UNDERSTANDING NEURODEGENERATION AND RESCUING PATHOLOGY ASSOCIATED WITH PARKINSON'S DISEASE IN *DROSOPHILA* MODEL

Thesis

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CERTIFICATE

This is to certify that the Thesis entitled "Understanding Neurodegeneration and Rescuing Pathology Associated with Parkinson's Disease in *Drosophila* Model" is a record of original research work done by Mr. Limamanen Phom under my supervision. He is a registered research scholar (Regd. No.565/2014) 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 degree or distinctions. The Thesis is therefore, forwarded for adjudication and consideration for the award of degree of Doctor of Philosophy in Zoology under Nagaland University.

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DECLARATION

I, Mr. Limamanen Phom hereby declare that the subject matter of this Thesis is the record of work done by me, that the contents of this Thesis did not form basis of the award of any previous degree to me or to the best of my knowledge 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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LIST OF ABBREVIATIONS

AADC	:	Aromatic amino decarboxylase
AchE	:	Acetylcholinesterase
Acp	:	Acyl carrier protein
Ad	:	Alzheimer's disease
AD	:	Autosomal dominant
ALS	:	Amyotrophic Lateral Sclerosis
AR	:	Autosomal recessive
AR-JP	:	Autosomal recessive-juvenile Parkinsonism
ATP132A	:	ATPase cation transporting 132A
ATXN2	:	Ataxin-2
CAT	:	Catalase
CNS	:	Central nervous system
DA	:	Dopamine
DAergic	:	Dopaminergic
DAMB	:	D1-like dopamine receptor
DJ-1	:	Daisuke-Junko-1
EIF4G1	:	Eukaryotic translation initiation factor 4 gamma 1
GSH	:	Glutathione
GST	:	Glutathion S-transferase
HD	:	Huntington's disease
HP	:	Hydroperoxides
HtrA2	:	HtrA serine peptidase 2
HTT	:	Huntingtin
K	:	Curcumin
LB	:	Lewy bodies
L-dopa	:	Levodopa
LN	:	Lewy neurites
LP	:	Lipid peroxides
LRRK2	:	Leucine-rich repeat kinase 2
MDA	:	Malondialdehyde
Mn	:	Manganese
MPTP	:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NDD	:	Neurodegeneration disease
NMS	:	Non-motor symptoms
OS	:	Oxidative Stress
PARK16	:	Parkinson disease 16 (susceptibility)
PARKIN	:	Parkin RBR E3 ubiquitin protein ligase
PC	:	Protein carbonyls
PD	:	Parkinson's disease
PINK1	:	Pten induced putative kinase 1
PLA2G6	:	Phospholipase A2 group VI
PQ	:	Paraquat, 1,1'-dimethyl-4,4'-pyridinium

PTEN	:	Phosphatase and tensin homolog
REM	:	Rapid eye movement
ROS	:	Reactive oxygen species
SN	:	Substantia nigra
SNCA	:	α-synuclein
SN*pc*	:	Substantia nigra *pars compacta*
TH	:	Tyrosine hydroxylase
UCH-L1	:	Ubiquitin carboxyl-terminal hydroxylase L1
VPS	:	Vacuolar protein sorting
VPS35	:	Vacuolar protein sorting-associated protein 35
α-synuclein	:	Alpha-synuclein

PREFACE

Parkinson's disease (PD) is the second most common neurodegenerative disease and it is characterized by degeneration of dopaminergic neurons in the substantia nigra of mid brain that lead to impairments of motor functions (Cacabelos *et al*, 2017). The loss of dopaminergic neurons give rise to motor symptoms like bradykinesia, rest tremor, postural instability, and shuffling gait; non-motor symptoms like impaired olfaction, constipation, depression, increased daytime sleep, rapid eye movement sleep disorder, and behavioral deficits are also commonly observed (Saleem *et al*, 2013).

Presently, the therapeutic strategies for PD include Deep Brain Stimulation and Levo-dopa supplementation. However the medication is limited only to improve the progressing symptoms and that too with more of side effects (Zrinzo *et al*, 2012; Fahn *et al*, 2000) and there is no therapy available that will cure the disease. Developing a therapeutic strategy for neurodegenerative disease such as PD remains a challenge till date. While attempting to understand the PD progression researchers have developed several animal models including *Drosophila* model. A suitable model for PD should show histopathologically characterizable progressive loss of dopamine neurons together with other neurons and significant reduction in dopamine level. Since PD is a late onset neurodegenerative disease, the symptoms depiction in the model organism should be in a stage of adulthood equivalent to the age where PD sets in. The model animal should also manifest disease in such a way that it would mimic the PD affected human motor symptoms.

Numerous case studies have reported that the subjects having exposure to pesticides, herbicides showed symptoms similar to Parkinsonism. Laboratory exposure of model organisms to environmental toxins like paraquat (PQ) is therefore productively employed to study the disease progressions. Studies on post-mortem brains from PD patients have

implicated the role of oxidative damage in the pathogenesis of PD (Yuan *et al*, 2016; Zeevalk *et al*, 2008; Bosco *et al*, 2006). Accumulation of free radicals and subsequent neurodegeneration in specific brain regions have been proposed as the underlying factors in neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Halliwell, 2006) suggesting that oxidative modifications of enzymes and proteins play a significant role in their pathogenesis.

Several researchers employ natural compounds with neuroprotective properties; try to explore ways for their therapeutic application. Curcumin, a natural active compound present in *Curcuma Longa* L. (Turmeric) has been shown to possess potent neuroprotective properties. It is largely used in food as spices, coloring agent, and traditional medicines in India, South Korea, China (Aggarwal *et al*, 2007) and properties of curcumin performing neuroprotective effect, anti-oxidant, anti-inflammatory and anti-cancer are well known. It crosses the blood-brain barrier and exerts protective action on neurons in central neurological disease (Hagl *et al*, 2015; Lee *et al*, 2013). In *Drosophila* model, curcumin has been shown to extend life span, sequester oxidative stress mediated free radicals, enhance locomotor ability and show chemo preventive property, improves characteristic symptoms associated with PD (Nguyen *et al*, 2018; Liu *et al*, 2013; Lee *et al*, 2010) suggesting its potential use in treatment applicability in higher organisms.

However, available investigations were performed in young model organisms. It is reported that there exists significant change of about 23% in genome-wide transcript profiles with age in *Drosophila* (Pletcher *et al*, 2002) and genotropic drugs would be effective only during those life cycle stages wherein target molecules are available (Soh *et al*, 2013), suggesting that targets of genotropic compounds under study may well not be present in all life stages. In spite of these important studies, no reports are available

regarding the efficacy of curcumin in PD models during later phases of adult life. Therefore, it is necessary to understand the neuroprotective efficacy of compounds at the adult phases like the transition phase in *Drosophila*, where the disease such as idiopathic PD sets in. Further, it is equally important to decipher mechanistic insights of these natural products in adult life in a stage specific manner.

A hard effort is made in this direction using *Drosophila* model of PD.

