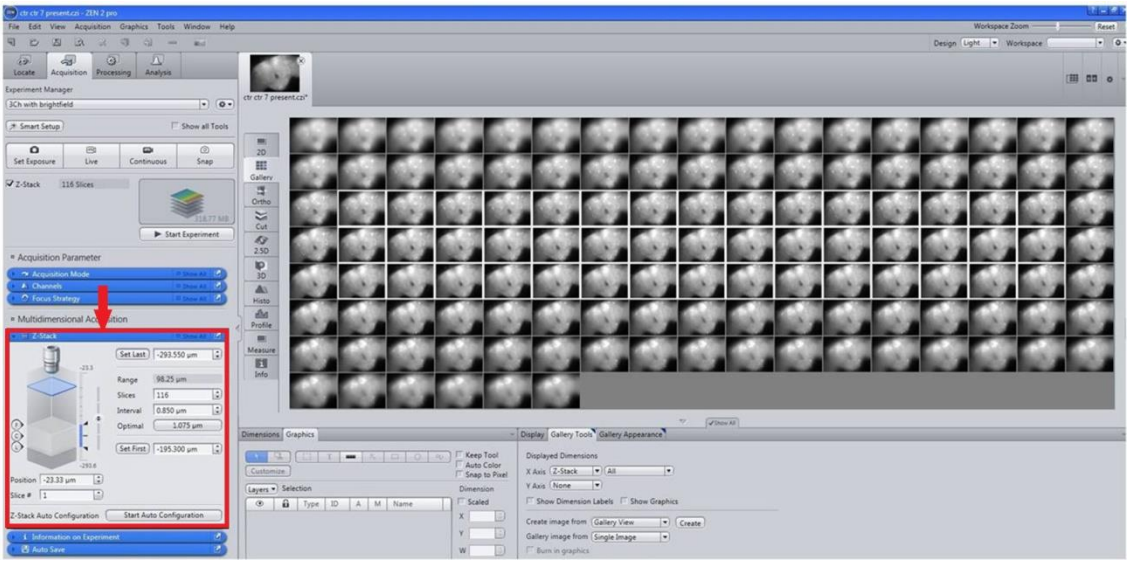


Figure 2.6. Selection of images and Z-Stacking.

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 2.7**).

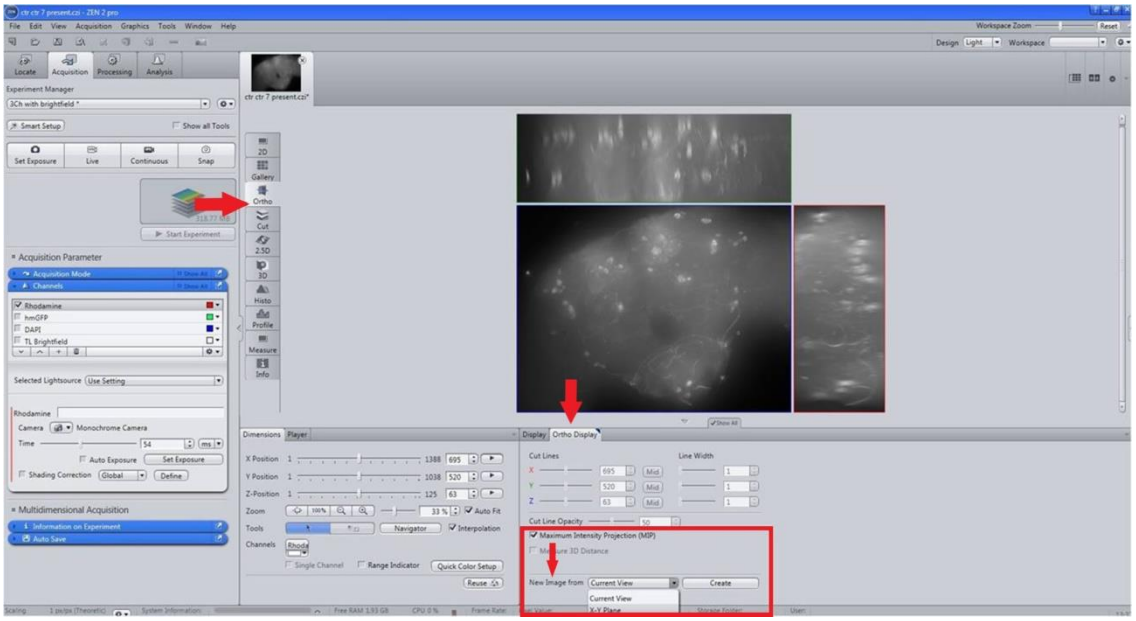


Figure 2.7. Creation of 2D image. For creating a 2D merged image, on the *Method* column, select *Maximum intensity projection (MIP)* with *X-Y Plane.*

6. The 2D image of the brain was exported in.jpg format for presentation (**Figure 2.8**).

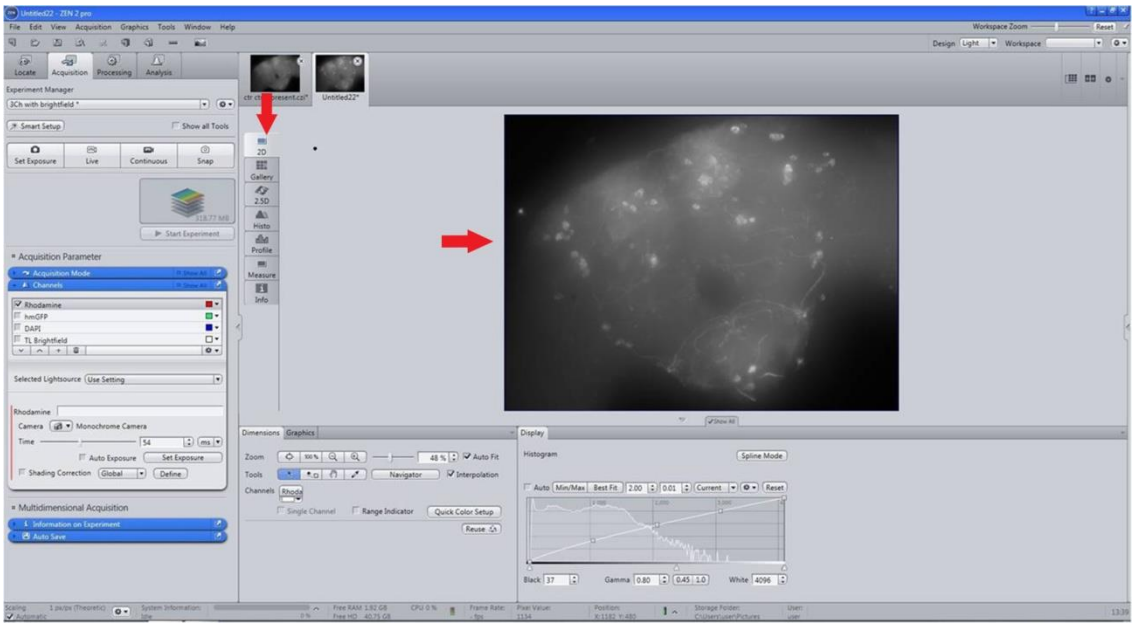


Figure 2.8. Export of 2D brain image to the required format

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 DAergic neurons.

D) Quantification of the fluorescence intensity (FI) of secondary antibodies to characterise neurodegeneration/ neuroprotection

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 α-synuclein-mediated *Drosophila* PD model that were similar to human PD. The DAergic neuronal system and their 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 the **Table 2.2**, and the *Drosophila* brain cartoon (**Figure 2.19A**) 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 2.2: The table briefs the anatomical location and number of DAergic neurons in the *Drosophila* brain, arranged in each hemisphere in different clusters. There is a total of 280 DAergic neurons in the *Drosophila* brain. While 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**		**References**
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 500 µM	-	-	Yes	Yes	Coulom and Birman, 2004
500 µM 500 µM	-	-	Yes	Yes	Coulom and Birman, 2004
	-	-	No	No	Meulener et al., 2005
	-	-	No	No	Navarro et al., 2014
10 µM 500 µM			No	No	Ayajuddin et al., 2022
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 2.3: Summarization 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 2.9**).

2. The image was enlarged to reveal the cell body/structure (**Figure 2.9**).

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.

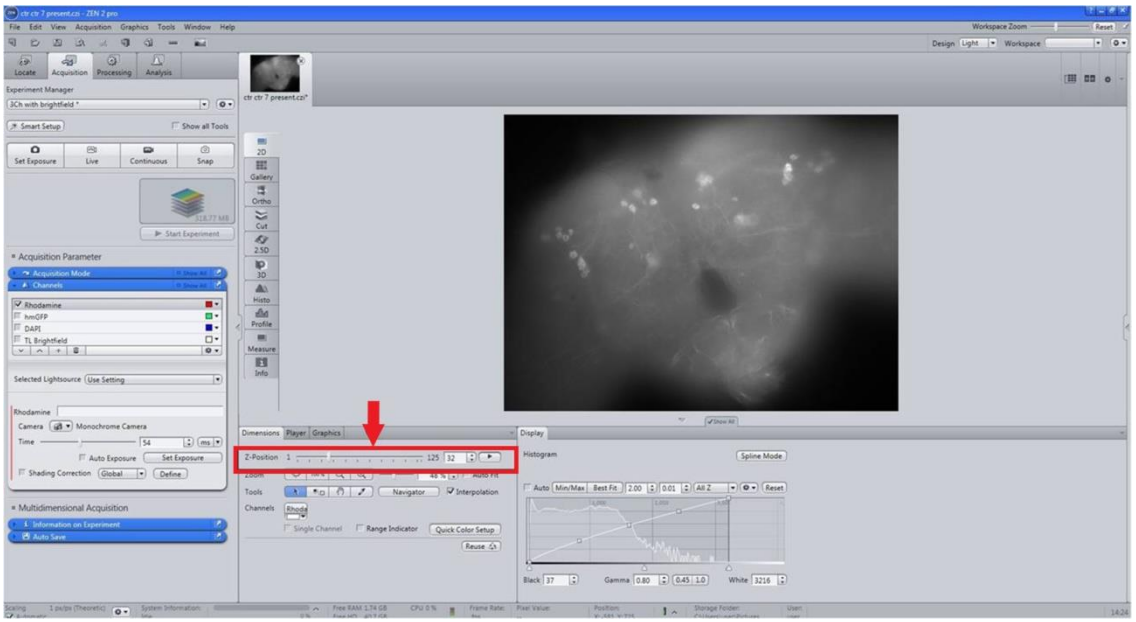


Figure 2.9. Quantification of dopaminergic (DAergic) neuronal number and fluorescence intensity (FI). For the quantification of DAergic neuronal number and FI, select 3D images/scans of Z-Stack with brain regions; PAL, PPL1, PPL2, PPM1/2, and PPM3 (PAL: Protocerebral anterior lateral; PPL: Protocerebral posterior lateral; PPM: Protocerebral posterior medial).

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 2.2). Though, 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 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) here, I attempted to investigate the DAergic neurodegeneration and *Leea asiatica*

(LA) mediated neuroprotection by measuring the FI of the fluorescently labelled 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 2.9**).

2. The brain images were enlarged to see the clear neurites (**Figure 2.10**).

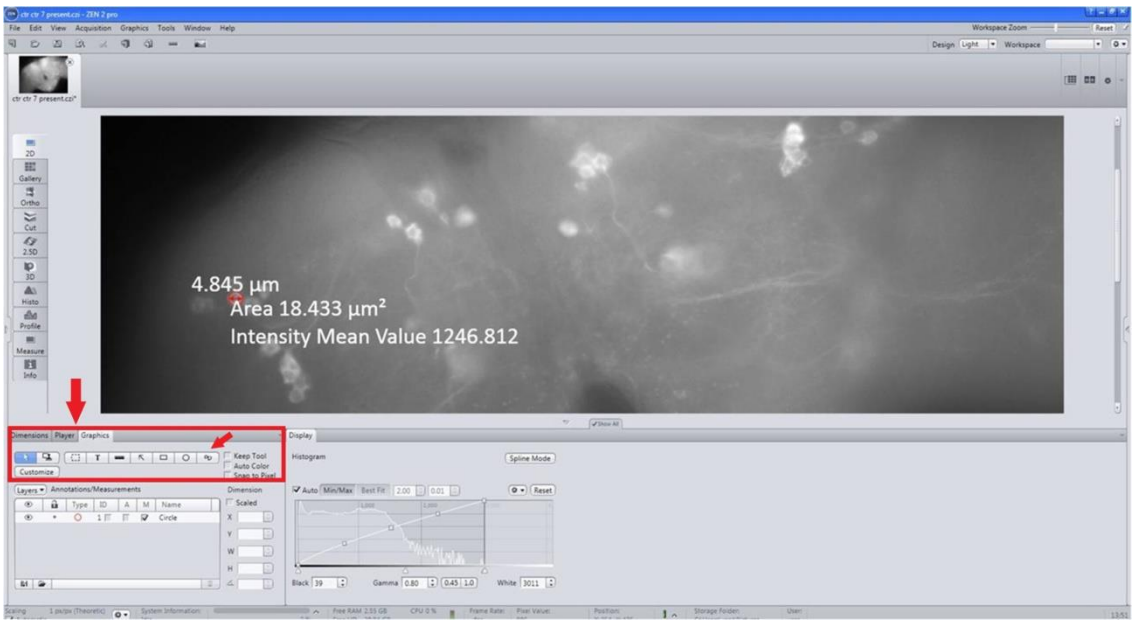


Figure 2.10. Details of the quantification of the fluorescence intensity (FI). Enlarge the images to see clear neurites, select appropriate tools, *draw spline contour* from graphics and draw a line around the neuron, and display intensity mean value and area.

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 2.10**).

4. *More measurement options* were selected and the *intensity sum* was chosen by right-clicking inside the neuron (**Figure 2.11**).

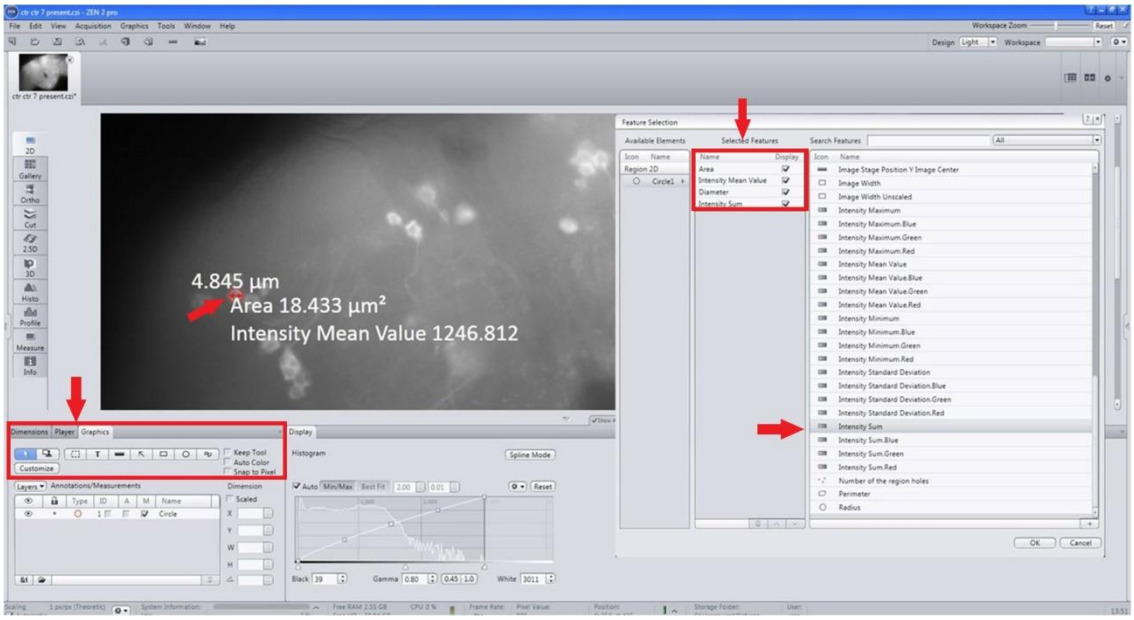


Figure 2.11. Measurement of fluorescence intensity (FI) sum. Select *intensity sum* by opting for *more measurement options* (software provides the pixel value upon right-clicking on the neuron).

5. *List*, *view all*, and *create document* were selected from the *measurement* tab on the left side of the panel (**Figure 2.12**).