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1. Introduction

Neurodegeneration refers to the progressive loss of structure or function of neurons that lead to severe neurological conditions. Neurodegenerative diseases (NDD) are a varied assortment of central nervous system (CNS) disorders accompanied by gradual deterioration in a person's cognitive abilities such as memory, characterized by the progressive loss of neural tissues (Ramanan and Saykin, 2013). This loss is due to either structural change that prevents neurons of brain cells from functioning normally, or to cell death. The most notable NDD are Alzheimer's disease (Ad), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). NDD pose an important threat to human health, as these age-dependent disorders are becoming more and more prevalent partly due to the increasing number of elderly populations in recent years (Heemels, 2016). Genetics and environmental factors and the aging process are believed to play important role, although neurodegeneration is frequently multifactorial in origin and the involvement of genes and environment remains debatable. For neurodegenerative disorders such as Ad and PD, both familial and sporadic forms exist whereas in HD, it is solely genetic in nature (Tan *et al,* 2014).

The main pathological hallmarks of neurodegenerative diseases consist of oxidative stress (OS), proteasomal impairment, mitochondrial dysfunction, and accumulation of abnormal protein aggregates. The cause and effect of inflammation in neurodegeneration are still unknown. There is no complete cure for this disorder since neurons of the CNS cannot regenerate on their own after cell death or damage. Thus, neurodegenerative diseases carry a risk of reduction in lifespan (L´opez-Ot´ın *et al*, 2013). Generally aging is a comprehensive phenomenon resulting from constant physiological degeneration over the lifetime of almost all organisms (Kirkwood and Austad, 2000). However, many studies have reported that the lifespan of patients with neurodegenerative diseases such as Ad, PD,

and HD is decreased (Steenland *et al,* 2010). For example, people with dementia are two to four times more likely to die at a given age than individuals without dementia of the same age (Guehne *et al,* 2005).

The mammalian nervous system is highly complex and a major challenge in understanding and designing therapeutic interventions in NDD is to reduce or eliminate the early symptoms and also to improve the inflammatory responses inside the CNS once the disease sets in. In spite of several years of investigations and scientific advancements in basic and clinical research, etiology and pathogenesis of these diseases still remain vague and neurodegenerative diseases represent a scientific challenge. Developing efficient treatments for such conditions where there are no concrete findings about its causes is a challenging and essential task for the scientific community.

2. Neurodegenerative Diseases

2.1. Alzheimer's Disease

Alzheimer's disease (Ad) is a most common devastating brain disease that generally affects people above the age of 65 years. It is characterized by progressive loss of neurons in the hippocampus and cortex that consequently led to reduction of brain mass. Essentially, the cells in the brain that is necessary for processing, storing and retrieving the neuronal information are destroyed which often is the case in neurodegenerative disorders (Singh, 2012). The outcome of which is manifested by loss of cognitive abilities and behavioral functions including memory, thinking and language skills (O'Brien and Wong, 2011). Ad is correlated with synaptic degeneration and neuronal death in limbic structures, such as the hippocampus and the amygdala, and related area of the cerebral cortex. Sporadic form of Ad wherein the cause is unknown make up majority of the cases and a complex interaction of environmental and genetic factors are believed to be associated in the

development of the disorder. With autosomal dominant (AD) inheritance, about 2-3% of Ad cases are early onset and familial in nature whereas bulk of them are late-onset with unknown cause.

One prominent neuropathological hallmark of Ad is an extensive buildup of extracellular plaques consisting of amyloid-β in the brain cerebral cortex. This accumulation has been reported to commence at 10-20 years prior to start of dementia (Bateman *et al*, 2012; Buchhave *et al*, 2012). It is suggested that at the initial extended period of disease progression, soluble amyloid-β oligomers and amyloid plaques change the nature and role of restricted neuronal circuits and large-scale networks via unsettling the stability of synaptic excitation and inhibition (*E*/*I* balance) in the brain (Busche *et al*, 2016).

Familial Ad can be caused due to mutations in amyloid precursor protein (APP), which is cleaved successively by α- and β-secretases, and presenilins 1 and 2 (PS1 and PS2), one or other of which is a part of each γ-secretases complex (Walsh and Selkoe, 2007; Selkoe, 1998). Presenilin is involved in regulation of Notch and Wnt signaling system and is responsible for progressive maturation of glia and neurons (Gaiano and Fishell, 2002). β-secretases consecutively cleave the Notch receptor to produce a Notch intracellular domain (Kojro and Fahrenholz, 2005), that in turn activate nuclear genes like hairy and enhancer of split 1 (HES1) and hairy and enhancer of split 5 (HES5) to assist in neurogenesis throughout development process and repairing of damage (Kopan *et al*, 2009). Most forms of sporadic late-onset Ad have complex etiology because environmental or genetic factors alone are not adequate enough for the disease development. Currently the main risk factor in sporadic Ad is acknowledged in the allele e4 of apolipoprotein E (ApoE4) (Holtzman *et al*, 2000). But in most of the late-onset Ad cases, the factors that are responsible for the disease are still unidentified and genetics factor most likely interact with environmental factors or with additional physiologic setting to exert the pathogenic outcome.

2.2. Huntington's Disease

Huntington's Disease (HD), another heritable devastating neurodegenerative disorder is caused due to an AD mutation in the Huntingtin (HTT) gene (Pavese *et al,* 2006, Gusella *et al,* 1995). At the molecular level, HD is caused by a CAG trinucleotide repeat expansion within exon 1 of the HTT gene. In patients the amount of CAG repeats expands from the normal population range of about 16 and 20 repeats to >35 (Munoz-Sanjuan *et al,* 2011; Warby *et al,* 2011), which results in extended polyglutamine region at amino terminal side of translated HTT protein which is linked to protein aggregation and toxicity associated with gain-of-function (Williams *et al,* 2008). Aggregation of mutated gene leads to neuronal injuries in the medium spiny neurons of the neostriatum and other neurons such as the cortex (Li and Li, 2004). Individuals usually experience progressive loss of muscular functions and decline in cognitive ability, decline in self and spatial alertness, depression, dementia, and high anxiety during the period of about 20 years prior to death. Impaired olfactory function was noticed in patients and pre-symptomatic gene carriers (Mochel *et al*, 2007). Among the number of mechanisms, a supposed toxic mechanism is because of the presence of toxic oligomers (Sathasivam *et al*, 2010). HD pathogenesis is very complex although it is monogenic in nature. The HTT interactome consists of proteins that are involved in transcription, preserving DNA integrity, regulation of cellular cycle, signaling and organization, transport and homeostasis of proteins, energy metabolism (Shirasaki *et al*, 2012). Long-term memory is often unaffected but it usually impairs decision-making ability such as organizing or adapting alternatives, and delay in the acquisition of new motor skills (Craufurd *et al,* 2002; Folstein, 1990), which becomes worse over time and communication deteriorates more rapidly than understanding. Manic and psychotic symptoms also develop in some HD patients (Folstein, 1990). Prevalence of HD is 4-10 per 100000 in the western world, with many more people at risk of the

disease. Mean age of onset of the disease is 40 years, with death occurring 15-20 years after the diagnosis of the disease (Ross *et al*, 2011). At present, management is limited to controlling chorea, which is the unintentional and uneven mobility of the arms and legs, and controlling mood changing aspects; there is no therapy to completely cure the disease (Munoz-Sanjuan *et al*, 2011).

2.3. Amyotrophic Lateral Sclerosis

ALS is the most common adult-onset motor neuron degenerative disease. It is identified by a progressive loss of motor neurons located in the spinal cord, brain stem and motor cortex which leads to reduced muscular function and ultimately respiratory failure. Normally the disease starts at about 55 years of age and patients on average dies within 3-5 years of diagnosis. About 90% of the reported disorders are sporadic (sALS) with unknown cause and 10% are reported to be familial (fALS) having a genetic component. The understanding of etiology and pathogenesis of the sALS are inadequate and there are no successful treatments accessible at present; as a result there is a high unmet need for novel therapeutic approach (Goyal and Mozaffar, 2014). Patients show the symptoms of progressive muscle degeneration and fatique, increased exhaustion and troubles with swallowing, which normally lead to respiratory malfunction and death (Sejvar *et al*, 2005; Rowland, 1994). Progressive functional deficits lead to a complete loss of independence (Ng *et al*, 2011), and individual become solely dependent on care and support. About 20% of familial cases happen due to mutations in the gene that code for the Cu/Zn-superoxide dismutase (SOD1) (Rosen *et al*, 1993). Despite of the difference in causal factor, approximately 95% of affected individuals are reported to follow similar pattern of molecular pathology, that includes cytoplasmic buildup of ubiquitinated, hyperphosphorylated and non-soluble TAR DNA-binding Protein 43 kDa (TDP-43

protein) aggregates and reduced levels of nuclear TDP-43 (Brettschneider *et al,* 2013; Geser *et al,* 2008; Mackenzie *et al,* 2007; Neumann *et al,* 2006).

2.4. Parkinson's Disease

Parkinson's disease (PD) is the second most common NDD and it is characterized by degeneration of dopaminergic (DAergic) neurons in the substantia nigra (SN) of mid brain that lead to impairments of motor functions (Cacabelos 2017; De Silva *et al,* 2000). Progressive loss of DAergic neurons and intraneuronal protein inclusions, called Lewy bodies (LB) are characteristic of PD (Olanow and Tatton, 1999; Lang and Lozano, 1998). The protein α-synuclein normally found in presynaptic terminals and nuclei are the most abundant in LBs. The deposition of LB and neurites has been demonstrated to occur years before the degeneration of the substantia nigra *pars compacta* (SN*pc*) and appearance of Parkinsonism (Braak *et al,* 2000).

The neuronal loss in this region gives rise to motor symptoms like bradykinesia, resting tremor, postural instability, and gait impairment where non-motor symptoms (NMS) like impaired olfaction, constipation, depression, increased daytime sleep, rapid eye movement sleep disorder, and behavioral deficits are commonly observed (Saleem *et al,* 2013). During the time of diagnosis, about 50% of the DAergic neurons have already been degenerated. Consequently, the main focus for effective therapies would be to sustain the remaining neurons. The majority of PD cases are sporadic with unknown cause but few of the cases are familial, inherited as a Mendelian trait. Medication of L-dopa, which is a precursor of dopamine (DA), is the existing treatment for PD. However, as the therapy progress, the effectiveness of L-dopa decline over the years following which the patient is required to consume DA agonists and activate the DA receptor. Compounds that slow down DA degrading enzymes are being tested in PD. Most of the therapeutic approach

for PD is aimed to increase or sustain the existing DA pool instead of finding means to inhibit the continuous neuronal loss. There is a need for additional approach that will sustain the neurons coupled with DA replacement therapy.

Current research focuses on developing model that reproduces the α-synucleinopathy of nigrostriatal pathway (Maries *et al*, 2003). However, it is vastly dependent on the capability to either overexpress different α-synuclein protein species locally in the SN or create relevant transgenic animal models (Niu *et al*, 2015). The whole picture of PD pathology is far more complicated than a DA depletion associated motor defect, because, the neurodegenerative progression is not limited to effect on the nigrostraital DAergic pathway but it is also involved in alteration of glutamatergic, serotonergic, noradrenergic, GABAergic and cholinergic systems, along with the deterioration of other DAergic structures in addition to SN*pc*, such as ventral tegmental area (Brichta *et al*, 2013).

2.4.1. Brief History of PD

PD is a disorder which is recognized since the ancient times in Indian and Chinese civilization (Manyam, 1990). In the Ayurveda, an ancient Indian medicine, the condition is termed as *Kampavata* ("*kampa*" means tremor in Sanskrit). An Egyptian papyrus during the 12th century BC recorded about a king drooling with age (Gracia Ruiz, 2004). In Western health and clinical text, the shaky sign was elaborated by a doctor named Galen in 175 AD. In 1817, James Parkinson elaborated in his description of the disorder considering the six cases as the basis which he had noticed during his own practice and observation around his neighborhood. The description about the condition was to propose and persuade others to study the disease in more detail for better understanding. This made to establish the disorder as an accepted medical condition. He named it as "*shaking palsy* or *paralysis agitans"* and wrote thorough descriptions in "An Essay on the Shaking Palsy". He explained the shaking palsy as an "Involuntary trembling movement, with reduced

controls over muscular activity at rest and including during on a support; with an inclination to turn the body trunk frontwards, and to go by a walking to a running speed: the mind and cognitive ability remain unharmed'' (Parkinson, 1817).

Later in 1865, William Rutherford Sanders coined the term "Parkinson's disease" and it was then popularized through the works of French neurologist Jean Martin Charcot with his colleagues (Lees, 2007). They narrated symptoms of the disorder considering two criterias: tremor and rigidity. They explained in detail the arthritic changes, dysautonomia, and pain that can come along with the disease but documented that individuals having PD may not be noticeably weak and tremor is not an essential symptom (Charcot, 1872).

2.4.2. Pathophysiology of PD

PD is the second most prevalent neurodegenerative disorder that affects about 1% of the population over age 50 (Modi *et al,* 2016). An essential pathological characteristic of PD is the selective degeneration of DA neurons in the SN*pc.* The DAergic neurons are necessary for accurate functioning of muscle and coordination; their loss is associated with tremor, rigidity, bradykinesia, and postural instability. A second neuropathological hallmark of PD is the formation of proteinaceous LB inclusion in the SN of mid brain which is reported to be full of a protein combination such as *α-synuclein,* ubiquitin, *parkin*, and neurofilaments (Jellinger, 2009). The mode of progression where *α-synuclein* and additional proteins combine for generating Lewy pathology is not well understood, however it possibly involve oxidative alteration and/or cross-linking. Though, degeneration of neurons appears first in the DAergic cell of SN*pc*, degeneration of non-DAergic neurons also take place in later stage of PD (Hornykiewicz and Kish, 1987). The cholinergic nucleus basalis of Meynert, the raphe nucleus serotoninergic neurons, and the hypocretin neurons of the hypothalamus undergo neuronal loss as the disease advance.

The DAergic neurons are necessary to perform right motor function and its deficiency or loss led to manifestation of popular condition called bradykinesia, tremors and rigidity which remain the characteristic features of PD. Also, hippocampal and cortical neurodegeneration can add up to dementia which is frequently linked with PD.

Several non-motor symptoms associated with PD are sleep instability, constipation, turning off cognitive ability, depression, fright, nervousness, bladder troubles, weight fluctuation, weariness, hypotension and sexual dysfunction. Such weakening conditions greatly affect the patient's quality of life (Pfeiffer, 2016) and with the passage of time as the disease and motor symptoms advance, the patient lose independence and cannot carry out basic function, thus they become fully dependent on care and support. The present available treatment methods deal with controlling the symptoms but by and large it cannot halt the disease development and incidence of increased mortality among PD patients still persist (Levy *et al*, 2002).