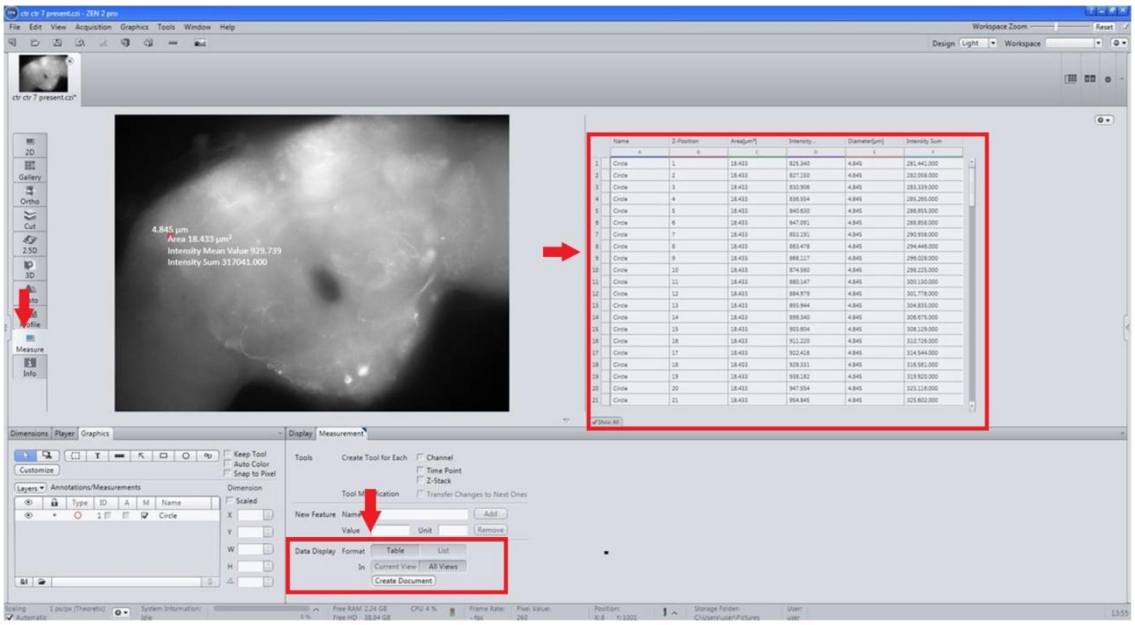


Figure 2.12. Fluorescence intensity (FI) compilation. From the *measurement* option select *list*, *All views*, and *create document*.

6. The area and FI sum were recorded for each scan of a neuron in .xml format (**Figure 2.13**).

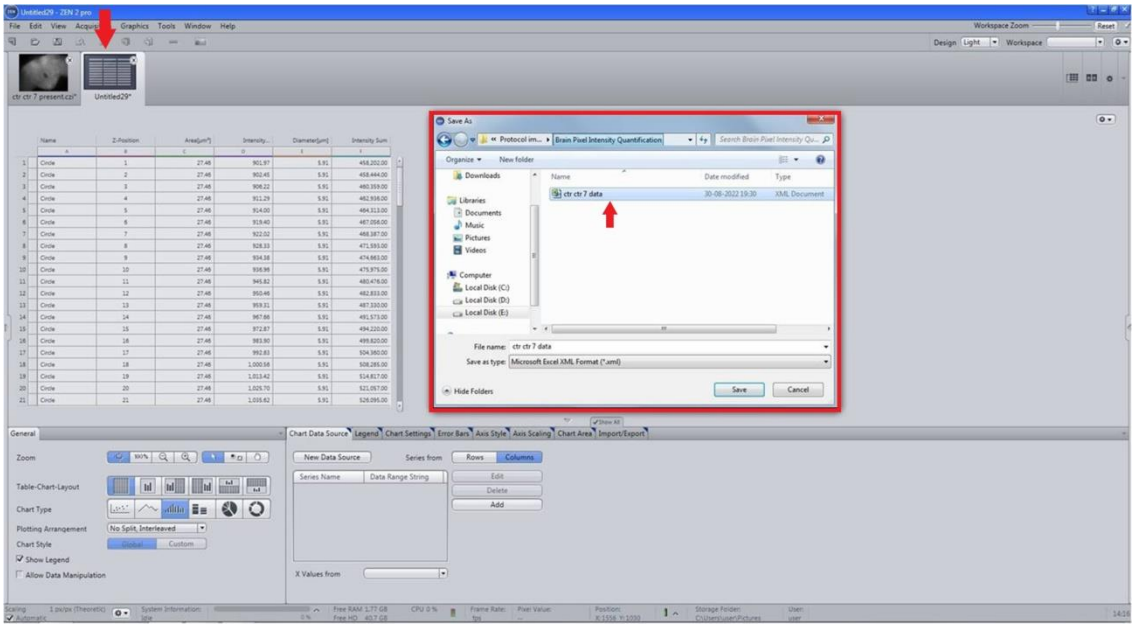


Figure 2.13. Measuring the FI sum for each scan of a neuron in .xml format

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 2.14**).

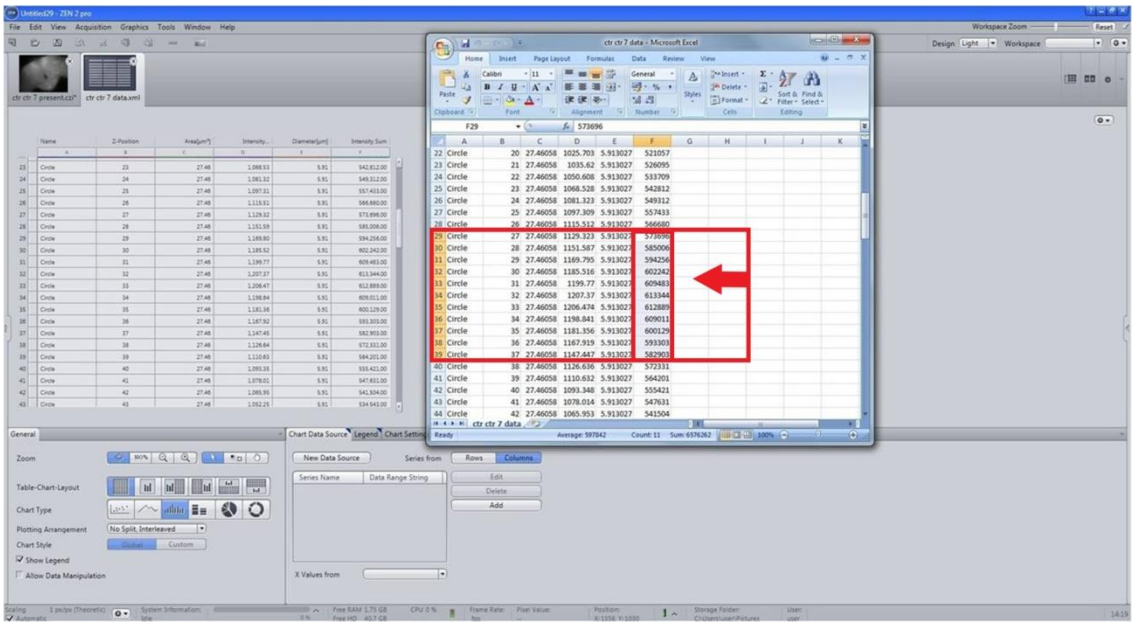


Figure 2.14. Compilation of fluorescence intensity (FI) of a single neuron and all the neurons of a cluster. For the characterization of FI of a single neuron, a total of 11 scans with an interval of 1.08 µm for each scan (cumulative 11.88 µm width) were considered. Take the average and find the standard 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.

2.6. 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.

2.7. Results

2.7.1. Survivability of *Drosophila*

The adult life span of *Drosophila* is typically divided into three stages: a health span, a transition span, and a senescent span (Arking et al., 2002). *Drosophila* 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 OK 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 2.15**; Phom et al., 2014).

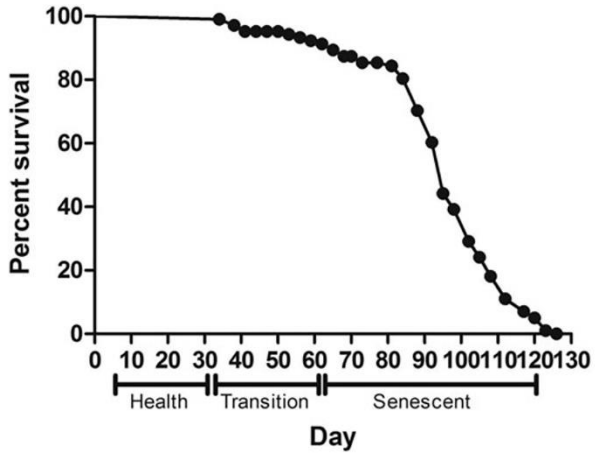


Figure 2.15. Survival curve of OK male flies in standard culture media. The flies were transferred to fresh media on every 3rd day. The mortality was recorded until all of the flies died. Healthspan extends from day 4/5 to 30 days; the transition phase is 31–60 days of adult span, and the senescent span is 61–120 days. The maximum life span is 121 days, and the median is 95 days. (Adapted from Phom et al., 2014)

2.7.2. *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 No. 1. 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 the 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 OS markers linked to PD after 24 hours of exposure, during which only 1%–2% of the flies died (**Figure2.16**; Phom et al., 2014; Phom, 2018).

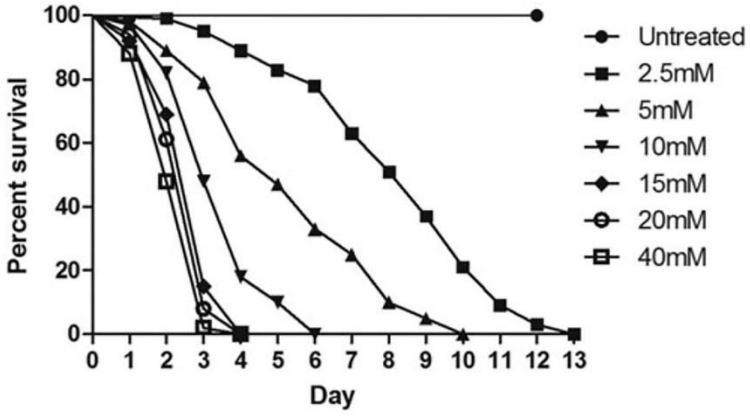


Figure 2.16: Concentration- and time-dependent mortality of *Drosophila melanogaster* (OK) exposed to paraquat (PQ). Adult male flies (4–5 days) were exposed to six different concentrations of PQ (2.5, 5, 10, 15, 20, and 40 mM). PQ exposure induced concentration-dependent lethality. A comparison of survival 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).

2.7.3. *Leea asiatica* (LA) leaf extract rescues the mobility defect induced by PQ under the co-treatment regime during the health phase (HP) in the *Drosophila* model of PD

To evaluate the mobility dysfunction/impairments in the PQ-mediated *Drosophila* model of PD during the HP 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, 48, and 72 hours of exposure to PQ or PQ along with LA (10%, 7.5%, 5%, 2.5%, and

1.25%). After 24, 48, and 72 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 2.17 A, B& C; ***P<0.001; **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 2.17 A, B& C; ***P<0.001; **P<0.001)**, suggesting the neuroprotective efficacy of LA (**Figure 2.17 A, B& C ***P<0.001; **P<0.001**) in *Drosophila* model of PD. 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 PD.

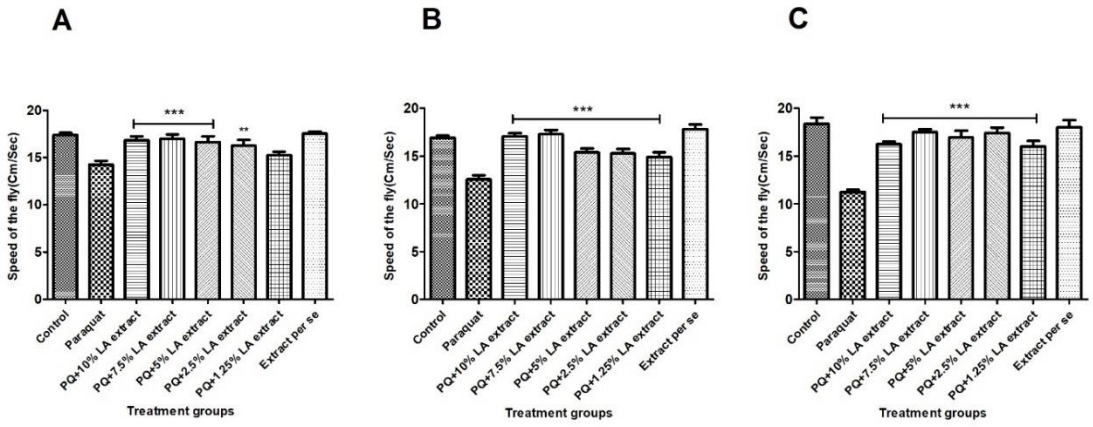


Figure 2.17: Negative geotaxis assay (NGA) for the co-treatment regime in the health phase (HP). LA leaf extract rescues the mobility dysfunction/defects induced by PQ under a co-treatment regime in the *Drosophila* model of PD. 10mM PQ induces mobility defect in *Drosophila* model at different time points, 24, 48, and 72 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**) & 72 hours (**C**). 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.

2.7.4. *Leea asiatica* (LA) leaf extract rescues the mobility defect induced by PQ under the pre-treatment regime during the health phase (HP) in the *Drosophila model* of PD

It is crucial to demonstrate that the neuroprotective effect of LA is not attributable to an antagonistic interaction with PQ. To rule out this possibility, the pre-feeding regime was ritualized, where flies were given LA along with 5% sucrose for 3 days, and switched to 10 mM PQ treatment for 24, 48, 72, and 96 hours. In the pre-treatment regime, flies exhibited similar mobility defects after PQ ingestion (**Figure 2.18 A, B, C & D; ***P<0.001; **P<0.01**) 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 2.18 A, B, C & D; ***P<0.001; **P<0.01)**. LA alleviates mobility defects in both the pre-and co-feeding regimens, as shown by a negative geotaxis assay in the *Drosophila* model of PD **(Figure 2.17 A, B & C; 2.18 A, B, C & D; ***P<0.001; **P<0.01)**. Based on these findings, LA may be effective as a treatment for NDDs such as PD.

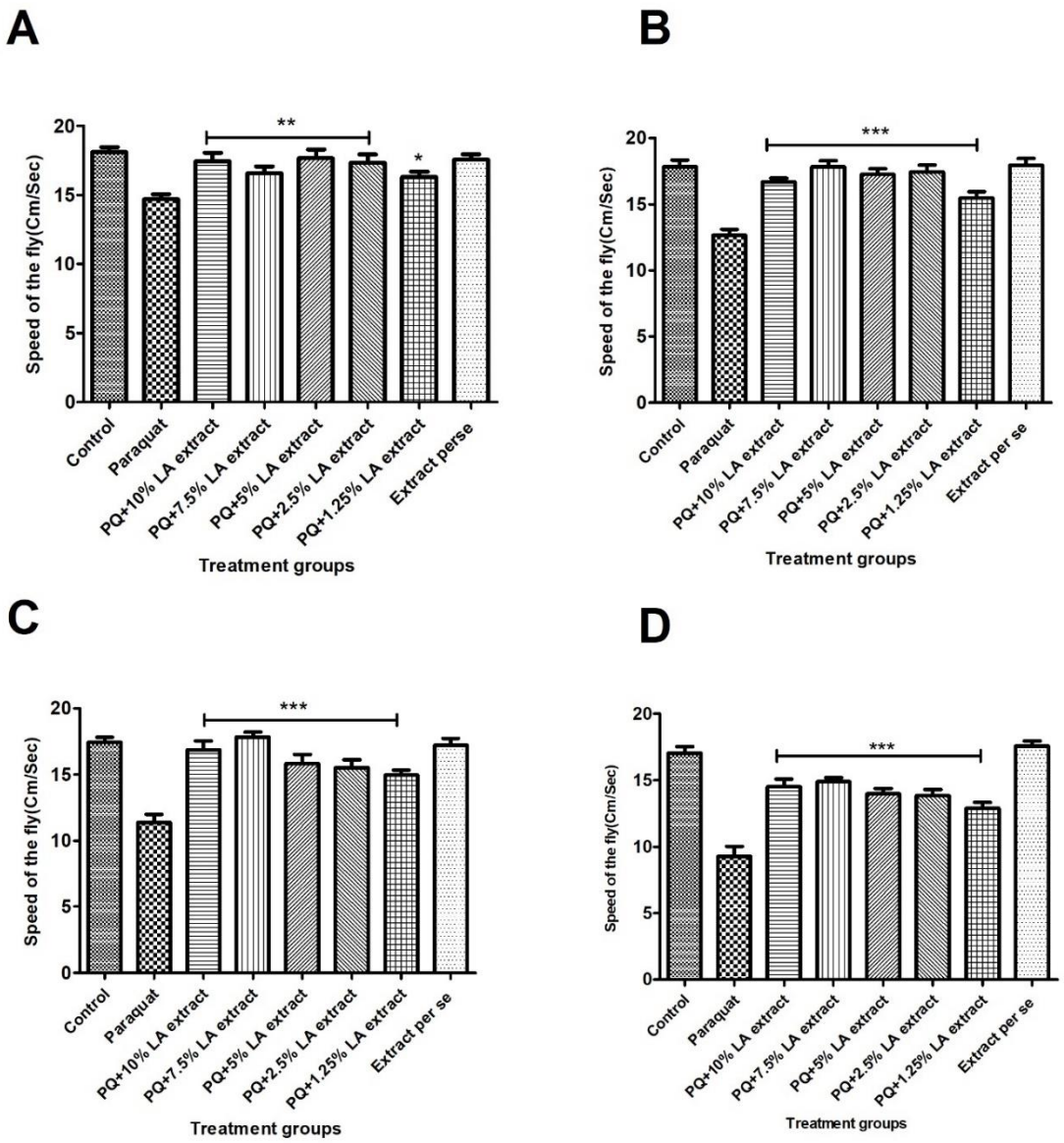


Figure 2.18: Negative geotaxis assay for Pre-treatment regime in HP. LA leaf extract rescues the mobility defects induced by PQ under the Pre-treatment regime in the *Drosophila* model of PD. 10mM PQ induces mobility defects in the *Drosophila* model after 24 hours (**A**), 48 hours (**B**), 72 hours (**C**), and 96 hours(**D**) 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 before PQ treatment for 24, 48, 72 & 96 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.