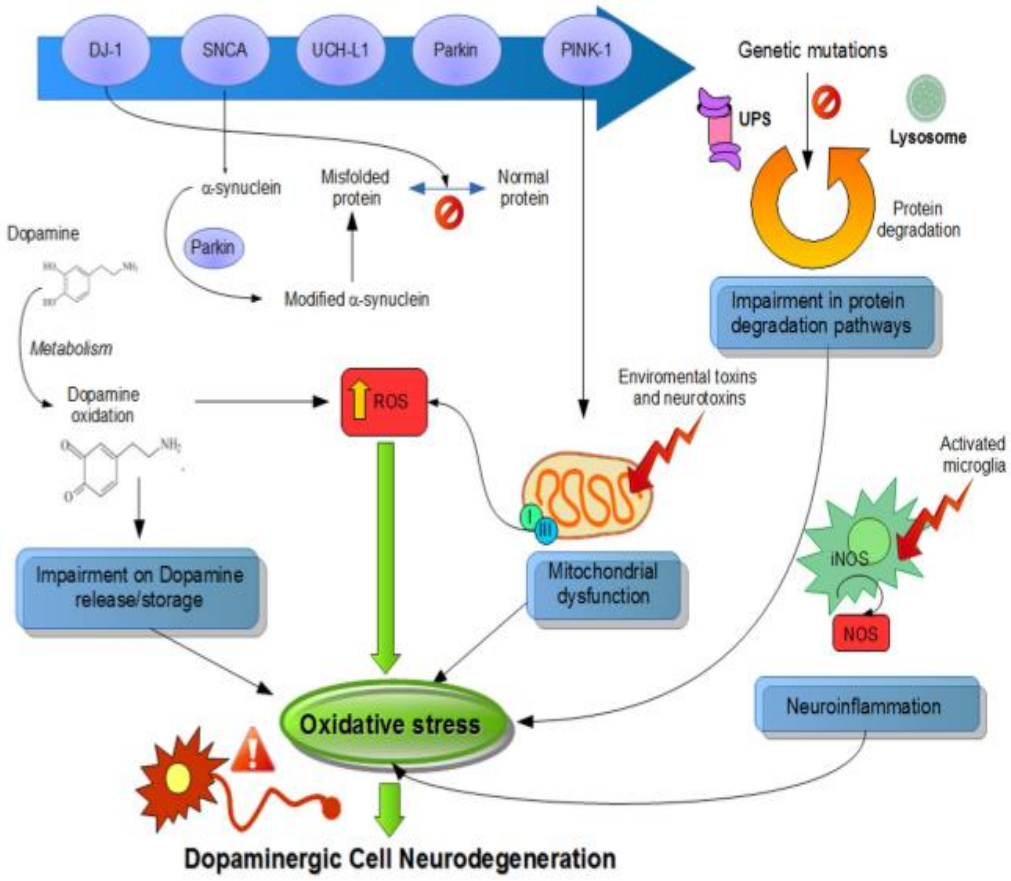


Figure.1. Suggested physiological processes related to pathogenesis of Parkinson's disease

Genetic alteration through various external influences affects pathways and lead to abnormal functions in genes that are associated with PD resulting in augmented OS, mitochondrial dysfunction and protein misfolding. Further neurotransmitter DA metabolism can be oxidized to reactive DA quinines that will contribute to high ROS. Conformational change in *α-synuclein* protein and its aggregation takes place. ROS affect the normal functioning of UPS resulting in decreased degradation of damaged and misfolded proteins consequently impairing the cell homeostasis and inducing cell death. Environmental stressors affect usual functioning of mitochondria, amplify the reactive free radicals and lead to accumulation of proteins like *α-synuclein*. Inhibition of mitochondrial complex I lead to decreased ATP synthesis which results in injury to intracellular mechanism and cell death. Furthermore, neuroinflammatory mechanisms might add up to process that cause the cells more susceptible to degenerate. All these numerous cellular mechanisms recognized to induce OS are implicated in the selective degeneration of DAergic neurons (Adapted from Blesa *et al*, 2015).

3. Symptoms of PD

3.1. Motor symptoms in PD

PD is associated with the following motor symptoms like resting tremor, bradykinesia or sluggish movements, stiffness, shuffling gait, postural unsteadiness. The setting in of PD is insidious where affected persons may point the symptoms to aging processes. The symptoms progresses over time however the speed at which motor problem develop are extremely variable (Fritsch *et al*, 2012).

Apart from the "classic" movement disorders that are previously described, there are several additional motor symptoms observed among the patients. They are masked facial expression (hypomimia), reduced eye blinking, hazy vision, lame upward gaze, dystonia, bent position, trouble rotating in bed, kyphosis, scoliosis, freezing and difficulty in verbal communication, such as hypophonia (progressively softer voice), or palilalia (recurrence of word or phrase) (Chou, 2013). One of the worst experiences the PD patients come across in severe condition is they lose the ability to do even basic works and become fully dependent on someone for support.

3.2. Non-motor symptoms in PD

Non-motor symptoms (NMS) are also widespread among PD patients. Independent studies have shown no less than one NMS in nearly all of the affected individuals (Kim *et al*, 2013; Krishnan *et al*, 2011). Additionally, NMS were reported among all the PD patients who were also under unstable motor ability (Witjas *et al*, 2002). Comparatively, aged individuals will experience atleast one NMS and thus among individuals without PD, it occurs normally in the process of aging. Therefore, it may be irrelevant to attribute all NMS experienced by PD subjects as caused by the PD (Kim *et al*, 2013; Krishnan *et al*, 2011). However, it is also reported that, PD patients have higher tendency to develop

diverse form of NMS which is often accompanied with increased occurrence and severity (Khoo *et al*, 2013).

Abnormalities of sensation: Various sensation abnormalities are reported with the olfactory impairment the most commonly described. Several other issues also arise such as vision and patterns of pain. During the point of diagnosis olfactory impairment is already familiar in about 90% of the cases. But the impaired sense of smell may go unnoticed to over 70% of affected individuals and that the impaired olfaction is independent of disease stage and duration (Doty *et al*, 1988). There are other report suggesting that impairment of olfaction is related to increased severity of the disease; acute hyposmia among PD individual scan signal the progress of PD dementia (Cavaco *et al*, 2015). NMS like olfactory testing are recommended for screening of PD although at the clinical level such testing can take a long time before concluding. As such, researchers conducted a study for just three odors (coffee, peppermint, and anise) and found comparable outcome to test with a complete odor panel (Casjens *et al*, 2013). Positive results in odor detection have been reported through olfactory exercise, but substantiation with further studies is required (Knudsen *et al*, 2015).

Behavioral changes: Depression is a regular phenomenon seen in PD. Occurrence of major depressive disorder in 17%, minor depression in 22% and dysthymia in 13% of individuals with PD have been reported (Riejnders *et al*, 2008). This behavioral change may occur prior to motor impairment as an early indication of PD. Medications including antidepressant are found to be efficient for treatment of Parkinsonian depression and beneficial role of transcranial magnetic stimulation is being developed.

The occurrence of anxiety in PD is 25-40% (Simuni *et al*, 2013). Anxiety may also emerge at any stage of the disease which often develops before the motor dysfunction and

persons with persistent anxiety are at higher risk group to develop PD. Among the PD patients generalized anxiety disorder, panic and one of the phobic disorders are common which may turn into noticeable wearing off occurrence. Recent meta-analysis indicates that increased incidence of apathy among patients is around 40% (den Brock *et al*, 2015) and a connection of apathy with impaired executive function particularly complexity with initiation is clear in PD.

Autonomic dysfunction: One of the common non-motor symptoms in PD is autonomic dysfunction that can be manifested at any point of time of disease and some facets like constipation may be developed with higher incidence earlier than the appearance of motor problem. Orthostatic hypotension which may be present in almost 60% of PD case is largely recognized feature of cardiovascular dysfunction in PD. Gastrointestinal dysfunction in PD may appear as dysphagia, impaired peristalsis, bacterial overgrowth in small intestine, and bowel dysfunction (Fasano *et al*, 2015). PD patients with gastroparesis may experience satiety, low appetite, bloating, abdominal stiffness, nausea, vomiting, and weight loss. Small intestinal bacterial overgrowth is described among PD patients and may be responsible for progression of motor fluctuations in certain case (Fasano *et al*, 2015).

Sleep disturbances: Sleep disturbance or impaired sleep is a common situation that occurs among the PD patients with its prevalence rate reaching about 90% (Kurtis *et al*, 2013). Sleep disturbance with recurrent nocturnal awakenings is one of the most regular forms of insomnia in PD. The basis is multifactorial that is associated with stiffness in sleep rendering difficulty in turning sides in bed. Identification and targeting the source of the problem will result in an effective treatment of this symptom. Substantial interest has generated in rapid eye movement (REM) sleep behavior disorder (RBD) distinguished by

constant capacity to move about in REM sleep, both as a symptom and as susceptible factor to develop PD. The occurrence of RBD in PD is not clearly identified and it may possibly be present years before classic motor features of PD appear.

Fatigue: Although very little is known about the mechanism involved and efficient treatment of fatigue is limited, it has been gradually accepted to be involved in PD progression. Fatigue is commonly recognized by individuals having PD as one of the most disabling symptoms that has negative impact on their daily life (Dogan *et al*, 2015).

4. Synthesis and Role of DA

Dr. Carlsson and colleagues showed that DA was a neurotransmitter in the brain and not just a precursor of norepinephrine (Carlsson *et al,* 1957). It is involved in a range of important CNS functions, including voluntary movement, feedback mechanism, sleep and awareness, effective memory and learning. In the periphery, DA act on essential physiological function in the regulation of olfaction, retinal processes, hormonal and sympathetic regulation, cardiovascular functions, immune system and renal functions, among others (McHugh and Buckley, 2015; Iversen and Iversen, 2007; Carlsson, 2001; Sibley, 1999; Missale *et al,* 1998).

DA synthesis in DAergic terminals requires tyrosine hydroxylase (TH) which, in the presence of iron and tetrahydropteridine, oxidizes tyrosine to 3,4-dihydroxyphenylalanine (Levodopa or L-dopa). L-dopa is decarboxylated to DA by aromatic amino acid decarboxylase (AADC), an enzyme which requires pyridoxyl phosphate as a coenzyme. Once released from presynaptic terminals, DA mediates its action by activating members of a family of G protein-coupled DA receptors named D1 to D5. The actions of DA are terminated through presynaptic reuptake. Some of the DA is then re-incorporated into vesicles, while the rest is metabolized.

DA receptors were classified on the basis of physiological (Cools *et al,* 1976), pharmacological and biochemical role they play (Trabucchi *et al,* 1975); alternatively, a dynamic equilibrium of a single DA receptor fluctuating between two state of configuration has been postulated (Creese *et al,* 1975). The demonstration of a DA sensitive adenylyl cyclase not only provided an in vitro model to study the properties of DA receptors but also suggested a mechanism for the generation of the physiological response to the amine (Kebabian *et al,* 1971). However not all the DA receptors have properties similar to those of the receptor linked to the adenylyl cyclase.

In a study employing rat model, fetal DA neurons could survive transplantation from the ventral midbrain to the 6-hydroxy DA lesioned dorsal striatum and could also perform motor functions (Bjorklund *et al,* 1980). This finding resulted in the experimental use of DA grafts in PD patients (Dunnett *et al,* 1999). Additionally, grafting studies in rats established the topographical cortico-striatal makeup of the dorsal striatum and the significance of this region of the DA system for understanding of complex motor behaviors (Dunnett *et al,* 1999; Dobrossy *et al,* 1996).

In *Drosophila*, DA is both a vital neuromodulator and a precursor of molecules that are necessary for hardening and pigmentation of the external cuticle (Riemensperger *et al,* 2011; Friggi-Grelin *et al,* 2003). DA released from fly neurons interacts with specific G protein-coupled DA receptors, either of the D1 or D2 subtypes (Hearn *et al,* 2002; Blenau *et al,* 2001), including dDA1 and D1-like DA receptor (DAMB), which play important function in arousal and memory (Berry *et al,* 2012; Lebestky *et al,* 2009). A long-term over expression of the DA-synthesizing enzyme TH in the nervous system prolongs the survival of adult PQ-intoxicated flies and a down-regulation of the DAMB receptor results in an enhanced PQ resistance in fly. DA signaling modulates OS resistance in the

Drosophila nervous system since an expansion of DAMB expression appears to trigger the age-related increase in PQ susceptibility of young adult flies.

5. Genes involved in DA metabolism and in metabolism of xenobiotics

A number of gene variants which code for proteins that are associated with DA transport and metabolism, metabolism of xenobiotics and in OS response, have mainly been under investigation as a probable cause for PD susceptibility. But results are conflicting or inconclusive for vast majority of them. Several authors support the opinion that there is no concrete result with regard to risk factor of PD because quite a few investigations have suggested relationship between gene polymorphisms and PD, just a handful have studied the gene-environment connection (Mellick, 2006). A large case control study across 5 European centers was conducted with the sole intention to understand the gene-environment interaction and the risk of developing PD (Dick *et al*, 2007). Exposure to solvents, pesticides and metals like iron, copper and manganese (Mn) was analyzed and linked to polymorphisms in a number of PD putative risk genes Cytochrome P450 2D6 (*CYP2D6*), *PON1* (Serum paraoxonase/arylesterase 1), *GSTM1&3* (Glutathione *S*-transferase Mu 1 & 3), *GSTT1* (Glutathione S-Transferase Theta 1), *GSTP1*(Glutathione s-transferase pi 1), *NQO1* (NAD(P)H Quinone Dehydrogenase 1), *CYP1B1* (Cytochrome P450 Family 1 Subfamily B Member 1), *MAO-A&B* (Monoamine oxidase A&B), *SOD2* (Superoxide dismutase 2), EPHX (Epoxide Hydrolase), *DAT1* (DA Transporter 1), *DRD2* (DA Receptor D2)and *NAT2* (N-Acetyltransferase 2). In male subjects, a modest association was found between *MAO-A* polymorphism and PD susceptibility and a greater part of gene-environment analysis did not show noticeable interaction effects. But, an increased risk of PD was seen among *GSTM1* null subjects heavily exposed to solvents. In a pesticide exposed environment *GSTP1* polymorphisms have been associated with PD among the population (Menegon *et al*, 1998), and there are evidence suggesting that

herbicide exposure modify the relation between *GSTP1* polymorphisms and PD age at onset (Wilk *et al*, 2006). There is no decisive confirmation found for an overall relationship of N-acetyl transferase 2 slow acetylator genotypes to PD in a meta-analysis of published papers. Also, the *CYP2D6* gene variants have also been broadly measured as genetic risk factors for PD with no concrete outcome. But some data suggests that the PD susceptibility might be modulated by exchange involving the *CYP2D6* genotype and environmental factors including contact with pesticides and cigarette smoking (Elbaz *et al*, 2004).