2.7.5. 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 HP-PD brain

In each brain hemisphere of an adult *Drosophila*, there are six quantifiable DAergic neuronal clusters (**Figure 2.19 A**) (Whitworth et al., 2006; Ayajuddin et al., 2023; Koza et al., 2023). 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 2.19 B**).

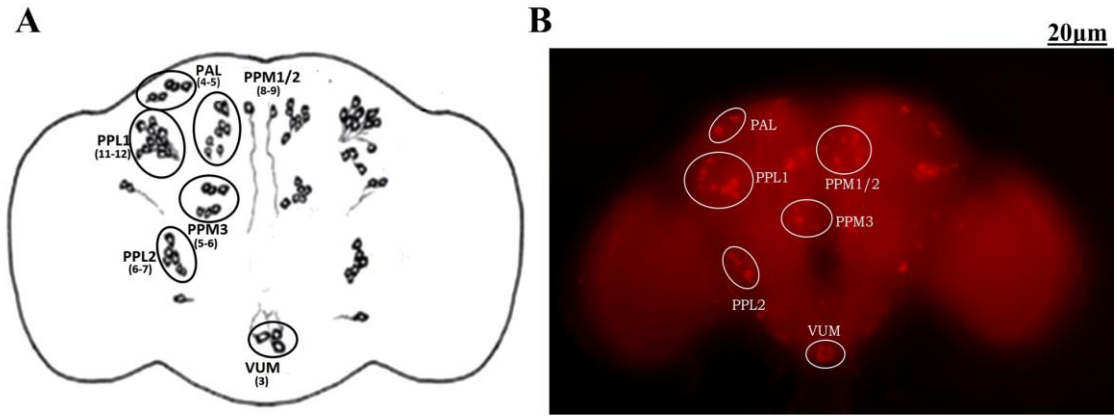


Figure 2.19: **(A)** Cartoon of *Drosophila* brain showing the position of different clusters of DAergic neurons. The brain of *Drosophila* has around 140 DAergic neurons in each hemisphere which are arranged into six different clusters. Some of them are PAL (4-5 neurons), PPL1 (11-12 neurons), PPL2 (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 labelled secondary antibody against the anti-TH primary antibody. There are other regions like 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 mounts of *Drosophila* captured using *ZEN software* of Carl Zeiss Fluorescence Microscope (Axio Imager 2, Carl Zeiss, Germany) (Adapted from Chaurasia et al., 2024).

The images of the various experimental groups in the HP of *Drosophila* brain are shown in **Figure 2.20 (A)**. Results indicate that the number of DAergic neurons quantified cluster-wise and brain-wise amongst all treatment groups evaluated throughout the HP (**Figure 2.20 B&C**) did not significantly differ from the control group. In the HP, the total number of DAergic neurons in the brains of the various treatment groups did not differ from the control group (**Figure 2.20 C**). The FI of the DAergic neurons was further assessed (a secondary antibody that is fluorescently labelled 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 the PAL, PPL1, PPL2, PPM1/2, and PPM3 clusters, the FI of the fly brain(s) DAergic neurons decreased significantly during the HP by about 53.42% (***$p<0.001$), 47.69% (**$p<0.01$), 54.77% (***$p<0.001$), 35.97% (*$p<0.05$), and 43.28% (**$p<0.01$), respectively, when compared to the control under the induced PD condition (10mM) (**Figure 2.20 D**). 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), who 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 HP led to depletion in the FI by around 35-40% (***$P<0.001$) (**Figure 2.20 E**) indicating decreased levels of TH protein synthesis at HP PD brain.

2.7.6 *Leea asiatica* (LA) intervention rescues the DAergic "neuronal dysfunction" by restoring tyrosine hydroxylase (TH) protein level in the HP-PD brain

To get insight into the neuroprotective efficacy of LA at the level of DAergic neurons during HP, LA co-feeding to the experimental groups was investigated. The cluster-wise depletion of FI was seen to be rescued during HP. 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 HP by about 38.48% (*p<0.05), 34.70% and 38.43% (*p<0.05;*p<0.05), 40.94% and 35.27% (*p<0.05; *p<0.05), 44.95% and 36.14% (**p<0.01; *p<0.05), and 66.81% and 83.03% (***p<0.001; ***p<0.001) (**Figure 2.20 D**). Similarly, group-wise merged FI of all the quantifiable DAergic neurons were also investigated upon LA co-treatments. 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 40~42% (***p<0.001) (**Figure 2.20 E**). LA intervention resuscitated the TH depletion significantly (***P<0.001) (**Figure 2.20 E**). These findings suggest that during the HP PD brain, LA can reverse the neurodegeneration brought on by 10mM PQ exposure. Further, these results substantiate the significance of nutraceutical LA in alleviating the DAergic neurodegeneration induced by PQ in the fly PD model. The findings of the current investigation corroborate the results of the negative geotaxis assay which showed that LA corrects the mobility defects during the HP of the *Drosophila* life span/PD brain.

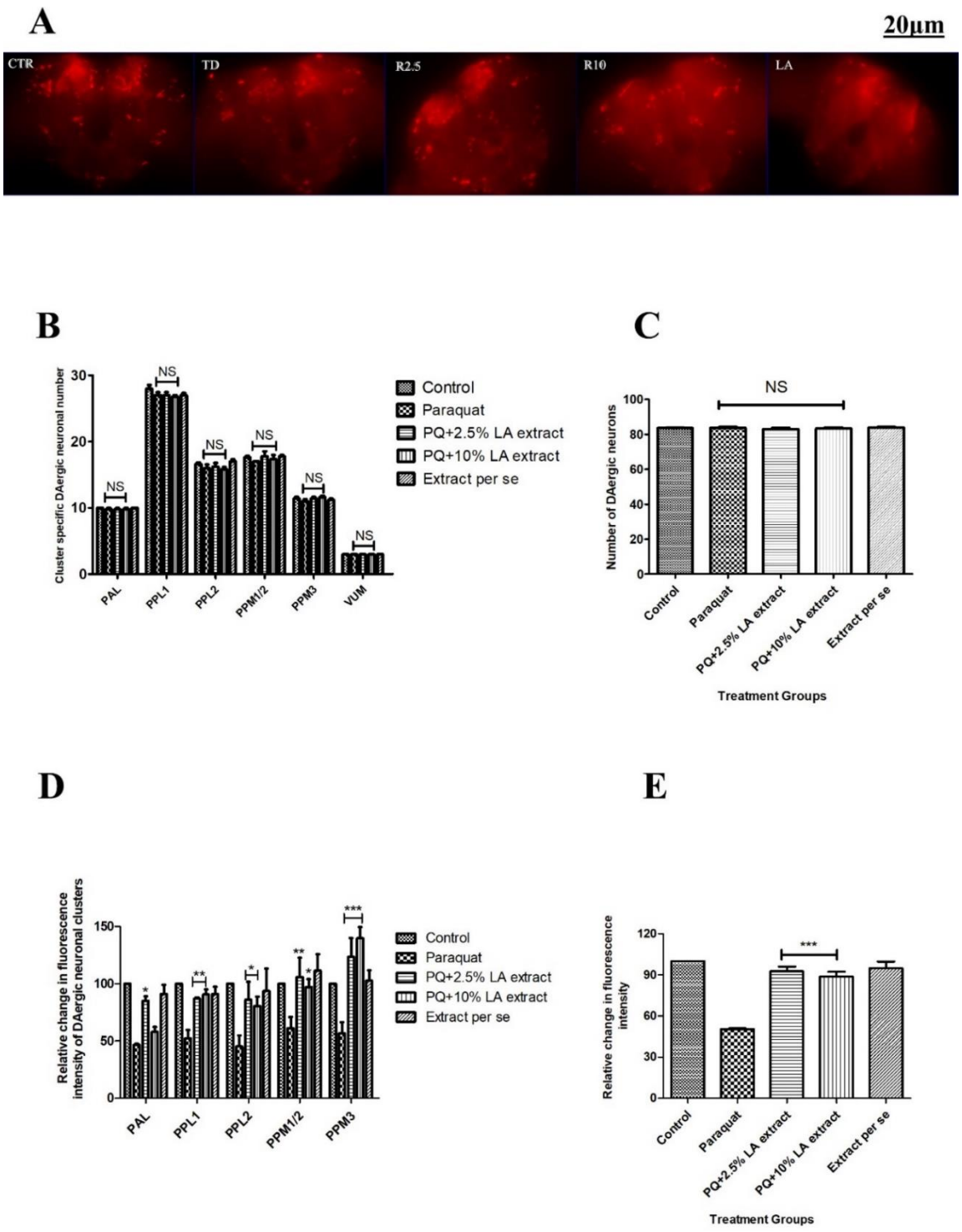


Figure 2.20: Characterization of DAergic neurodegeneration in the whole fly brain of *Drosophila* during the health phase (HP) through anti-TH antibody immunostaining **(A)** reveals that there is no loss in the number of DAergic neurons upon exposure with neurotoxin PQ (10mM) alone and, among all the studied groups **(B&C)**. However, feeding the *Drosophila* during the HP with PQ (10mM) leads to "neuronal dysfunction" characterized by quantification

of DAergic neuronal fluorescence intensity (FI) that is directly proportional to the amount of TH protein synthesis by around ~40-50 %, which could be significantly rescued upon co-feeding with LA **(D&E)**. The reduction in fluorescence intensity (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 brains 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+7.5% LA Extract; Perse7.5% - Sucrose + LA extract alone).

2.8. Discussion

Loss of DAergic neurons in *SNpc* remains the main determinant of PD in humans. Studies have shown that the fly model of PD likewise showed gradual age-tied mobility deficit, and a decline in the DA level of the brain when the DAergic neurons start to disintegrate (Feany and Bender, 2000; Chaudhuri et al., 2007; Ayajuddin et al., 2022). So, using fluorescence microscopy, the entire DAergic neurons present throughout the brain of *D. melanogaster* were quantified before "neuronal dysfunction" was defined. After quantifying the numbers of DAergic neurons, "neuronal dysfunction" (if any) was identified by measuring the FI of a secondary antibody that binds the anti-TH primary antibody specifically. So, the amount and synthesis of TH will directly be reciprocated through the FI that is emitted. Through this study, the level of DAgeric neurons can be evaluated, and at the same time by inducing the PD brain conditions the dysfunction can be analyzed. Furthermore, the therapeutic efficacy of *L. asiatica* can be assessed. The outcome result shows, that the numbers of DAergic neurons of the HP remain unchanged in both the control and induced PD brains (**Figure 2.20 B&C).** This insight aligns with a previous definitive result where there was no diminishment observed in the neuronal

number (Menzies et al., 2005; Meulener et al., 2005; Pesah et al., 2005; Navarro et al., 2014; Ayajuddin et al., 2022; 2023). In light of the "dying back" phenomenon, which posits that neurodegeneration begins at the farthest end of the neuron, called the axonal terminus, and advances towards the cell body, all of these results are coherent. (Wong et al., 2019). This further clarifies why the popular L-Dopa supplementation is often ineffective and with long-term use, leads to dyskinesia, irregular activation of DA receptor and signalling pathways, and toxicity from plasma L-DOPA due to irregular absorption (caused by DAergic neuronal terminals degeneration of axons) (Nakmodde et al., 2023). Ayajuddin et al. (2022) demonstrated that in a mitochondrial complex I inhibition-mediated PD model, there is a significant alteration in the level of TH protein synthesis, while the number of DAergic neurons remains unaffected in the HP PD *Drosophila* brain. In *Drosophila,* the loss of DAergic neurons in the fly PD models has been a topic of controversy. Initial investigators, Feany and Bender (2000) showed that there was a loss of DAergic neurons at an adult onset in the *Drosophila* model of PD. Subsequently, many researchers used that fly model to delve into and depict the different degrees of DAergic cell death in different DAergic clusters (Auluck et al., 2002; Chen and Feany, 2010; 2005; Pesah *et al.*, 2005; Cooper et al., 2006; Trinh et al., 2008; Barone et al., 2011; Song et al., 2017; Sur et al., 2018; Bordet et al., 2021; Maitra et al., 2021; Rai and Roy, 2022). Auluck and Bonini (2002), Auluck et al. (2002), and Yang et al. (2003) carried out studies using the same fly model, and all of them observed a 50% loss of DAergic neurons in the fly brain. 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 (Cha et al., 2005; Whitworth et al., 2005; Trinh et al.,2008, 2010; Kim et al., 2012). Conversely, Pesah et al., 2004 detected no neuronal loss in the PPM1/2 cluster, suggesting that not all but a particular DAergic cluster in *PARKIN* may be sensitive to degeneration. Considerable differences have been observed in

studies utilizing the *PINK 1 Drosophila* model of PD. These differences include a substantial reduction in neurons across multiple DAergic clusters in RNAi knockdown flies (Park et al., 2006) to a discrete loss of two to four neurons in the PPL1clusters in a null mutant (Wang et al., 2006; Yang et al., 2006). Apart from genetic models, toxicity-induced PD models, or PQ-based models have garnered significant attention by offering insights into different degrees of DAergic neuronal degeneration. Exposure to 5mM PQ for 12-48 hours, by Maitra et al. (2019; 2021) and Chaouhan et al. (2022), indicates the loss of DAergic neurons in the PPM and PPL1 clusters in the *Drosophila* model of PD. A subsequent investigation by Shukla et al. (2014) showed that flies treated with 10mM and 20mM PQ for 24 hours experienced a cluster-wise selective loss of DAergic neurons. Furthermore, in *HSP70*-expressed flies, PQ-induced cluster-wise selective loss of DAergic neurons is inhibited for 12 and 24 hours, respectively, by *HSP70* overexpression (Shukla et al., 2014). Similar to this, mutant flies with decreased *Aux* expression exhibited changes in the PPM ½ cluster neuronal count, which could be caused by α-synuclein toxicity (Song et al. 2017). Analogously, it has been discovered that loss of Catsup function which is an ortholog of *Drosophila* to mammalian zinc transporter *SLC39A7*(ZIP7), postpones the death of DAergic neurons following exposure to PQ (Chaudhuri et al. 2007). In the study carried out by Inamdar et al., (2012), the DAergic neuronal clusters ((PAL, PPL1, PPM2, and PPM3) showed statistically significant neuronal loss, after the fly received PQ exposure for 24 hours. On extending the period of PQ exposure up to 48 hours, neurodegeneration in PPM1 and PPL2 was also observed, while the already afflicted neuronal clusters continued to deteriorate (Inamdar et al., 2012). The PQ-induced fly model showed no change in the neuronal numbers (Navarro et al., 2014; Ayajuddin et al., 2023) or specific loss of DAergic neurons at various concentrations of the toxin (Chaudhuri et al., 2007; Wang et al., 2007; Inamdar et al., 2012; Shukla et al., 2014; Maitra et al., 2019, 2021; Chaouhan et al., 2022).

Therefore, it may be considered that the research on the loss of DAergic neurons in the *Drosophila* model of PD undertaken in the past and those conducted in the present are inconsistent or contradictory. This matter was previously thoroughly investigated in several genetic and sporadic fly models of PD, and it was discovered that there is no loss in the structure of DAergic neurons, just a reduction in the production of GFP (GFP reporter) / TH protein, which is correlated with a decline in the FI of neurons (Navarro et al. 2014; Ayajuddin et al., 2022; 2023). In this conducted study, flies treated with 10mM PQ alone resulted in a significant decrease in the FI in all the studies of DAergic neuronal clusters. This reduction in the FI could be significantly reversed when LA was co-fed to the flies during the HP **(Figure 2.20 D)** of the *Drosophila* PD brain. These results were further assessed when the total FI of all the DAergic neurons in the fly brain(s) of the various experimental groups was quantified **(Figure 2.20 E)**. These groups were analyzed independently and produced comparable outcomes. Because the fluorescence of the neuronal cell body strongly correlates with the rate at which the rate-limiting enzyme TH is synthesised, decreased levels of FI reflect reduced levels of TH protein synthesis (TH signals). According to the current study in the *Drosophila* model PD, "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 HP of the *Drosophila* PD brain.