6. Epidemiological Studies on PD

A case study involving drug user patients was performed in 1980s. Patients showed symptoms of visual hallucinations, limb jerking and stiffness. At the initial use of the synthetic heroin, within 4 to 14 days they experienced a generalized slowness and difficulty in moving. Examination of each subject showed almost complete loss of mobility, noticeable tone increase, failure to converse clearly, fixed glancing, reduced eye blinking, facial seborrhea, regular drooling, responsive to glabellar tap test and cogwheel inflexibility in the upper extremities and one subject exhibited a pill rolling tremor at rest on the right hand. One could perform walking and showed a diminutive stepping, sluggish, shuffling gait and bradykinesia. All the subjects showed a flexed position distinctive of an advanced stage of PD. It was described to be linked with the chemical substance 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and suggested that a product of Meperidine-analog synthesis lead to chronic Parkinsonism in humans (Langston *et al*, 1983). Almost immediately after the discovery of MPTP neurotoxicity, potential pesticides and herbicides involvement in development of PD was studied since there is close structural resemblance between MPP^+ (an active toxin, a product of MPTP metabolism) and herbicide 1,1'-dimethyl-4,4'-pyridinium (paraquat). Interestingly, in the

1960s, MPP^+ itself was experimented as an herbicide under the marketable name of cyperquat (Di Monte, 2002).

Since the Second World War, several pesticides, insecticides, herbicides, fungicides, have been extensivelyin use. Farmers were exposed to pesticides because of direct handling and contact skin, consumption of contaminated food and water or aerial inhaling of sprayed pesticides or fumigants. Later on, these farmers developed Parkinsonian symptom and in the recent decades, relationship between individuals contact with pesticide and frequency of PD occurrence has been well documented and recognized. A meta-analysis of epidemiological studies conducted around the world during 1989-1999 showed the relationship between exposures of pesticides to the incidence of PD (Priyadarshi *et al*, 2000).

A study in Taiwan investigated the role of environmental factors for PD. They considered 120 PD patients and 240 hospital control subjects having age and sex matched. Researchers undertook a standardized interview to gather the subjects' history of exposure to environmental factors. On analysis, the use of pesticides and herbicides in their rural occupational farming were found to be associated with an increased PD risk with highest among those subjects who had used paraquat (PQ) and other herbicide/pesticides. Therefore, concluding that exposure to environmental factors like PQ and other herbicides/pesticides, may take significant part in the developing PD (Liou *et al*, 1997).

Another study from southwestern region of Taiwan investigated whether functional variants of genes interacted with occupational pesticide exposure to increase PD risk. A total of 153 patients with sporadic PD and 155 healthy subjects were genotyped for genetic variants. This study found significant differences in genotypes between PD patients and the control subjects. Besides this the combined Mn dependent superoxide dismutase

(MnSOD*)* and NAD(P)H Quinone Dehydrogenase 1 (NQO1) variant genotype was notably linked to a 4.09-fold increased risk of PD in pesticide exposed subjects (Fong *et al,* 2007), suggesting the role of environmental influence on susceptible genes. Another study conducted in Val Camonica (Italy), a geographic area with higher prevalence of individuals affected by Parkinsonism, probably related to increased exposure to Mn. On investigating whether polymorphism in genes regulating Mn metabolism and toxicity could modify neurophysiological effects of Mn exposure, it was found that Mn exposure significantly impaired motor coordination in the elderly people (Rentschler *et al*, 2012).

PQ exposure in *Drosophila* model shows OS induced neurodegenerative phenotype like motor dysfunction as seen among human PD subjects. It has been observed that *Drosophila* mutant for DA regulating genes show an uneven susceptibility to PQ. Exposure of PQ in *Drosophila DJ-1* mutants lead to impairment of motor ability but found resistant to its toxicity in loss of function of *DJ-1β* mutants and overexpression of *DJ-1α* in DA neurons confers protection. PQ induced DAergic neuronal loss are protected and confers delay in symptoms when there is mutation in down regulator of DA production Catecholamines-up (Catsup) (Chaudhuri *et al*, 2007). Combined exposure of PQ and Maneb on E1 ligase knockdown *Drosophila* resulted in considerable loss of DA neuron (Martin *et al,* 2014) suggesting that environmental agents like pesticides may act in synergy and develop as risk factor for PD.

7. Sporadic Parkinson's Disease

In sporadic PD there is no known cause and studies implicate the role of environmental factors with susceptible genetic background as one of the main causes of the disease. Sporadic PD pathogenesis is thus believed to have multiple factors which involve

interaction between the environment and gene. Environmental factors like neurotoxicants are thought to be involved in the pathogenesis of nigrostriatal degeneration, which support idea that there is relationship between environment agents and PD (Di Monte *et al,* 2002).

Furthermore, the report that mutation in *α-synuclein* gene causes familial PD (Polymeropoulos *et al,* 1997) and its recognition as a possible risk factor for sporadic form of PD (Simon-Sanchez *et al,* 2009) provides an association of genetic background that causes the disease. Several studies have suggested that among the rural people, regularly consuming well water, exposure to farm pesticides and agricultural farm occupation, mining and welding are more prone to gradually develop PD (Ritz *et al,* 2009; Elbaz *et al,* 2009; Dhillon *et al,* 2008; Kamel *et al,* 2007). Epidemiological studies indicate the link between PD and environmental toxic factors. Further several other results propose that contact with environmental pesticide like bipyridyl, organochlorine, PQ and carbamate derivatives could add to developing PD (Liou *et al,* 1997; Seidler *et al,* 1996).

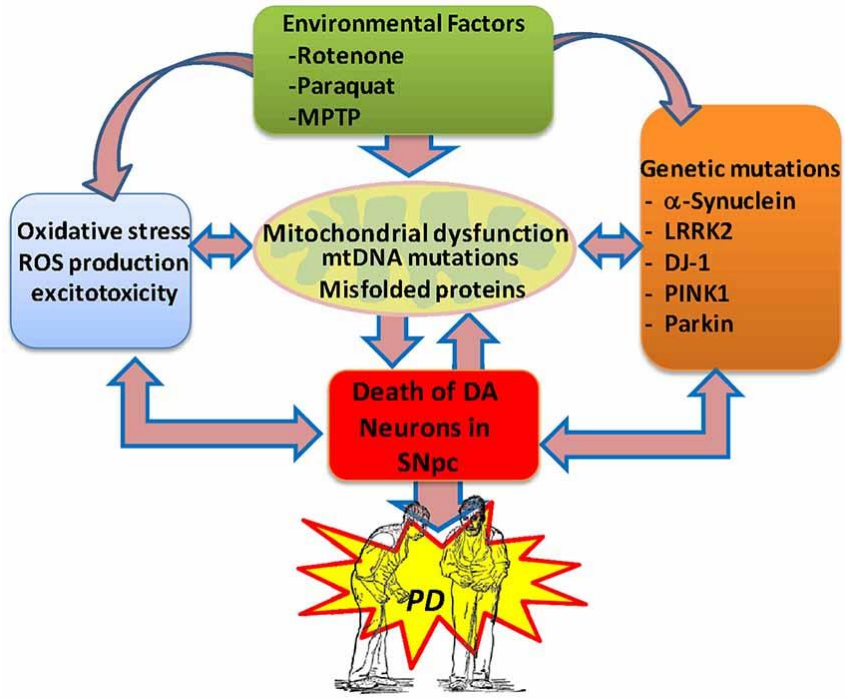


Figure.2. Potential etiological factors of Sporadic Parkinson's disease. Current evidence suggests that environmental factors and genetic factors provoking OS, excitotoxicity and mitochondrial dysfunction in the brain which lead to the degeneration of the midbrain DAergic system, resulting in PD (Adapted from George *et al*, 2014).

8. Genetics of Parkinson's Disease

There are about 15 genes identified till now and 21 loci that are associated with genetic form of PD. Human PD genes and their *Drosophila* homolog are depicted in Table 1. Some of them are related below.

Symbol	Gene locus	Gene	*Drosophila* homolog	Inheritance	Disorder	Status and remarks
PARK1	4q21-22	*SNCA* (Polymeropoulos *et al*, 1997)	no homolog	AD	Early-onset Parkinsonism	Confirmed
PARK2	6q25.2-q27	*PARK2* encoding Parkin (Kitada *et al*, 1998)	parkin	AR	Early onset Parkinsonism	Confirmed
PARK3	2p13	Unknown	-	AD	Classical Parkinsonism	Unconfirmed
PARK4	4q21-q23	*SNCA*	no homolog	AD	Early-onset Parkinsonism	Erroneous locus (identical to PARK1)
PARK5	4p13	*UCHL1*	Uch	AD	Classical Parkinsonism	Un-confirmed
PARK6	1p35-p36	*PINK1*(Valente *et al*, 2004)	pink1	AR	Early onset Parkinsonism	Confirmed
PARK7	1p36	*PARK7* encoding DJ-1 (Bonifati *et al*, 2003)	Dj-1α and dj-1β	AR	Early onset Parkinsonism	Confirmed
PARK8	12q12	*LRRK2* (Paisán-Ruíz *et al*, 2004)	Lrrk	AD	Classical Parkinsonism	Confirmed
PARK9	1p36	*ATP13A2* (Ramirez *et al*, 2006)	CG32000	AR	Kufor-Rakeb syndrome, a form of juvenile-onset atypical Parkinsonism with dementia, spasticity and supranuclear gaze palsy	Confirmed

PARK10	1p32	Unknown	-	Risk factor	Classical Parkinsonism	Confirmed susceptibility locus
PARK11	2q36-27	Unknown (maybe *GIGYF2*)	-	AD	Late onset Parkinsonism	Not independently confirmed
PARK12	Xq21-q25	Unknown	-	Risk factor	Classical Parkinsonism	Confirmed susceptibility locus
PARK13	2p12	*HTRA2*	HtrA2	AD or risk factor	Classical Parkinsonism	Un-confirmed
PARK14	22q13.1	*PLA2G6* (Paisan-Ruiz *et al*, 2009)	iPLA2-VIA	AR	Early-onset dystonia-Parkinsonism	Confirmed
PARK15	22q12-q13	*FBXO7* (Shojaee *et al*, 2008)	no homolog	AR	Early-onset parkinsonian-pyramidal syndrome	Confirmed
PARK16	1q32	Unknown (maybe *RAB7L1*)	-	Risk factor	Classical Parkinsonism	Confirmed susceptibility locus
PARK17	16q11.2	*VPS35*	Vps35	AD	Classical Parkinsonism	Un-confirmed
PARK18	6p21.3	*EIF4G1*	eIF4G	AD	Late onset Parkinsonism	Un-confirmed
PARK19	1p31.3	*DNAJC6* (Edvardson *et al*, 2012)	auxillin	AR	Juvenile-onset Parkinsonism	Confirmed
PARK20	21q22.11	*SYNJ1* (Krebs *et al*, 2013; Quadri *et al*, 2013)	Synj	AR	Early-onset Parkinsonism	Confirmed

AD, autosomal dominant; AR, autosomal recessive.

Table: 1. Monogenetic forms of PD and its fly homolog(s). (Adapted from Modi *et al*, 2016).

8.1. α-Synuclein (*SNCA*)

The *SNCA* gene provides instructions for making a small protein called α-synuclein. A missense mutation in the *SNCA* gene was identified in a large Italian family by Polymeropoulos and colleagues. Their study involved a traditional linkage approach through which they could track the underlying genetic damage in the long arm of human chromosome number 4 (Polymeropoulos *et al*, 1996) which brought about a FRAME SHIFT in the PD genetic study. Another study showed that the main composition of LB, pathological hallmark of PD was the α-synuclein protein (Spillantini *et al*, 1998). Thus, these two important scientific results suggested an association of sporadic form with familial forms of PD.

Patients with *SNCA* mutation have early age onset of PD with initially good response to L-dopa treatment however dementia develop in increased pace at regular feature as the disease advance. Then case such as cognitive decline, hallucinations and alteration in self-awareness becomes apparent and upon histopathological investigation reveals a rich LB pathology (Sheerin *et al*, 2014). Of the two-point mutations, H50Q was identified with late onset PD showing a L-dopa responsiveness and cognitive impairment (Proukakis *et al*, 2013; Appel-Cresswell *et al*, 2013). The G51D point mutation which was initially described in French kin having Parkinsonian-pyramidal syndrome was accompanied by an early-onset Parkinsonism with mild to modest response to L-dopa coupled with and fast disease development (Lesage *et al*, 2013). Two additional copies of the genomic region consisting the *SNCA* gene as a basis of PD have been reported. Thus, duplications and triplications of *SNCA* locus serve as a ground of causing PD as well which is more common. In case of triplication, carriers have early-onset disease development whereas *SNCA* duplication patients have later onset of symptoms (Nishioka *et al*, 2006).

8.2. Leucine-rich repeat kinase 2 (*LRRK2*)

The LRRK2 gene provides information for making a protein called dardarin that are involved in kinase and GTPase activity. A study involving a large Japanese family reported link between autosomal-dominant Parkinsonism and chromosome number 12 (Funayama *et al*, 2002). It was then recognized that mutations in *LRRK2* gene results in fundamental genetic cause of chromosome number 12 associated with PD (Paisan-ruiz *et al*, 2004; Zimprich *et al*, 2004). Although numerous *LRRK2* mutations have been reportedly suggested as a cause of PD, only few mutations are found to have high degree of proof. G2019S is the most common mutation of *LRRK2* which is identified in about 1% and 6% case of the sporadic and familial PD respectively (Healy *et al*, 2008). The second frequent *LRRK2* mutation is the R1441G which is very normally found among the Basques (Di Fonzo *et al*, 2006).