2.9. Conclusion

The findings of the present investigation demonstrate that LA mediates neuroprotection in the *Drosophila* PD model, indicating its potential upstream role in DA anabolism, as suggested by the observed restoration of reduced TH levels. Additionally, the impact of LA on DA

catabolism and turnover rate warrants further investigation, as it may provide insights into its efficacy in DAergic neuroprotection. Therefore, in Chapter 3, DA catabolism and oxidative metabolism to its immediate downstream metabolites (DOPAC and HVA) will be analyzed using high-performance liquid chromatography with electrochemical detection (HPLC-ECD). This will deepen our understanding of the neurochemical mechanisms underlying “neuronal dysfunction” and LA-mediated neuroprotection.

Chapter 3

Leea asiatica (LA) mediated alteration of Brain Dopamine (DA) Metabolism in the *Drosophila* Model of Parkinson's Disease; Implications to its therapeutic efficacy

3.1. Introduction:

DA is a monoamine brain neurotransmitter with a multifaceted role in both physiological and psychological processes. In 1910 DA was first synthesized and described as a weak sympathomimetic (Barger and Dale, 1910). Over the following four decades, it was largely disregarded until Katharine Montagu's research in 1957 revealed that DA naturally existed in the brain (Montagu 1957). Soon after, it was discovered that DA functions as a neurotransmitter and was seen distributed variably across the brain, plasma, and other parts of the body tissues of humans (Carlsson et al., 1957, 1958; Weil-Malherbe and Bone, 1957; Imai et al., 1970). A pivotal study by Ehringer and Hornykiewicz 1960 showed that DA deficiencies in Parkinsonian were linked to the loss of extrapyramidal activity, solidifying its role in PD (Ehringer and Hornykiewicz, 1960, 1998). DA-producing neurons were first mapped by Annica Dahlström and Kjell Fuxe in 1964 (Dahlström and Fuxe, 1964; Andén et al., 1966). DA is certainly one of the oldest neurotransmitters, its role is remarkably conserved in almost all animal species, from roundworms to humans (Yamamoto, K., and Vernier, P. 2011; Costa, K. M., and Schoenbaum, G. 2022). DA has a wide range of roles, and its levels must be tightly regulated. Both elevated and DA levels are associated with pathological conditions. Although DA is crucial in the brain, a significant portion of its production occurs outside the brain, particularly in the mesenteric organs (Eisenhofer et al., 1997). In the brain, it is synthesized by DAergic neurons in the substantia nigra (SN), which have axonal projections to the striatum. DA is released from the presynaptic membrane into the synaptic gap, where it binds to and activates DA receptors on the postsynaptic membrane. It regulates movement, learning, memory, cognition, sleep, emotion, and pleasurable reward (Zhou et al., 2023; Tai et al., 2024). In addition, studies conducted in the last few decades have shown that DA significantly affects immune cell function in both the peripheral blood and central nervous system (Channer et al., 2023). DA exerts its effects through G-protein-coupled receptors (GPCRs), which are divided

into D1-like (D1, D5) and D2-like (D2, D3, D4) receptor families, based on their regulation of the second messenger cAMP (cyclic adenosine monophosphate) (Seeman and Van Tol, 1994; Beaulieu and Gainetdinov, 2011; Channer et al., 2023; Lauretani et al., 2024). DA contains 80% of catecholamines in the CNS, even though DAergic neurons make up less than 1% of all cell subtypes in the brain (Lauretani, et al., 2024). The loss of these neurons is directly linked to PD. In healthy ageing human beings, the gradual natural degradation of neurons is reported to be 4 % (Fearnley and Lees, 1991). In PD patients, the death of neurons is aggravated, with a 70% reduction in the terminal and a 40- 50% in the striatal DAergic neurons compared to the age-matched healthy individuals (Cheng et al., 2010). In addition, people who had a family history of PD or were at risk of developing the disease had lower levels of DA in their cerebrospinal fluid (CSF) (Goldstein et al., 2018). These results unambiguously imply that dysregulated DA is not the only contributor to motor and non-motor behaviours linked to PD, but also that an early reduction in DA levels may precede the onset of PD. There is more than one manner in which DAergic neurodegeneration and DA level depletion are related. Currently, it is unknown how or why the DAgeric system is selectively (or mainly) affected in PD. In recent years, a prominent hypothesis has emerged to explain the increased sensibility of DAergic neurons in PD pointing to DA metabolism as a central factor for the progressive loss of DAergic neurons in familial as well as sporadic cases of the disease (Bayersdorfer et al., 2010). It is claimed that the degeneration of DAergic neurons in certain conditions is caused by impaired DA metabolism and oxidation, which can exacerbate the release of ROS. (Masato et al., 2019). DA is synthesized in the cytosol by TH (the rate-limiting enzyme) which converts the amino acid tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA). L-DOPA can then be converted into DA by the action of aromatic amino acid decarboxylase (AADC) (Klein et al., 2019; Chakrabarti and Bisaglia, 2023). When DAgeric neurons are at rest, vesicular monoamine transporter 2 (VMAT2) in the cytosol moves and stores produced DA in vesicles

with the help of a vesicular ATPase-dependent H^+ gradient. Under neuronal stimulation, DA is released from pre-synaptic membrane vesicles to synaptic clefts, where it binds to and activates post-synaptic DA receptors (Zhou, et al., 2023; Yi, et al.,2024). The presynaptic neuron can reabsorb excess DA, which MAO can then catabolize to create DOPAC. Moreover, DOPAC can be transferred to astrocytes, a particular type of glial cell, where it will undergo additional degradation by COMT activity to produce HVA. (Winner et al., 2017).

Although DA dynamics and signalling in *Drosophila* and mammals are nearly identical, there are three main differences: viz. Mammalian DA metabolism solely employs oxidation and methylation, while in flies, N-acetylation and β-alanylation can also be employed. Moreover, flies do not possess the genes required to manufacture norepinephrine and epinephrine from DA. Lastly, DA is essential for the synthesis of epidermal melanin in flies but not in mammals (Yamamoto and Seto, 2014).In flies, it is reported that acetylation by Dopamine N-acetyltransferase, also known as arylalkyl amine N-acetyltransferase (aaNAT) (Paxon et al., 2005), and enzymes coded by *pale*, *ebony*, *tan*, is one mechanism by which DA is metabolized in flies (Wright, 1987). In flies, NADA (N-acetyl dopamine) and NBAD (N-β-alanyl dopamine) are the end products of DA breakdown. The enzymes like Tan can revert NBAD back to DA (Yamamoto and Seto, 2014). Fly epidermal cells and the brain share a similar DA metabolic cycle. From DA to NBAD, NADA is generated in epidermal cells and is then transferred to the cuticle for hardness and coloring. In contrast, glial cells in the fly brain break down DA to produce NBAD and NADA, and DAgeric neurons convert NBAD back to DA. It is noteworthy that *Drosophila* still lacks an orthologue for the COMT coding gene and the orthologue of the human MAO gene. However, in addition to NBAD and NADA, fly brains have been found to contain DA oxidative products such as DOPAC and HVA (Zhang et al., 2005; Chaudhuri et al., 2007; Wakabayashi-Ito et al., 2011; Freeman et al., 2012). This finding raises the possibility that the fly brain has metabolic components and pathways similar to those

seen in mammals (Yamamoto and Seto, 2014). A study conducted by Stefani et al., (2017) reports that CSF DOPAC and HVA levels were increased in patients with mild PD symptoms. The increase in DA metabolites is directly correlated to motor impairment (Stefani et al., 2017). Further in PQ-induced sporadic fly and mice models of PD, DA depletion in brain tissue was accompanied by enhanced DA degradation to its metabolite(s) as reported in various independent studies (Rudyk et al., 2015; Shukla et al., 2014; Inamdar et al., 2012; Chaudhuri et al., 2007). These models further illustrate the importance of looking into DA catabolism together with the DA pool in the brain to understand PD pathogenesis.

The most reliable method for determining the concentration of catecholamines in a model system is the HPLC-ECD. SDS-PAGE (Sodium dodecyl sulfate–polyacrylamide gel electrophoresis) (Rees et al., 2007) and the protein pull-down assay (Plotegher et al., 2017; Liu et al., 2014) are used to quantify catechol-modified proteins. O-quinones and other charged proteins are also found in cells and tissues, and NIRF (Near-infrared fluorescence imaging) scanning is used to identify and measure them (Jinsmaa et al., 2018; Burbulla et al., 2017). The optimum method for catecholamine measurement is HPLC-ECD because all other methods besides HPLC-ECD are less sensitive. The benefits of HPLC-ECD are its speed, and accuracy in detecting catecholamines that are particular to the brain. Further, greater adaptability for detecting additional related catecholamines cements the technique's flexibility (Allen et al., 2017).

In Chapter 2 it was demonstrated that PQ-mediated PD onset and progression underlies "neuronal dysfunction" during the health phase. Further, *Leea asiatica* (LA) -mediated neuroprotection involves the inhibition of neuronal dysfunction in HP. The process of neuronal dysfunction is a term coined to describe the diminished synthesis of TH which is the rate-limiting enzyme for DA anabolism (Navarro et al., 2014). Therefore, employing the HPLC-

ECD method, the objective of current Chapter 3 is to decipher the effect of neuronal dysfunction and HP neuroprotection concerning the DA pool in the fly brain. Further, as the role of DA catabolism is evident in NDD, I also tried to investigate the DA catabolism associated with PD symptoms and LA-mediated HP neuroprotection.

3.2. Material and Methods

3.2.1. Modelling PD in *Drosophila* and therapeutic intervention

(Described in Chapter-2 please refer the section/subheading 2.2.3 to 2.2.5)

3.2.2. 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. 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 were done following the protocols of Ayajuddin et al., (2021).

Catecholamine quantification

Dopamine (DA, Sigma-Aldrich, Cat: H8502); 3,4-Dihydroxyphenylacetic acid (DOPAC; Sigma-Aldrich, St. Louis, USA, Cat: 11569); Homovanillic acid (HVA; Sigma-Aldrich, St. Louis, USA, Cat: 69673); Phosphate-buffered saline (PBS; HiMedia, Thane, India, Cat: ML-023); Trichloro Acetic Acid (TCA; SRL, Mumbai, India, Cat: 204842); MDTM mobile phase (Thermo-scientific, Waltham, USA, Cat: 701332); HPLC grade water (JT Baker, Radnor Township, USA, Cat: 4218-03); Acetonitrile (JT Baker, Radnor Township, USA, Cat: 9017-03); Methanol (JT Baker, Radnor Township, USA, Cat: 9093-68) were used for quantification

of DA and its metabolites using HPLC-ECD 3000 RS system (Thermo-scientific Dionex Ultimate 3000).

Miscellaneous: Sterilized Eppendorf tubes, Pipette (cleaned with 70% ethanol before and after use), Sterilized pipette tips, Nanodrop® 2000c Spectrophotometer (Thermo- Scientific, Waltham, USA).

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 health 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 equipment 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 3.1. For loading the standard, 200 ng/L of the concentration was used.

Standard	PBS	Concentration	Stock Name
2 mg	2mL	1000 µg/mL	S
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 3.1: 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 a 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

1. On the ice, the tissues were homogenized and sonicated (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 were 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. Fresh pipette tips were used to prepare serial dilutions of the standard and to transfer tissue extract.

C) Quantification of protein:

The 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 3.2**, 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 3.2: 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 minutelevel 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	:	40C
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:

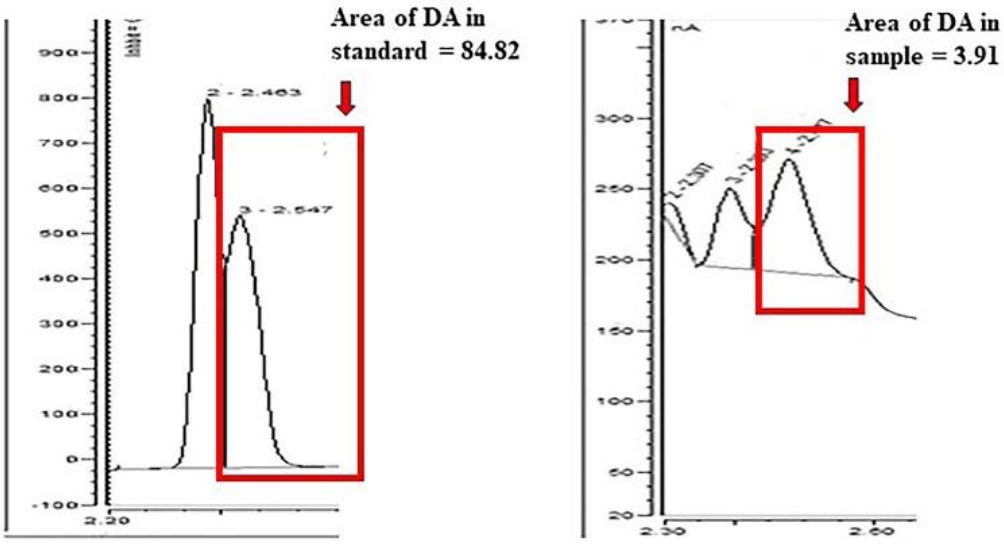


Figure 3.1: Image of chromatogram showing the area of the standard and sample

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 3.1**)

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 HP_{Samp} = 0.143µg/ µL.

viii. The following steps were followed for calculating the actual amount of the catecholamines in tissue extract (Table 3.3).

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 X20)/1000 = 4 ng	$DOPAC_{Std}$ XI_{Std}/1000 i.e. (200 X20)/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= 0.18ng	(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 X4)/60.41 = 0.65 ng ,
Step III: Determining the total protein in 50 µL that was injected into column	HP_{Samp} X I_{Samp}) i.e. (50 X 0.143) = 7.15µg	(HP_{Samp} X I_{Samp}) i.e. (50 X 0.143) = 7.15µg	(HP_{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] = 0.025ng	[0.15/7.15] = 0.021ng	[0.65/7.15)] = 0.091 ng
Step V: Determining the actual amount of catecholamine per head as injected tissue	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

extract solution had brain tissue extract + TCA in 1:1 ratio			

Table 3.3: Steps for calculation of the amount of catecholamines for single fly brain.

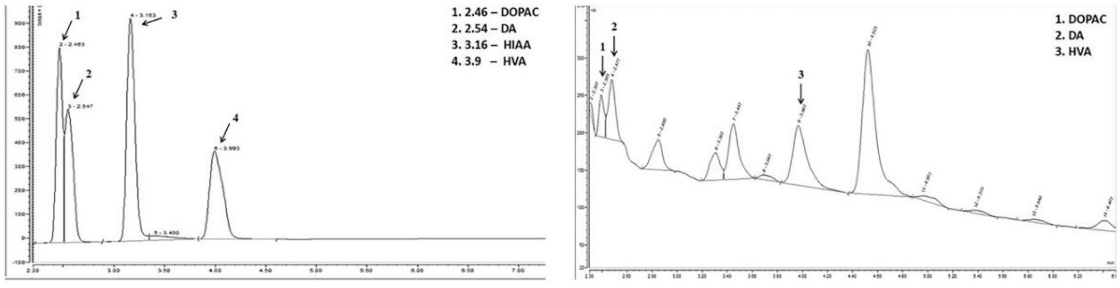
3.2.3. Statistical analysis

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.

3.3 Results

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

To understand DA metabolism, the concentration of brain DA and its metabolites (DOPAC and HVA) were evaluated using the standard and sample chromatogram that was obtained from the HPLC-ECD equipment (**Figure: 3.2**)



(A) (B)

Figure 3.2: 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.

In the fly PD brain, the DA level was reduced by 32.48% (**P<0.01) (**Fig 3.3A**). As seen in chapter 2, the whole brain immune staining for the quantification of DAergic neurons has shown reduced FI on exposure to PQ which reflects the reduction in the TH protein synthesis and neuronal dysfunction (**Fig 2.20E**). So, a reduction in the TH level is directly correlated to a reduced DA level. Phom et al. (2014) have demonstrated that the peak of these events may lead to motor defects in the PD model (Phom et al., 2014). The immediate metabolite of DA, i.e., DOPAC level was also reduced in the HP PD brain by 19.53% (**P< 0.01) (**Figure 3.3 B**). Reduced DA and DOPAC levels are also observed in the post-mortem brains of PD patients (Goldstein et al., 2011). HVA is the final product of DA metabolism, wherein the *Drosophila* through the MAO/COMT analogous pathway, DA and DOPAC can be degraded to HVA (Yamamoto and Seto, 2014; Meiser et al., 2013). Hence, HVA levels were also measured and it was found that in PD brain HVA level is increased by 37.39 % (*P<0.05) (**Figure 3.3C**). The DA depletion was seen to be higher compared to DOPAC and HVA. This suggests higher DOPAC and HVA synthesis than DA in the PD brain. Higher synthesis of DA downstream DOPAC and HVA in the PD brain suggests that these monoamines might have a role in PD onset and progression as they are considered endogenous neurotoxins.

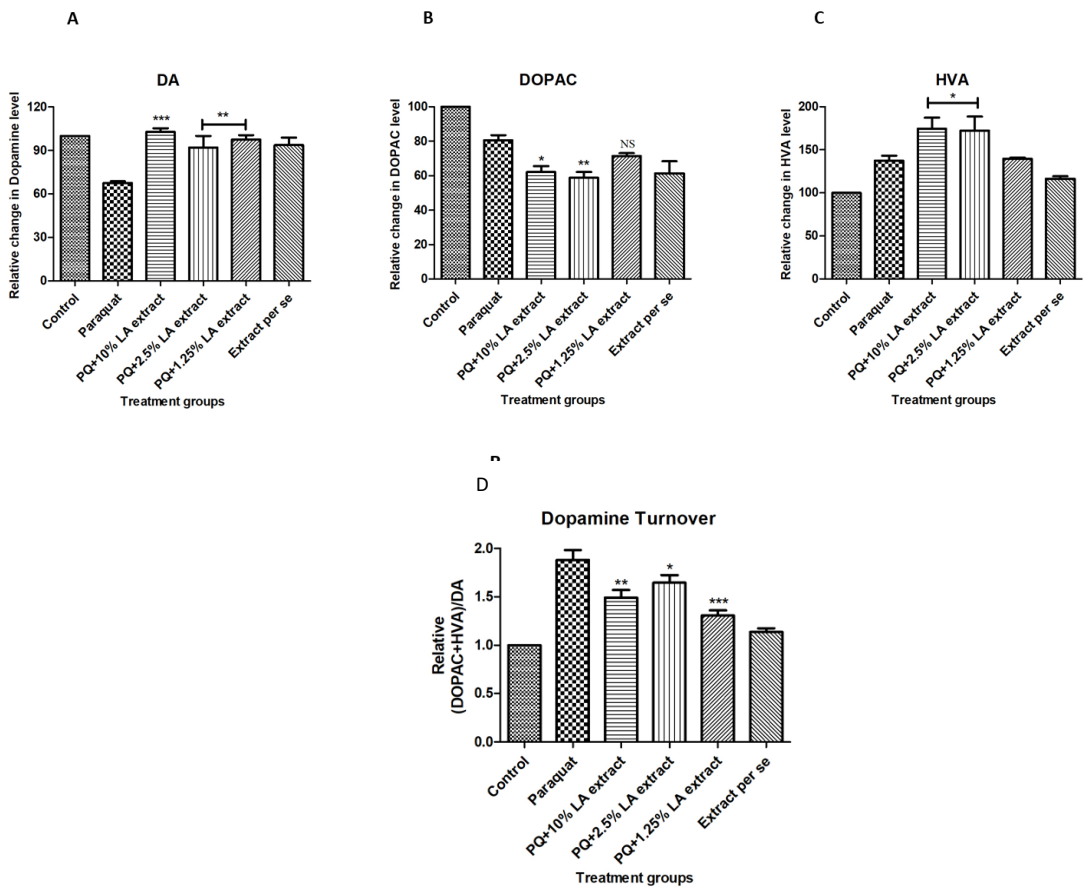


Figure 3.3: Quantification of DA and metabolite levels in fly brain using HPLC-ECD. During the HP, feeding the flies with PQ alone led to a significant reduction in brain DA, and DOPAC, and a significant elevation of HVA levels (**A**), (**B**), & (**C**) respectively. The level of DA depletion is higher compared to downstream metabolite DOPAC and HVA, suggesting an enhanced DA turnover rate (**D**). LA aqueous extract intervention during HP showed a significant increase in the DA level (**A**), it further diminished the level of DOPAC (**B**) but increased in HVA level (**C**). The intervention was able to significantly prevent DA turnover (**B**). Insight suggests that the aqueous leaf extract of LA was able to ameliorate DA depletion and inhibit DA turnover rate in the HP PD brain. Significance was drawn by analyzing the data of a minimum of three replicates with one-way ANOVA followed by Tukey Post-Test for each age group. [*p<0.05; **p<0.01; ***p<0.001; NS: Not significant - compared to PQ treated group].

3.3.2. *Leea asiatica* leaf extract replenishes diminished DA levels and adjust DOPAC but not HVA level in the HP-PD brain

On co-feeding with LA the motor deficit was shown to be rescued in the *Drosophila* model of PD (**Chapter 2**). In the present study, LA co-feeding could rescue diminished DA levels (***P<0.001, **P<0.01) which were depleted in the PD brain (**Figure 3.3A**). The DOPAC (Depleted in the PD brain) was further inhibited (**P<0.01, *P<0.05) (**Figure 3.3B**), whereas the elevated HVA level in the PD brain was further increased (*P<0.05) (**Figure 3.3C**). Interestingly, in HP PD brain neuroprotection by LA extract intervention involved the rescue of diminished DA level, whereas DOPAC level was inhabited and HVA level was further enhanced. The insights suggest that LA extract-mediated neuroprotection in early-onset PD may be through modulating DA metabolic pathway. To further investigate the implication of LA extract in DA metabolism and neuroprotection I looked into the DA turnover rate in the *Drosophila* model of PD.

3.3.3. *Leea asiatica* (LA) rescues enhanced levels of DA turnover rate in the HP *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 *Drosophila* model of the PD brain was found to be higher than in the healthy brain (**Figure 3.3 D**). It implies that in the *Drosophila* model of the 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 in the *Drosophila* model of PD brain (***p<0.001; **P<0.01; *P<0.05) (**Figure 3.3 D**). Overall insight implies that 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-onset PD.

3.4. Discussion

Results demonstrated that neurotoxicant exposure leads to the depletion of DA levels in the HP-PD brain (**Figure 3.4**). The depletion of DA level in the brain of HP is accompanied by the moderate depletion of DOPAC (**Figure 3.4**) and increment of HVA levels (**Figure 3.4**), resulting in increased DA turnover in the PD brain of HP (**Figure 3.4**). In the young mice (6-7 weeks old) extra nigrostriatal DA in nuclear accumbens depletes under PQ-mediated stress. In the same region of the brain, depleted DOPAC levels and enhanced HVA levels were observed with increased DA turnover when PQ-intoxicated mice were further subjected to psychological stress (Rudyk et al., 2015). Motor and non-motor symptoms of PD patients are further aggravated by the psychological stress resulting in depression. The study by Rudyk et al., (2015) in a mice model demonstrated that enhanced HVA level with decreased DA, DOPAC level, and enhanced DA turnover in some extra nigral brain regions is associated with PD mice having psychological impairment. In the current study on *Drosophila* with PQ intoxication alone, the HP PD brain exhibits similar changes in the monoamine pools with increased DA turnover (**Figure 3.4**). Further insight is needed to conclude if such change is associated with the onset of psychological disorder in the fly model along with observed PD motor symptoms. In fly models, it was observed that 10 mM or 20 mM PQ exposure on filter paper for 24 hrs reduces DA level and enhances DOPAC level in the brains of adult young flies (2-4 days old) belonging to CS and white eye strains {*y W1118, Df(1)w,y*} (Inamdar et al., 2012, Chaudhuri et al., 2007). The enhanced DOPAC level and lower DA level with the neurotoxicant exposure were postulated to be the enhanced oxidation and degradation DA. In the present study also

significant depletion of the DA pool is observed and degradation is manifested in the HP PD brain, owing to a higher HVA pool and a lower depletion of DOPAC compared to DA. The differences in the observation between the present study and Inamdar et al., in regards to DOPAC level modulation in PD brain, although apparent, a closer look suggests otherwise. As observed by Inamdar et al., a similar concentration (10mM PQ) of the neurotoxicant exposure enhances DOPAC levels in adult young fly brains (2-4 days old). However, in the current study in HP PD brain depletion of DA level is higher than that of DOPAC level (**Figure 3.4**). This observation in the present study suggests higher DOPAC synthesis from DA oxidation (Therefore lesser DOPAC depletion and higher DA depletion) which corroborates with the hypothesis of Inamdar et al., (2012). Further, from the current observation it can be postulated that during HP there is a relatively lower level of DOPAC degradation to HVA. Instead, the HVA may be more likely to be synthesized from DA through an alternate route i.e., DA>3-MT>HVA (**Figure 3.4**). Although to a different degree, the synchronous depletion of DA and DOPAC (**Figure 3.4**), during the HP PD condition can be explained by the fact that DOPAC is the primary metabolite of DA, and as such changes in the DA pool may immediately be reflected on DOPAC pool. In fact, it has been reported that deficiency of DOPAC in the nigrostriatal region and CSF highlights DA deficiency in the central brain, and therefore DOPAC pool in CSF is also used as a reliable marker of DA deficiency in the case of human PD (Goldstein et al., 2018; 2011). Owing to the neurotoxic natures of the DA catabolites and the generation of ROS/peroxides due to the catabolic process (Cao et al., 2021; Zhang et al., 2019) neurodegeneration ensues.

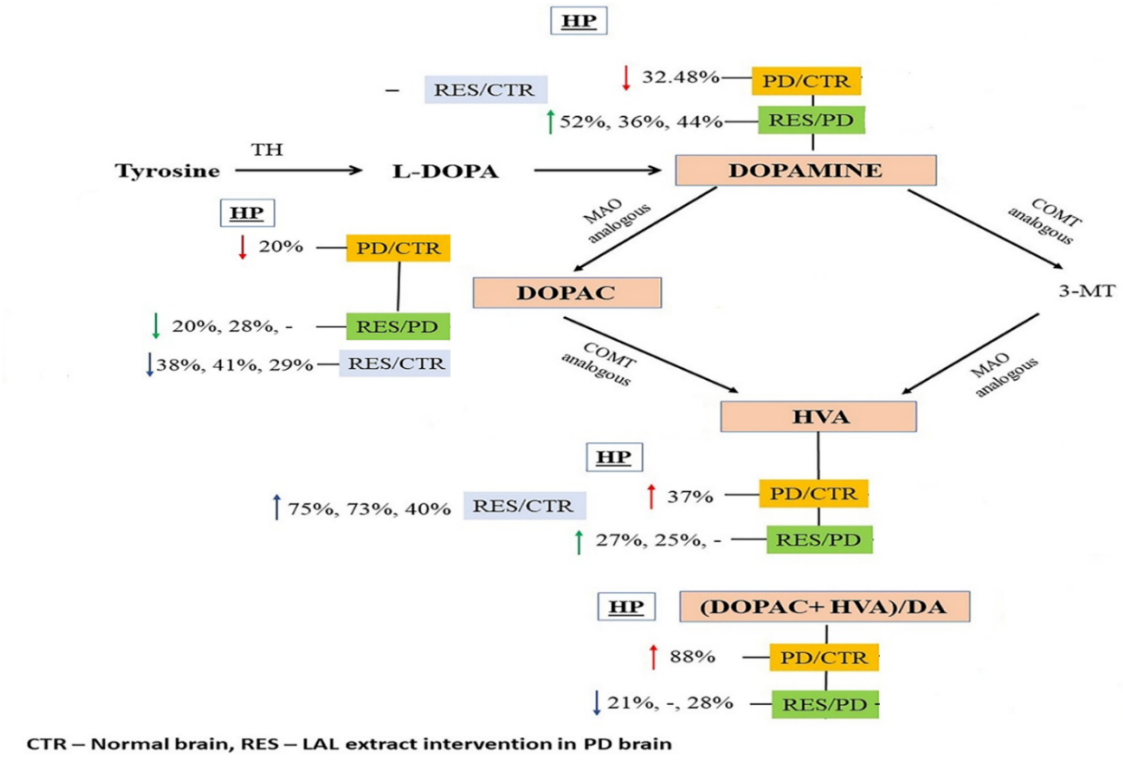


Figure 3.4: Schematic representation of DA metabolism in PD brain and with LA extract interventions: During HP DA and DOPAC levels are decreased, while HVA level is increased under PQ-mediated PD condition. The relatively higher DA depletion compared to DOPAC and enhanced HVA suggests, higher DA oxidation in the PD condition in the fly brain. LA extract intervention rescued diminished DA levels and altered DA turnover during HP.

Insight on the neuroprotective efficacy of LA extract suggests that LA effectively rescues diminished DA levels during HP. On the other hand, while in HP PD brain LA intervention inhibits DOPAC level, the HVA level is further enhanced (**Figure 3.4)**. Previously I discussed that oxidative turnover of DA in the PD model may be more towards direct HVA synthesis than DOPAC. Similarly, it is observed with LA intervention that although DOPAC level is differentially modulated in HP PD brain, upon resuscitation of DA level in HP PD brain, HVA level is further enhanced respectively. This suggests that LA extract-mediated regulation of DA metabolism is more focused on 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.

However, as mentioned before, elevated DA synthesis may concomitantly increase the risk of producing high ROS and generation of OS. It is plausible that the LA extract due to its highly potent antioxidant may counter additive ROS generation and OS in the PD brain, thereby preserving the integrity of the brain neurons. So, based on the speculation in chapter 4 I explored the antioxidant capacity of LA extract in *Drosophila* model of PD.

Chapter 4

Oxidative stress-mediated DAergic neurodegeneration and its sequestration by *Leea asiatica* (LA) in the *Drosophila* model of Parkinson's disease

4.1. Introduction

4.1.1 Oxidative Stress and its impact on Human health

Despite making up only 2% of the total body mass, the adult human brain weighs an average of 1,400 grams, however it uses around 20% of total basal oxygen (Bayliak, 2023; Pittella, 2024; Hou, 2024). It voraciously takes up O_2 to fuel its ~86 billion neurons and their incredibly intricate connectome, which consists of trillions of synapses. This process is aided by approximately 250–300 billion glia (Cobley et al., 2018; Bayliak, 2023; Luppi et al., 2024). Owing to its high oxygen consumption, high quantities of lipids and iron, oxidative phosphorylation dependence, extreme metabolic activity, and lack of energy reserve, the brain is particularly vulnerable to oxidative damage. Due to these circumstances, neurons are more susceptible to NDDs, which can result in oxidative stress (OS) (Jelinek et al., 2021). OS was originally defined in 1985 (Sies, 1985) and is described as the imbalance between oxidants and antioxidants in a biological system. Oxidants such as reactive oxygen species (ROS)/ reactive nitrogen species (RNS) are normally generated as a result of metabolic activities in the cell (mitochondria and NADPH oxidases) and external stimuli like air pollution, UV radiation, smoking, alcohol, infections, non-steroidal anti-inflammatory drugs (NSAIDs), and inflammation. It becomes detrimental when ROS/RNS overpowers its reducing agents, which is ultimately termed OS, or “bad stress” (Forman and Zhang, 2021; Hajam et al., 2022; Reddy, 2023; Orfali et al., 2024). Low-to-moderate levels of oxidants also referred to as "good stress," are what characterize ROS/RNS in a normal physiological state. These oxidants play a role in regulating various biochemical transformations such as hydroxylation, carboxylation, peroxidation, as well as modulation of signal transduction pathways such as nuclear factor-κB (NF-κB), phosphoinositide-3-kinase(P13K), nuclear factor erythroid 2–related factor 2 (Nrf2), and the like (Forman and Zhang, 2021; Hajam et al., 2022;

Reddy, 2023). However, high ROS concentrations cause redox equilibrium to be upset, which then results in OS and ROS-mediated damage to all significant biomolecules, such as lipids, proteins, and DNA, which impairs cell function (Liguori et al. 2018; Jomova et al., 2023; Orfali et al., 2024). The initial three complexes of the electron transport chain (ETC) permit an electron to break free and attach to oxygen during cellular respiration, resulting in the production of superoxide anions (O^{2-}). The mitochondrial membrane contains three different types of superoxide dismutase (SOD): manganese superoxide dismutase (Mn-SOD), copper superoxide dismutase (Cu-SOD), and zinc superoxide dismutase (Zn-SOD). O^{-}_{2} is changed by Mn-SOD into hydrogen peroxide (H_2O_2), which the enzyme aconitase uses in the Fenton reaction to produce a hydroxyl radical reaction (Vásquez-Viva et al., 2000; Hajam et al., 2022; Reddy, 2023). Cu-SOD and Zn-SOD use the intermembrane gap to convert O^{-}_{2} into H_2O_2 and oxygen, respectively (Okado-Matsumoto et al., 2001; Hajam et al., 2022; Chidambaram et al., 2024). The mitochondrial cytochrome catalytic cycle, which involves the enzyme Cytochrome P450 and several organic compounds like steroids, lipids, and xenobiotics, can also produce ROS. It produces a range of reactive byproducts, such as superoxide radicals and H_2O_2 (Hajam et al., 2022). The synthesis of H_2O_2 in the mitochondria is also increased due to the increased activity of monoamine oxidase (MAO) and mitochondrial glyceraldehyde-3-phosphate dehydrogenase (mGDPH). Pesta and Roden (2017) state that H_2O_2 inhibits the absorption of glucose (hyperglycemia) and causes a malfunction in the Insulin signaling pathway by activating the mitogen-activated protein kinase (MAPK) and C-Jun-N-terminal kinase (JNK) pathways. By allowing calcium to enter the mitochondria, ER stress also contributes to mitochondrial OS (Pesta and Roden, 2017). Increased ROS generation results from elevated calcium levels in the mitochondria, which block electron transport and energy production (Pesta and Roden, 2017).

Moreover, elevated generation and accumulation of ROS within the cell activates pro-inflammatory transcription factors such as NFκB and activator protein-1 (AP-1), which in turn activates a range of pro-inflammatory chemokines/cytokines and adhesion molecules (Rendra et al., 2019). Similarly, ROS are produced by a number of protein complexes in mammals, such as MAOs, succinate dehydrogenase (SDH), dihydroorotate dehydrogenase (DHODH), and nicotinamide adenine dinucleotide (NADH)-cytochrome b5 reductase (b5R) (Hey-Mogensen et al., 2014; Hajam et al., 2022; Jamova et al., 2023). These protein complexes cause damage to DNA.

ROS can damage the nitrogenous bases and sugar-phosphate backbone of nucleic acids, breaking DNA and causing premature aging (Hastoy et al., 2018). The mutagenic base8-oxo-2′-deoxyguanosine is a biomarker for OS that is elevated in tissue as a result of OS-induced DNA damage (Hastoy et al., 2018). Furthermore, OS generates a complex mixture of lipid peroxidation products, some of which, including malondialdehyde (MDA) and 4-hydroxy-trans-2-nonenal (HNE), are primarily in charge of DNA changes and the subsequent development of cancer (Wei and Yin, 2015). According to Wang et al. (2023), OS also weakens the blood-brain barrier (BBB), exacerbating neuronal impairment in AD and TBI (traumatic brain injury) patients (Wang et al., 2023). OS is a catch-all word for a variety of chronic illnesses, even though it is widely acknowledged that their causes are multifaceted. A persistently elevated OS raises the chance of acquiring several life-threatening pathologies, including aging, cardiovascular disease, diabetes, lung diseases (lung cancer, chronic pulmonary obstruction), mental illnesses (bipolar disorder, schizophrenia, depression), and neurodegenerative disorders (AD, PD, ALS) (Halliwell, 2020; Forman and Zhang, 2021; Mangione et al., 2022; Jomova et al., 2023). Neurodegenerative processes are triggered by neuroinflammation caused by OS, and apoptosis and cell damage caused by mitochondrial malfunction (**Figure 4.1**) (Selivanov et al., 2011; Ashok et al., 2022). Comprehending the basic

mechanisms behind the pathophysiology of numerous clinical illnesses stemming from OS can facilitate the development of therapeutic approaches aimed at combating these ailments and advancing human health.

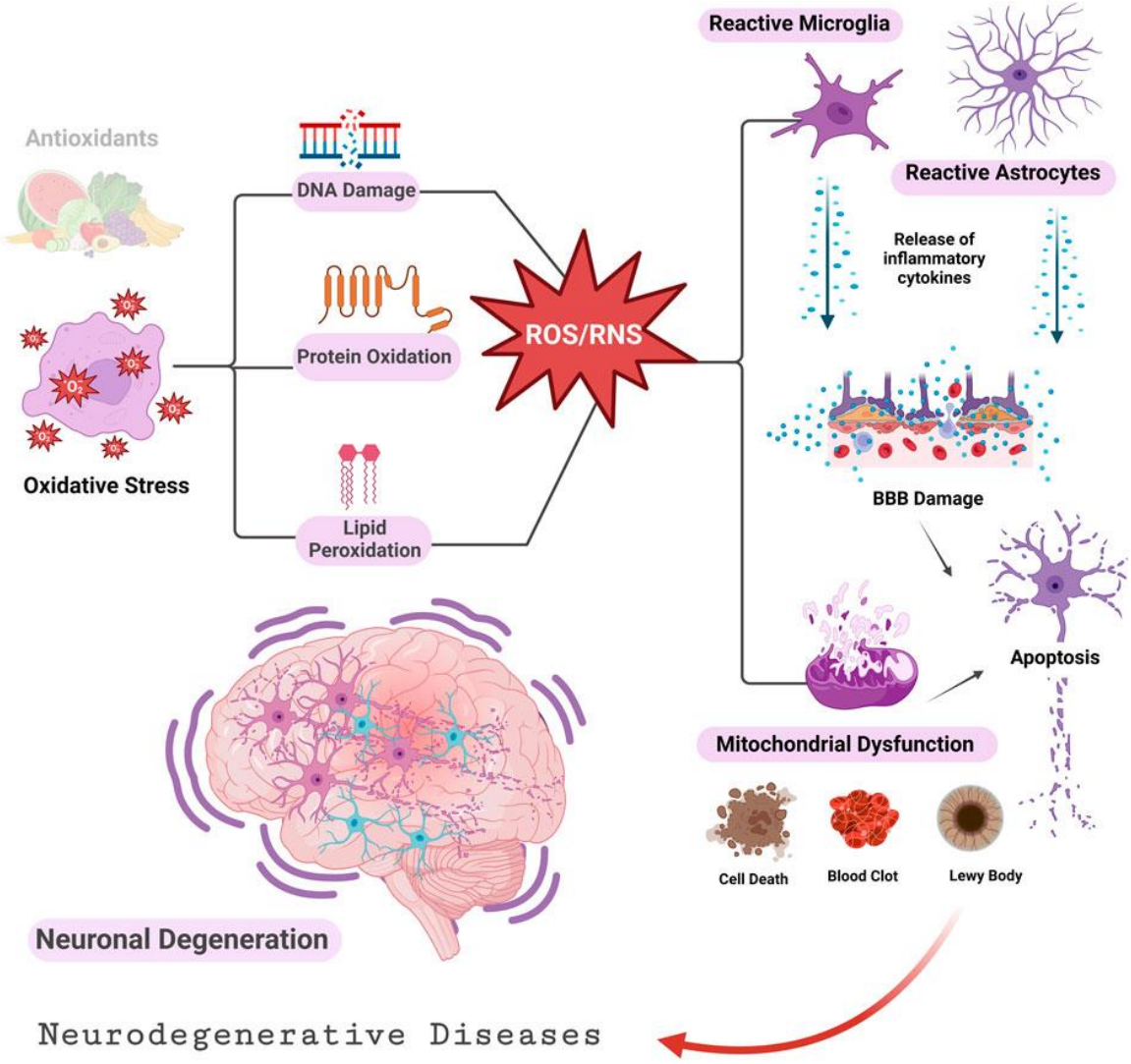


Figure. 4.1: Oxidative stress (OS) and neurodegenerative diseases (NNDs). An imbalance between ROS/RNS and antioxidants can cause damage to lipids, proteins, and DNA The accumulation of activated astrocytes and microglia, along with impaired mitochondrial activity, causes cellular apoptosis and tissue death. (BBB- Blood Brain Barrier) (Ashok et al., 2022).

4.1.2 Oxidative stress (OS) and Parkinson's disease

The etiology of OS-induced pathology encompasses multiple processes, including DA metabolism, mitochondrial failure, neuroinflammation, and environmental variables that

produce ROS. ROS plays a crucial role in the onset and progression of PD (Houldsworth, 2024). In the brain of PD patients, DA autooxidation 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 (Kuhn et al., 1999; Whitehead et al., 2001), as well as modification of several proteins, such as *α-synuclein (α-syn)*, *parkin*, *DJ-1*, *SOD2*, and *UCH-L1*, alterations of brain mitochondria, and failure in Complex I activity (Jana et al., 2007; Zhou et al., 2014; Blesa et al., 2015; Houldsworth, 2024). Recent reports demonstrated that PD patients exhibit reduced complex I activity in the substantia nigra pars compacta, which leads to an excessive generation of reactive ROS and the degradation of DAergic neurons (Hauser and Hastings, 2013; Hajam et al., 2022). 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 (Simon et al., 2010; Ellmore et al., 2020; Ma et al., 2021). Reduced complex I activity, increased ROS, and increased DA toxicity through OS was linked to heightened α-syn accumulation in DAergic neurons (Martin et al., 2006; Dryanovski et al., 2013; Xiang et al., 2013; Moradi Vastegani et al., 2023). Reduction of glutathione (GSH) in the substantia nigra pars compacta of PD is caused by ROS-induced OS (Oakley et al., 2007). GSH and glutathione disulfide (GSSG) levels are lower in the brain tissue of PD patients than in the brain tissue of healthy individuals (Sian et al., 1994; Pearce et al., 1997). 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 (Chen et al., 2012; Sarva and Shanker, 2014; Braga Neto et al., 2016; Picher-Martel and Dupre, 2018). Elevated non-enzymatic OS markers (ROS, LP, HP, and PC) are also demonstrated in the PQ-mediated *Drosophila* model of sporadic PD. Similarly, the enzymatic OS markers, viz., SOD, CAT, and GST, are found to be elevated in the PQ-mediated *Drosophila* model of sporadic PD (Phom et al., 2014; Phom, 2018). 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 HP-PQ-mediated *Drosophila* model of sporadic PD (**Figure 4.2 & Figure 4.3**). These pathways eventually lead to the degradation of DAergic neurons in the PD brain.

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

4.1.3.1. Non-enzymatic oxidative stress marker

4.1.3.1.1. 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. Lipid peroxidation (LP) is caused when ROS interacts with lipids within cell membranes (Su et al., 2019). The major degradation products of LP are 4-HNE and 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 a reliable marker of OS (Ayala et al., 2014; Gaschler and Stockwell, 2017). 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 (Gaschler and Stockwell, 2017; Bilska-Wilkosz et al., 2022). LP is a well-studied molecular pathway engaged in PD pathogenesis (Reddy, 2023; Angelova et al., 2021). However, whether LP products are the cause or consequence of neurodegenerative diseases like PD remains unclear (Ayala et al., 2014). According to the reports 4-HNE-induced neuronal loss and α-Syn aggregation (Peña-Bautista et al., 2019). Furthermore, it is said 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, an end product of LP, increased in PD patient's blood. Other end products of LP, such as 4-HNE, are also found in the Lewy bodies of post-mortem PD brain tissues (Castellani et al., 2002). In the brain, LP gains importance as the brain is particularly rich in polyunsaturated fatty acids (PUFA). Higher levels of MDA, a marker of OS, have been reported in the *SNpc* of PD patients (Dexter et al., 1989). The study concluded by Sharma et al. (2008) demonstrated a 2-fold increase in the plasma MDA levels of PD patients compared to healthy age-matched controls (Sharma et al., 2008). Yoritaka et al. (1996) found that there is an increased level of MDA and 4-HNE modified proteins in 58% of the SN neurons (vs 8% in the control group). Additionally, research showed that the Parkinsonian SN had multiple times higher levels of LP products (Dexter et al., 1994; Munch et al., 1998). Fedorova et al. 2019 studies revealed an increase in LP levels by 20 % on average, irrespective of the PD stage (stages 2-4). Patients with more severe disease who were in the later phases of the illness (stages 3 and 4) had elevated MDA levels. (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* flies, MDA levels were significantly elevated in PQ-treated PD brains (20 mM PQ for 48 hours) (Shukla et al., 2014; Srivastav et al., 2018;2020). A study concluded by Maitra et al. (2021) and Chaouhan et al. (2022) showed that exposure to 5 mM PQ for 48 Hrs 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 Hrs) in the *Drosophila* PD flies. Similarly, prolonged exposure to PQ (20mM for 24 Hrs) 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* flies (Salim et al., 2020; Soares et al., 2017). Also, a study conducted by Phom et al. (2018) exhibited elevated MDA levels when the flies were exposed to 10 mM PQ for 24 and 48 hours (Phom et al., 2018). Since MDA and 4-HNE are well-known biomarkers of LP (Tsikas, 2017), the results above suggest that elevated OS leads to increased lipid peroxidation in the brain.

4.1. 3.2. Enzymatic oxidative stress markers

4.1.3.2.1. 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 (Zelko et al., 2002; Nozik-Grayck et al., 2005). A study on the PD brain showed an increased SOD-like activity most prominent in the SN and basal nucleus (Marttila et al., 1998). Studies by 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. Another 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, higher SOD activity levels showed a positive correlation with results from a recent blood analysis 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 Hrs) in the *Drosophila* PD flies. Similarly, Phom et al. (2018) found that flies treated with 10 mM PQ for 24 hours have higher SOD levels (Phom et al., 2018). Also, prolonged exposure to PQ (20mM for 24 Hrs) 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 of 5 mM PQ for 48 Hrs causes reduced SOD in the *Drosophila* PD brain. Similarly, Shukla et al. (2014) reported that PQ-mediated toxicity (20 mM for 24 Hrs) 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 (Marttila et al., 1988; Saggu et al., 1989; Younes-Mhenni et al., 2007). These findings indicate that PQ induces oxidative damage by reducing the antioxidant defense.

4.1.3.2.2. 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 (Schapira et al., 1990; Hattori et al., 1991; Hattingen et al., 2009). Furthermore, mitochondrial complex I deficiency has been demonstrated in various brain regions, fibroblasts, blood platelets, skeletal muscle, and lymphocytes of PD patients (Mizuno et al., 1989; Parker et al., 2008; Mytilineou et al., 1994; Krige et al., 1992; Blandini et al., 1998; Blin et al., 1994; Yoshino et al., 1992; Haas et al., 1995). 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 (Schapira et al., 1990; Parker et al., 2008). Furthermore, a recent 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 paraquat dication (PQ^{2+}) enters cells, it goes through redox cycling. During this process, it interrupts the oxidation of NADPH by accepting electrons. This forms paraquat mono-cation radical ($PQ^{\bullet+}$), facilitated by NADPH-cytochrome P450 reductase. Ultimately, this inhibits the activity of mitochondrial complex I (Fukushima et al., 1993; Fussell et al., 2011). 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 hrs) 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. According to Choi et al. (2008), the PQ (50 μM for 24 hrs) did not cause the DAergic neurons from NDUFS4 mutant mice to become more sensitive. The present study demonstrated that complex-I-III activity was reduced in HP PD brains.

4.1.3.2.3. Mitochondria Complex II (Succinate dehydrogenase)

Encoded by nuclear DNA (Hattori et al., 1999), succinate dehydrogenase (SDH) is a key component of the mitochondrial oxidative phosphorylation (OXPHOS) system, linking the electron transport chain, which produces ATP, with the Krebs cycle (Bezawork-Geleta et al., 2017; Goetzman et al., 2023). 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). SOD 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 Complex II was modestly reduced in both platelets and lymphocytes in PD compared to age-matched controls (Yoshino et al., 1992; Haas et al., 1995). 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 Hrs reduced the SDH levels in PD mice (Hosamani et al., 2016). SOD 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 &40mM PQ for 24 & 48Hrs) in young *Drosophila* PD brains exhibited reduced SDH/Complex-II activity (Hosamani and Muralidhara, 2010). Another independent investigation by Rao et al. (2016) demonstrated the reduced SDH activity upon exposure to rotenone (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 (20mM for 48 Hrs), 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, oxidative damage, 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 HP 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 (Vanova et al., 2020; Goetzman et al., 2023).

4.1.4. *Leea asiatica* (LA)and oxidative stress (OS)

Studies have shown the presence of terpenoids, phenolic, flavonoid compounds, triterpenes, and glycosides in methanolic, ethyl acetate, and petroleum ether extract of LA leaves (Nair et al., 2014; Sen et al., 2013; Kil et al., 2019). 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 lipid peroxidation 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 has 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 lipid peroxidation inhibition, superoxide anion radical ($O_2^{let^-}$), hydroxyl radical (OH˙), nitric oxide radical (NO˙), hydrogen peroxide (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). Another laboratory study by 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. On the other hand, Nair and colleagues have observed LA methanolic extract mediated potent anti-inflammatory and wound healing activity on male albino Wistar rats (Nair et al., 2014). 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 and colleagues also tried to address the extract's dermal and oral toxicity *in vivo* in a dose-dependent manner. They found no side effects 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 (Antioxidant ability) 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.

4.2. Materials and Methods

4.2.1. Modelling PD in *Drosophila* and therapeutic intervention

(Described in Chapter-2 please refer the section/subheading 2.2.3 to 2.2.5)

4.2.2 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).

4.2.3. 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.

4.2.3.1. 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 to measure 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).

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

100 heads were homogenized with 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 centrifuge under the same conditions. The resulting clear supernatant was stored at -80˚C/used for biochemical assay.

4.2.4. 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 change 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 for the protein concentration of the unknown sample. Bovine serum albumin was used as the standard prepared in concentration range of 0.5µgP to 3.5µgP (Table 5.1). The measurement was performed using Nano Drop 2000 (Thermo Scientific).

4.2.5. 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 the 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.

4.2.5.1. Mitochondria 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).

4.2.5.2 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 4.1: Preparation of serial dilutions using standard BSA.

4.2.6 Biochemical Assays

4.2.6.1 Assessment of Lipid Peroxidation (LP)

LPwas 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) (Ohakawa et al., 1979; Phom et al., 2014; Phom, 2018)

4.2.6.2. Assessment of Superoxide Dismutase (SOD)

SOD 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 (Kostyuk & Potapovich, 1989; Phom et al., 2014; Phom, 2018).

4.2.6.3. Assessment of electron transport enzyme activity: Complex I-III (NADH and Cytochrome C reductase)

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 $mM^{-1}cm^{-1}$)

4.2.6.4. Assessment of electron transport enzyme activity: Complex II-IV (Succinate dehydrogenase (SDH))

The SDH activity was determined by reducing p-iodonitrotetrazolium violet (INT) (Pennington, 1961; Girish and Muralidhara, 2012). 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 top colour phase was taken and measured at 490 nm. The SDH activity was expressed as optical density (OD)/mg protein.

4.2.7 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.

4.8 Results

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

LP, a potential biomarker for most NDDs, including AD, PD, and other numerous neurological disorders, could be facilitated by metabolic failure in antioxidant mechanisms, leading to

activation of phospholipases and promotion of signalling cascades that tenet neuronal cell death (Angelova et al., 2020; Phom et al., 2014; Hosamani and Muralidhara, 2009, 2010). In the present study, in the PD brain LP levels were upregulated by 100% (**Figure 4.2**). LA significantly attenuated the LP upregulation (***P<0.001) **(Figure 4.2)** when co-administered in *Drosophila* model of PD brain. The observations suggest that the LA has effective free radical sequestering properties. LA *per se* studies confer no toxic effect on *Drosophila* model of PD brain **(Figure 4.2)**.

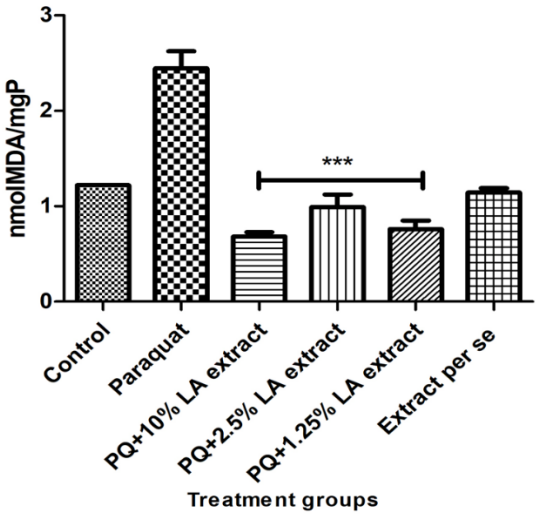


Figure. 4.2: Measurement of MDA levels in *Drosophila* of model. In PD brain the MDA level was upregulated by 100%. Upon co-feeding with *Leea asiatica* (LA), enhanced levels of MDA were significantly diminished, suggesting the effective modulatory action of LA against PQ induced Oxidative stress (OS) in 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 Newman-Keuls Multiple Comparison Test (***p<0.001; **p<0.01; *p<0.05; NS-Not Significant), when compared to PQ (10mM) treated group.

4.8.2 *Leea asiatica* (LA) abates the PQ-mediated SOD levels in the HP-PD brain

SOD, a redox equilibrium regulator, serves as a first line of defense against reactive oxygen species (ROS) and reactive nitrogen species (RNS) 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 et al., 2014; Hosamani and Muralidhara, 2009, 2012). In the present study, in the *Drosophila* model of PD brain SOD levels were upregulated by 50% (***P<0.001) **(Figure 4.2)**. LA significantly reduced the SOD upregulation (***P<0.001, **P<0.01) **(Figure 4.2)** when co-administered in the *Drosophila* model of PD brain. The observations suggest that the LA has effective free radical sequestering properties by modulating the OS. LA *per se* studies also confer the sequestering properties by diminishing the level when compared with the control brain-specific SOD (***P<0.001) **(Figure 4.2)**.

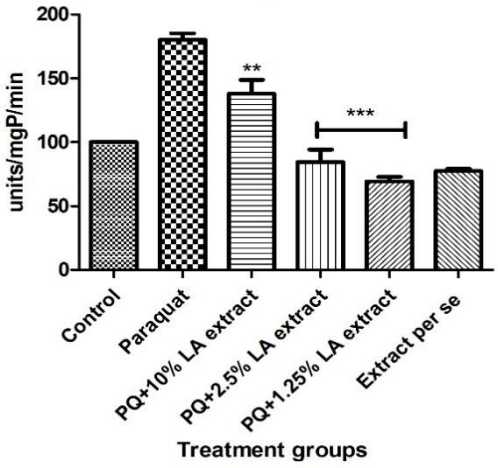


Figure. 4.3: Measurement of SOD activity in *Drosophila* model. In PD brain the SOD activity was upregulated by 50%. Upon co-feeding with *Leea asiatica* (LA), enhanced/increased activity of SOD was significantly diminished, suggesting that LA has a potent anti-oxidative activity against PQ induced OS in 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 Newman-

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

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

Mitochondrial complex I-III enzyme activity is significantly reduced in the substantia nigra, skeletal muscles, and platelets of PD patients (Bose and Beal, 2016). The present study inhibited the *Drosophila* PD brain NADH-Cytochrome C reductase activity by 55% (***P<0.001) **(Figure 4.4)**. LA significantly upregulates the inhibited NADH- Cytochrome C reductase activity (***P<0.001, **P<0.01) **(Figure 4.4)** when co-administered in the *Drosophila* model of PD brain. The observations suggest that the LA has effective free radical sequestering properties by modulating mitochondrial dysfunction. LA *per se* studies also confer the sequestering properties by upregulating the *Drosophila* model control brain-specific NADH-Cytochrome C reductase activity (***P<0.001) **(Figure 4.4)**.

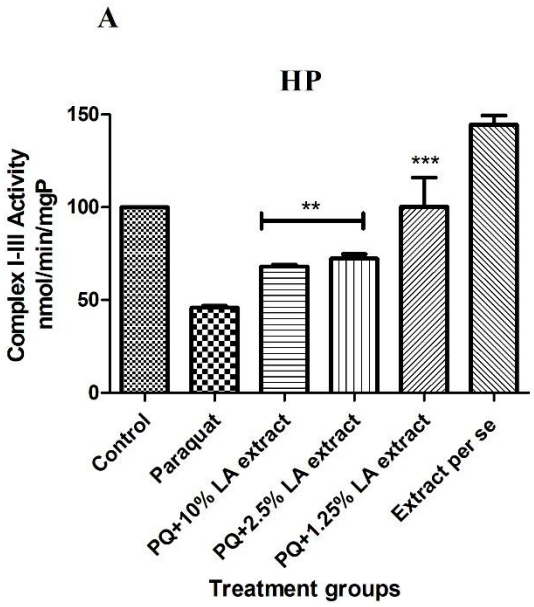


Figure.4.4: The mitochondrial complex I-III activity was assayed to explore the inhibition of mitochondrial complex I in PQ-treated flies. In the PD brain, the complex I-III activity was inhibited by 55%. This reduction in complex I-III activity clearly indicates the impairment of

complex I of the electron transport chain. Upon co-feeding with *Leea asiatica* (LA), the complex I-III enzyme activity was significantly upregulated in the fly model (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 Newman-Keuls Multiple Comparison Test (***p<0.001; **p<0.01; *p<0.05; NS-Not Significant), when compared to PQ (10mM) treated group.

4.8.4 *Leea asiatica* (LA) rescues the inhibited PQ-mediated Succinate dehydrogenase (SDH) enzyme activity in HP-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 neurodegenerative disorders (NDDs) like PD, AD, and HD (Jodeiri Farshbaf et al., 2016; Schwall et al., 2012).

The present study inhibited the *Drosophila* model of PD brain SDH activity by ~30-35% (**P<0.01) **(Figure 4.5)**. LA significantly upregulates the inhibited succinate dehydrogenase activity (**P<0.01; *p<0.05) **(Figure 4.5)** when co-administered in the *Drosophila* model of 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 *Drosophila* model control brain-specific succinate dehydrogenase activity (***P<0.001) **(Figure 4.5)**.

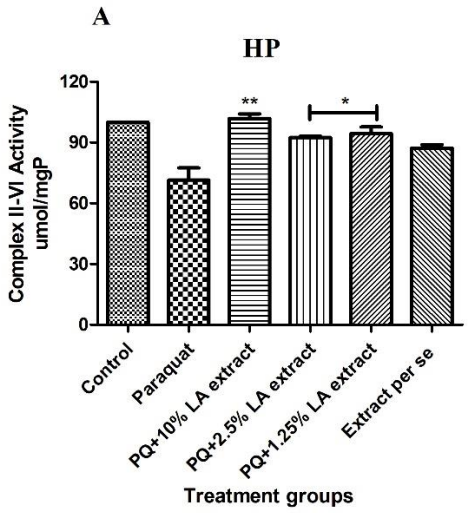


Figure.4.5: The mitochondrial complex II-IV activity was assayed to explore the inhibition of mitochondrial complex II-IV in PQ-treated flies. In the PD brain, the complex II-IV activity was inhibited by ~30-35%. This reduction in complex II-IV activity clearly indicates the impairment of complex II-IV of the electron transport chain. Upon co-feeding with *Leea asiatica* (LA), the complex II-IV enzyme activity was significantly upregulated in the fly (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 Newman-Keuls Multiple Comparison Test (***p<0.001; **p<0.01; *p<0.05; NS-Not Significant), when compared with the PD group.

4.9. Discussion

Cellular aging and the onset of age-related diseases are both underpinned by OS (Kurtishi et al., 2019; Yang et al., 2024). 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 (Kuilman et al., 2010; Luceri et al., 2018). 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 oxidative stress-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 superoxide dismutase/catalase 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-*Drosophila* model of PD brain SOD levels was upregulated by 50% (***P<0.001) **(Figure 4.3)**, which is significantly reduced (***P<0.001, **P<0.01) when co-administered with LA in the *Drosophila* model of PD brain. The observations suggest that the LA has free radical sequestering properties which modulate the OS. LA *per se* studies also conferred the sequestering ability by diminishing SOD when compared with the control brain (***P<0.001) **(Figure 4.3)**. Similarly, studies on post-mortem PD brains have shown LP was elevated in the substantia nigra of PD parkinsonian brain tissue 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-*Drosophila* model of PD brain LP levels was upregulated by 100% (***P<0.001) **(Figure 4.2)** when compared with the *Drosophila* model of healthy brains. LA significantly attenuated the LP upregulation (***P<0.001) when co-administered in the *Drosophila* model of 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

brain **(Figure 4.2)**. It has been demonstrated that PQ mediates mitochondrial dysfunction by inhibiting electron Transport Chain (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 (Bender et al., 2006; Schapira et al., 1990; Parker et al., 2008). Meanwhile, the reduced level of complex-I activity (65%) was demonstrated in the SN neurons of PD patients. Recent studies elucidated the reduced complex-I activity throughout the brain of PD patients and the mis assembly 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 transfer 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 paraquat mono-cation radical ($PQ^{\bullet+}$) through NADPH-cytochrome P450 reductase, ultimately inhibiting mitochondrial complex I activity (Fukushima et al., 1993; Fussell et al., 2011). It was reported that the level of ATP was reduced upon PQ exposure (20mM for 48 hours) to *Drosophila* flies (Srivastav et al., 2018). Studies suggested that inhibiting mitochondrial complex I is not pivotal in PQ-mediated DAergic neuronal death. It was demonstrated that the DAergic neurons from NDUFS4 knockout mice did not show increased sensitivity to PQ when exposed to a concentration of 50 μM for 24 hours (Choi et al.,2008). The present study inhibited the *Drosophila* model of PD brain NADH-Cytochrome C reductase activity by 55% (***P<0.001) (Figure 4.4). LA significantly upregulates the inhibited NADH- Cytochrome C reductase activity (***P<0.001,**p<0.01) (**Figure 4.4**) when co-administered in the *Drosophila* model of PD brain. The observations suggest that the LA has effective free radical sequestering properties

by modulating mitochondrial dysfunction. LA *per se* studies also confer the sequestering properties by upregulating the *Drosophila* model control brain-specific NADH-Cytochrome C reductase activity. Complex II [succinate dehydrogenase (SDH) or succinate: ubiquinone oxidoreductase (SQR)] includes four subunits, which are encoded by nuclear DNA (Hattori et al., 1999). 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 succinate dehydrogenase 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 neurodegenerative disorders, abnormalities of SDH activity have been reported. Lipid accumulation and excitotoxicity are the main hallmarks of neurodegenerative disorders like PD. 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 inhibited the *Drosophila* model of PD brain SDH activity by ~30-35%. LA significantly upregulates the inhibited succinate dehydrogenase activity (**P<0.01; *p<0.05) when co-administered in the *Drosophila* model of PD brain. The observations suggest that the LA has effective free radical sequestering properties by modulating mitochondrial dysfunction. Most previous studies' evidence could candidate SDH as an effective target for therapeutic interventions in neurological diseases like PD and aging. SDH overexpression or manipulation of its activity could control lipid overloading in neurodegenerative disorders. 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. About the present evidence, the sequestration of OS in the *Drosophila* model of PD could be one of the potential underlying mechanisms for the observed DAergic neuroprotective efficacy of the LA.

4.11 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 *Drosophila* model PD by elevating the MDA and SOD levels and inhibiting the complex-I-III and complex-II-IV enzyme activity. Feeding LA to *Drosophila* 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 insights/results suggest that the sequestration of OS in the *Drosophila* model of PD is one of the possible underlying mechanisms for the observed DAergic neuroprotective efficacy of the LA.

Summary

Parkinson's disease (PD) is the second most prevalent and the fastest-growing neurodegenerative disease (de Lau and Breteler, 2006; Phom et al., 2014; Dorsey et al., 2018). It is an enigmatic, multifactorial disease marked by the gradual and targeted loss of DAergic neurons (Meissner et al., 2011; Miranda et al., 2022; Tsalenchuk et al., 2023). PD affects 1% of those over 60 years of age and more and an increase of 1% to 3 % of senior citizens over 80 are affected and is predicted to double by 2030 (Driver et al., 2009; WHO, 2023).

Currently, there is no cure for PD, and treatment is limited to pharmacological interventions aimed at alleviating motor symptoms by restoring striatal DA levels. This is achieved through the use of DAgeric drugs, such as the DA precursor viz., L-DOPA, DA agonists like apomorphine and bromocriptine, and inhibitors of MAO-B (e.g., selegiline, rasagiline) and COMT (e.g., entacapone, tolcapone), as well as decarboxylase inhibitors such as carbidopa and benserazide. Among these, L-DOPA revolutionized PD management and remains the gold-standard therapy due to its significant impact on symptom relief, quality of life, and life expectancy, even for late-stage PD (Tambasco et al., 2018; Nakmode et al., 2023). However, L-DOPA therapy has limitations, primarily due to its short half-life (90-120 minutes), which leads to fluctuating DA levels and subsequent changes in clinical symptoms, particularly in advanced stages. Long-term L-DOPA use is also associated with adverse effects, including motor complications and L-DOPA-induced dyskinesia (LID), characterized by involuntary movements (Rascol et al., 2003). Therefore, disease-modifying treatments, such as small molecules, nutraceuticals, plant-based compounds, and nano-formulations, along with drug repurposing, are considered promising tools to target the underlying pathophysiology of PD (Nakmode et al., 2023). In particular, plant extracts and nutraceuticals offer a viable alternative for disease modification due to their affordability, safety profile, and multi-targeted mechanisms of action, making them a promising avenue for PD therapy (Lama et al., 2020)."

The review of the literature on *Leea asiatica* (LA) has provided deeper insight into understanding ethnomedicinal use, anthelmintic (Sen et al., 2011, 2012), antioxidant-related nephroprotective (Sen et al., 2013), hepatoprotective properties (Sen et al., 2014), and anticancer activities (Ali et al., 2021).

Thus, in Chapter 2, I employed the Health Phase (HP) fly model of PD established in our laboratory to assess the neuroprotective potential of *Leea asiatica* (LA) leaf extract. The results demonstrate that PQ exposure induces locomotor impairments in HP-PD flies, which are mitigated by LA treatment in both pre-and co-treatment regimens. These findings indicate that the aqueous extract of LA shows promise for further investigation into key PD-related phenotypes using the *Drosophila* PD model.

Additionally, I explored the DAgeric neuroprotective effects of LA in the *Drosophila* PD model. Anti-TH immunostaining of whole Drosophila brains revealed that PQ exposure does not result in DAgeric neuron loss, but rather reduces TH protein synthesis. TH protein levels, as indicated by the FI of secondary antibodies targeting anti-TH, were diminished in HP-PD brains. LA treatment reversed this 'DAgeric neuronal dysfunction' by restoring TH protein synthesis levels in the HP-PD brain. These results suggest that LA exerts neuroprotective effects in the HP-PD model.

In Chapter 3, I aimed to investigate the impact of 'neuronal dysfunction' on DA metabolism in the fly PD brain. The findings demonstrate that PQ exposure reduces brain DA levels and alters the concentrations of its metabolites, DOPAC and HVA, leading to an increase in DA turnover in the HP-PD brain. Conversely, treatment with LA restored DA levels and normalized DOPAC and HVA concentrations, resulting in decreased DA turnover, in the PD model. These results suggest that one potential mechanism of DA restoration involves the upregulation of TH. The overall findings indicate that the neuroprotective effect of LA extract may be mediated

by modulation of DA catabolism, whereby the enhanced breakdown of DA into DOPAC and HVA in the PD brain is mitigated. This mechanism could potentially prevent endogenous neurotoxicity in PD.

The onset and progression of numerous NDDs are significantly influenced by OS, which drives alterations in enzymes and structural proteins. The accumulation of reactive oxygen species (ROS) and the ensuing neurodegeneration in specific brain regions have been identified as key contributors to NDDs, including PD (Jellinger, 2010). In the PD brain, DA undergoes auto-oxidation via MAO, producing ROS and DA quinones (Zucca et al., 2014; Segura-Aguilar et al., 2014; Ayajuddin et al., 2022). DA quinones are known to inactivate the DAT and TH, disrupt mitochondrial function, and impair Complex I and II activities (Blesa et al., 2015; Houldsworth, 2024). Studies have also demonstrated reduced Complex I and II activity in the *SNpc* of PD patients, resulting in excessive ROS generation and degeneration of dopaminergic neurons (Hauser and Hastings, 2013). Elevated MDA levels and increased SOD activity have also been reported in the *SNpc* and basal nucleus of PD patients (Dexter et al., 1989; Marttila et al., 1998).

In this study (Chapter 4), PQ exposure led to increased LP, elevated SOD levels, and reduced Complex-I-III and Complex-II-IV enzyme activity, resulting in heightened OS in the HP-PD brain. Feeding with LA in HP-PD flies reduced OS markers, as evidenced by lower MDA and SOD levels. Moreover, LA supplementation restored the activity of mitochondrial complexes that were impaired in the PD brain. These findings indicate that OS sequestration in the HP-PD brain may underlie the observed dopaminergic neuroprotective effects of LA.

This study demonstrates that LA aqueous leaf extract ameliorates mobility deficits, rescues dopaminergic neuronal dysfunction, and normalizes altered DA metabolism in the brain. Collectively, these results highlight the neuroprotective potential of LA. The findings provide

a foundation for future investigations into the genetic and molecular mechanisms involved and may contribute to the development of improved therapeutic approaches for neurological disorders such as PD.

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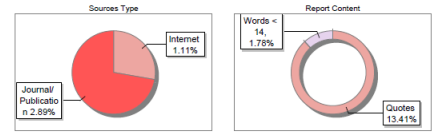

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Source: Excluded < 14 Words	Excluded
Excluded Source	0 %
Excluded Phrases	Excluded

Database Selection

Language	English
Student Papers	Yes
Journals & publishers	Yes
Internet or Web	Yes
Institution Repository	Yes

A Unique QR Code use to View/Download/Share Pdf File

DrillBit

DrillBit Similarity Report

A-Satisfactory (0-10%)
B-Upgrade (11-40%)
C-Poor (41-60%)
D-Unacceptable (61-100%)

4	15	A
SIMILARITY %	MATCHED SOURCES	GRADE

LOCATION	MATCHED DOMAIN	%	SOURCE TYPE
1	Thesis Submitted to Shodhganga Repository	1	Publication
2	Thesis Submitted to Shodhganga Repository	1	Publication
3	link.springer.com	1	Internet Data
4	Succinate dehydrogenase in Parkinsons disease by Farshbaf-2017	1	Publication
5	Succinate dehydrogenase in Parkinsons disease by Farshbaf-2017	<1	Publication
7	Animal Models of Parkinsons Disease A Gateway to Therapeutics by Le-2013	<1	Publication
8	moam.info	<1	Internet Data
9	coek.info	<1	Internet Data
10	Thesis submitted to shodhganga - shodhganga.inflibnet.ac.in	<1	Publication
11	moam.info	<1	Internet Data
12	innovareacademics.in	<1	Publication
13	cisejournal.org	<1	Internet Data
14	Thesis Submitted to Shodhganga Repository	<1	Publication
15	aanda.org	<1	Internet Data
18	spandidos-publications.com	<1	Internet Data

EXCLUDED PHRASES

1	et al.,

CONFERENCES/SEMINARS/SYMPOSIUM

International

- Oral Presentation at the International Conference on Impacts & Consequences of Environmental Degradation on Animal Health and Human Wellbeing, 2-4 September 2021, Abhayapuri College in association with Department of Zoology, Gauhati University and Aaranyak, Assam, India, "Oxidative Stress Marker Based Assessment of Neuroprotective Efficacy of Nutraceuticals: Insights from Drosophila Model".

National

- Poster Presentation at the 107th Indian Science Congress, 3-7 January 2020 , Bengaluru, Karnataka, India, entitled "Utility of Paper Microscope –Foldscope in Biomedical Research".
- Oral Presentation at the National Conference on Contemporary Excitement in New Biology (CENB), 30-31 October 2018, Department of Zoology, Nagaland University, Lumami, Nagaland, India, entitled "Foldscope: A Useful Tool in Basic Science and Biomedical Research"

Symposium/Workshops

- **Participated** in the International webinar on "Recent Trends in Biomedical Sciences" from 24th-26th August 2020 held at Guru Nanak College Autonomous, Velachery, Chennai.
- **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.

AWARDS

- Availed **DBT for the Junior Research Fellowship**, **through Nagaland University, Nagaland, India**
- Awarded **non-NET fellowship from the University Grant Commission (UGC) through Nagaland University, Nagaland, India**.
- Awarded **Nagaland Research Scholarship**, **Sponsored by the Government of Nagaland.**

Three Day Virtual International Conference on

"Impacts & Consequences of Environmental Degradation on Animal Health and Human Wellbeing"

Aaranyak

Certificate

This is to certify that **Kelevikho Neikha** of Nagaland University (Central), Lumami, Nagaland participated and presented his research paper titled **"Oxidative stress marker-based assessment of neuroprotective efficacy of nutraceuticals: Insights from Drosophila model"** in the three day virtual International Conference on **"Impacts & Consequences of Environmental Degradation on Animal Health and Human Wellbeing"** held on 2nd to 4th September, 2021, organized by Abhayapuri College in association with Department of Zoology, Gauhati University and Aaranyak, Assam.

We wish him/her all success in life.

Prof. Jogen Chandra Kalita	Dr Sadananda Nath	Dr Pori Devi
(Chief Adviser)	(President)	(Convener)
Head, Dept. of Zoology, GU	Principal, Abhayapuri College	Asst. Prof., Abhayapuri College

UGC Sponsored National Conference

Contemporary Excitement in New Biology

Certificate of participation

This is to certify that Prof./Dr./Mr./Ms. KELEVIKHO NEIKHA of NAGALAND UNIVERSITY participated/presented a paper (Key Note Talk/Invited Talk/Presentation Talk/Platform Talk) titled: FOLDSCOPE: A USEFUL TOOL IN BASIC SCIENCE AND BIOMEDICAL RESEARCH 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	Prof. Sarat C. Yenisetti	Prof. L. N. Kakati	Prof. Bendang Ao
Dean	Organising Secretary	Convener	Treasurer
School of Sciences	CENB-2018	CENB-2018	CENB-2018

THE INDIAN SCIENCE CONGRESS ASSOCIATION

(Professional Body under Department of Science & Technology, Ministry of Science & Technology, Government of India)

14, Dr. Biresh Guha Street, Kolkata – 700 017

Paper Presentation Certificate

This is to certify that Prof./Dr./Shri/Smt. Kelevikho Neikha of Nagaland University has presented a Paper (Oral/Poster) entitled Utility of Paper Microscope in the Section of Anim., Vet and Fishery Sciences during 107th Indian Science Congress held at University of Agricultural Sciences, Bangalore from January 3 to 7, 2020.

His/Her Membership Number is LSO.SLM.407

Date 07/01/2020

Office Seal

(Signature)
Sectional President

GURU NANAK COLLEGE AUTONOMOUS

(Re-accredited at 'A' Grade by NAAC)
Affiliated to the University of Madras
Guru Nanak Salai, Velachery, Chennai – 600 042

INTERNATIONAL WEBINAR
ON
"RECENT TRENDS IN BIOMEDICAL SCIENCES"
24th - 26th August, 2020

Certificate of Participation

This is to certify that

Mr. KELEVIKHO NEIKHA
Research Scholar, NAGALAND UNIVERSITY LUMAMI

has actively participated in the International Webinar on "Recent Trends in Biomedical Sciences" held from 24th to 26th August, 2020 (Three Days) organized by the PG & Research Department of Advanced Zoology and Biotechnology, Guru Nanak College (Autonomous), Guru Nanak Salai, Velachery, Chennai - 42.

Dr. M. G. RAGUNATHAN
PRINCIPAL

Mr. MANJIT SINGH NAYAR
GENERAL SECRETARY & CORRESPONDENT

Subject Code: 174053H01

Registration No. 2015150794385

Nagaland University

GIAN
GLOBAL INITIATIVE OF ACADEMIC NETWORKS

Government of India
Ministry of Human Resource
Development

Global Initiative of Academic Network (GIAN)

This is to Certify that Prof./Dr./Mr./Ms. Kelevikho Neikha *from* Nagaland University, Lumami

participated in the course Anti-Inflammatory Life Style for Prevention and Treatment of Cancer and Neurodegeneration: Facts and Fiction

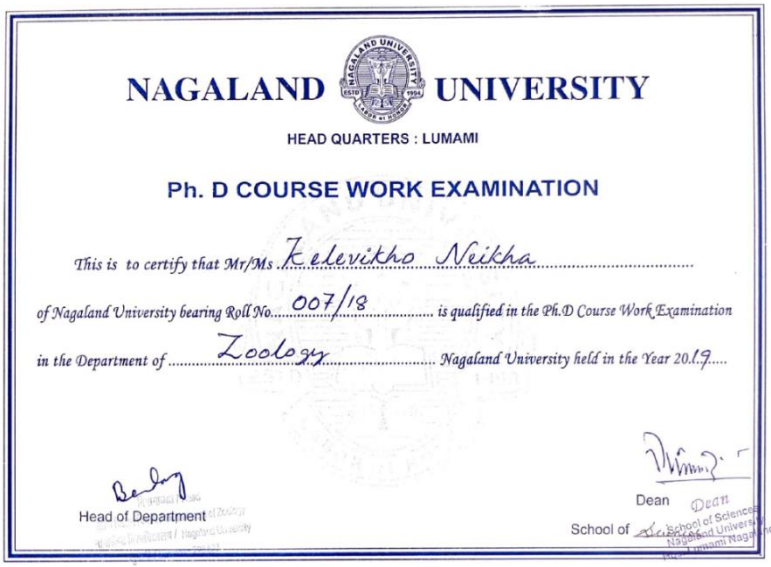
conducted during 1st – 5th October 2019

Date: 05/10/2019

Place: Department of Zoology, Nagaland University, Lumami, Nagaland, India

Dean
Prof.(Dr.) T Sangyu Yaden
Nagaland University

Local & Course Coordinator
Prof.(Dr.) Sarat C. Yenisetti
Dept. of Zoology,
Nagaland University



NAGALAND UNIVERSITY

HEAD QUARTERS : LUMAMI

Ph. D COURSE WORK EXAMINATION

This is to certify that Mr/Ms Kelevikho Neikha

of Nagaland University bearing Roll No. 007/18 is qualified in the Ph.D Course Work Examination

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

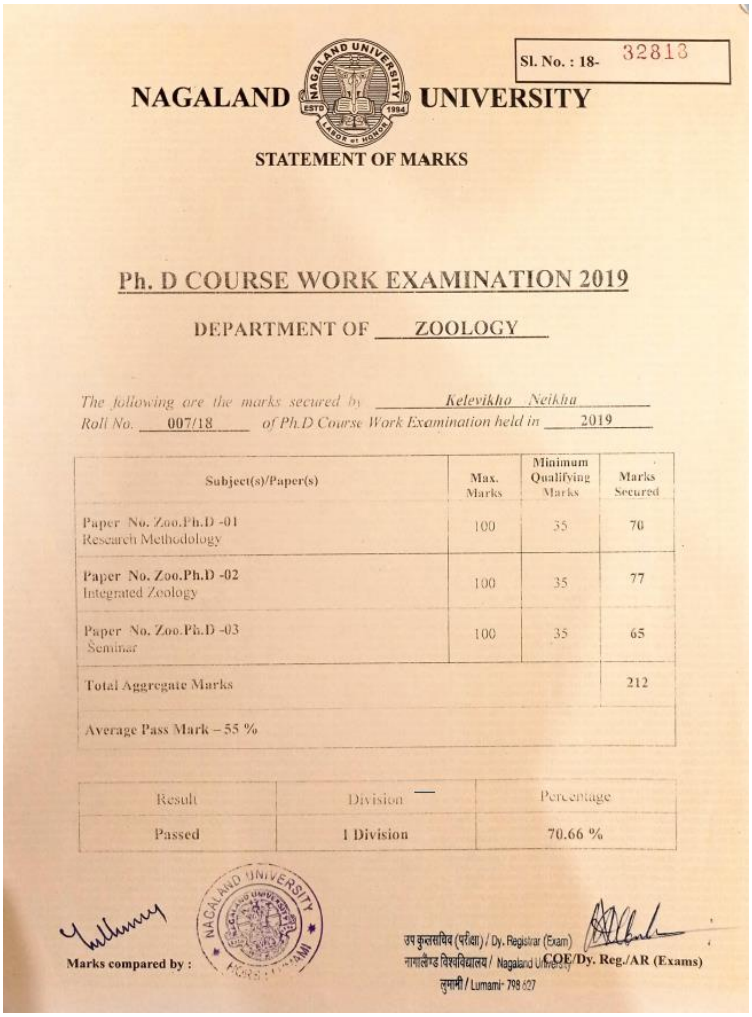
Head of Department

Dean

School of Sciences

Publications

Research Articles



NAGALAND UNIVERSITY

Sl. No. : 18- 32813

STATEMENT OF MARKS

Ph. D COURSE WORK EXAMINATION 2019

DEPARTMENT OF ZOOLOGY

The following are the marks secured by Kelevikho Neikha

Roll No. 007/18 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	70
Paper No. Zoo.Ph.D -02 Integrated Zoology	100	35	77
Paper No. Zoo.Ph.D -03 Seminar	100	35	65
Total Aggregate Marks			212
Average Pass Mark – 55 %			

Result	Division	Percentage
Passed	1 Division	70.66 %

Marks compared by :

उप कुलसचिव (परीक्षा) / Dy. Registrar (Exam)
नागालैण्ड विश्वविद्यालय / Nagaland University
लुमामी / Lumami- 798 627

COE/Dy. Reg./AR (Exams)

- 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

- Kelevikho Neikha, Nukshimenla Jamir, Abuno Thepa, Bendangtula Walling and Sarat C. Yenisetti (2021) Utility of paper microscope (Foldscope) in class room teaching of genetics. In: Experiments with Drosophila for Biology Courses (eds: S.C. Lakhotia & H.A. Ranganath). Indian Academy of Sciences, Bangalore, India. pp. 97-102. ISBN 978-81- 950664-2-1.

Book Chapters

- Kelevikho Neikha, Bendangtula Walling, Abuno Thepa, Nukshimenla Jamir and Sarat C. Yenisetti (2020) Utility of paper microscope (Foldscope) in biomedical research. Current status of research in biosciences. 193-200. Ed: Joshi PC, Joshi N, Reshman Yasmin, Mansotra DK (Today and Tomorrow publishers, New Delhi, India). ISBN 10:81- 7019-661-5.
- Bendangtula Walling, Neikha Kelevikho, Abuno Thepa, Nukshimenla Jamir and Sarat C. Yenisetti (2020) Utility of paper microscope (Foldscope) in classroom teaching of genetics 397-403. Ed: Joshi PC, Joshi N, Reshman Yasmin, Mansotra DK (Today and Tomorrow publishers, New Delhi, India). ISBN 10:81-7019-661-5.

Publications