8.3. Parkin RBR E3 ubiquitin protein ligase (*PARKIN*)

PARKIN was the second identified PD gene. *PARKIN* plays a role in the cell machinery that degrades unneeded proteins by tagging damaged and excess proteins with molecules called ubiquitin. Initially, a disorder which is denoted by early dystonia and L-dopa medication induced complication, osteotendinous hyper-reflexia with relatively sluggish motor development was reported in Japan in 1973, later it was called the Autosomal-recessive juvenile Parkinsonism (AR-JP) (Khan *et al*, 2002). The condition was recognized as due to mutations in *PARKIN* gene (Kitada *et al*, 1998). It was then suggested that AR-JP locate to long arm of chromosome number 6; associated with pointer *D6S305* and *D6S253* and the former is reported to be deleted in an AR-JP Japanese patient (Matsumine *et al*, 1998). Mutations in *PARKIN* are the main reason of autosomal-recessive (AR) early-onset Parkinsonism. In most of the cases, the disorder is associated with heterozygous *PARKIN* mutations where the

transfer is through dominant mode, thereby indicating that even carting a single *PARKIN* mutation may serve as possible factor for a person and ultimately develop PD (Lohmann *et al*, 2003; Lincoln *et al*, 2003). Clinically, slow disease progression coupled with better response to L-dopa treatment and early dyskinesia has been reported in the profiling of patients carrying the mutated *PARKIN* gene. Uncharacteristic symptoms like key psychiatric manifestations, cerebellar, neuropathy, hyper-reflexia and dystonia signs were also observed (Lohmann *et al*, 2003).

8.4. Daisuke-Junko-1 (*DJ-1*)

DJ1 is involved in cell protection, particularly brain cells, from OS; serve as a chaperone molecule that helps fold newly produced proteins into the proper 3-dimensional shape and helps refold damaged proteins and involved in delivering selected proteins to proteasomes. *DJ1* gene mutation was identified as a basis of AR early onset PD (Bonifati *et al*, 2003) with the locus for this form of PD localized to 1p36 in the Netherlands. This type of mutations is rare and only about 1% *DJ-1* mutation has been identified in early-onset PD individuals (Abou-Sleiman *et al*, 2003). Clinically, the symptoms seen among of *DJ-1* subject are similar to *PARKIN* and *PINK1* associated Parkinsonism (Massano *et al*, 2012).

8.5. PTEN induced putative kinase 1 (*PINK1*)

The *PINK1* gene provides instructions for making a protein called Phosphatase and tensin homolog (PTEN) induced putative kinase 1, which help protect mitochondria from malfunctioning during periods of cellular stress, such as unusually high energy demands. In a study involving Sicilian family with AR Parkinsonism, mutations in the *PINK1* was first recognized (Valente *et al*, 2004). Most of the cases observed were of

missense mutation but copy number mutations, genetic and exonic shifting was also reported (Samaranch *et al*, 2013). Homozygous and compound heterozygous form of *PINK1* mutations have been identified in sporadic and genetic case of PD. This suggests possible part of lone *PINK1* heterozygous mutation as causal factor for PD (Nuytemans *et al*, 2010). *PINK1* mutation is related to early-onset PD in most cases and its connection in sporadic cases is about 2%–4%. Clinically, the phenotype is similar with those patients of *PARKIN* and *DJ-1* mutations. The affected individuals show L-dopa-responsive pattern steadily (Samaranch *et al*, 2013). At neuropathology, neuronal loss in the SN*pc*, LB and aberrant neuritis in the reticular nuclei of the brainstem, SN*pc* and nucleus basalis of Meynert are reported in the *PINK1*-linked PD (Valente *et al*, 2004).

8.6. Vacuolar protein sorting-associated protein 35 (*VPS35*)

This gene belongs to a group of vacuolar protein sorting (VPS) genes. The encoded protein is a component of a large multimeric complex, termed the retromer complex, involved in retrograde transport of proteins from endosomes to the trans-Golgi network. Two separate studies using exome sequencing described that mutation in *VPS35* causes monogenic form of PD. They detected p.D620N mutation in *VPS35* in Swiss family members with a late-onset, AD PD (Vilarino *et al*, 2011). Later study in a large multigeneration and two Austrian PD families reported the identification of the p.D620N mutation and two other families screened for *VPS35* mutations was published (Zimprich *et al*, 2011) but details of this case are inadequate.

8.7. ATPase cation transporting 13A2 (*ATP13A2*)

This gene encodes a member of the P5 subfamily of ATPases which transports inorganic cations as well as other substrates. Study in consanguineous Jordanian

family initially reported loss of function in a neuronal P-type ATPase gene, *ATP13A2* (Najim al-Din *et al*, 1994). At clinical level, the patients showed an early disease ons*et al*ong with signs of rigid-akinetic phenotype and less trembling at rest, pyramidal syndrome, gradual cognitive dysfunction, vertical gaze palsy, small myoclonus, sleeplessness and L-dopa receptive features (Najim al-Din *et al*, 1994). Loss of function in this gene mapping on chromosome 1p36 lead to an uncommon form of PD recognized as Kufor-Rakeb syndrome (Hampshire *et al*, 2001). Using homozygosity mapping and positional cloning, compound heterozygous mutations in *ATP13A2* have been identified in juvenile Chilean kin (Ramirez *et al*, 2006). Clinical features of such early-onset pallidopyramidal syndrome show varying effect of severity and barely few cases have been identified (Schneider *et al*, 2010; Ning *et al*, 2008).

8.8. Ubiquitin carboxyl-terminal hydrolase L1 (*UCH-L1*)

The *UCHL1* gene provides information for making an enzyme called ubiquitin carboxyl-terminal esterase L1. Ubiquitin carboxyl-terminal esterase L1 is possibly involved in the cell machinery that breaks down (degrades) unneeded proteins. In a German family with AD transmission PD, reported a missense mutations of gene that code for the ubiquitin carboxyl-terminal hydrolase L1 (*UCH-L1*), a ubiquitin recovery enzyme found on chromosome 4p14 (Leroy *et al*, 1998). The subjects show phenotype similar to those of idiopathic PD patients. Later a connection between the *UCH-L1* gene S18Y variant and PD (Ragland *et al*, 2009) was established through an epidemiological study. Overexpression of *UCH-L1* gene causes upregulation in formation of aggresomes by impairment of proteasome system (Ardley *et al*, 2004).

8.9. *HtrA* serine peptidase 2(*HtrA2*)

This gene encodes a serine protease, a protein that is found in the endoplasmic reticulum and interacts with an alternatively spliced form of mitogen-activated protein kinase 14. The protein has also been localized to the mitochondria with release to the cytosol following apoptotic stimulus. The protein is thought to induce apoptosis by binding the apoptosis inhibitory protein. Loss of function of the gene coding for *Omi/HtrA2* (high temperature requiring A2 mitochondrial protein) have been suggested to be one of the risk factors for PD through several studies employing German (Strauss *et al*, 2005) and Belgian PD patients (Bogaerts *et al*, 2008). At molecular level, mitochondria release the *Omi/HtrA2* and promote apoptosis and mutations in *Omi/HtrA2* gene interrupt its protease action associated to mitochondrial impairment (Bogaerts *et al,* 2008; Strauss *et al,* 2005). *Omi/HtrA2* functions on *PINK1/PARKIN* pathway downstream of *PINK1* and perform function free from *Parkin* influence (Whitworth *et al,* 2008). However, apart from *PINK1* or *PARKIN* null mutants, this is not established in *Omi/HtrA2* knock-out mutants (Yun *et al,* 2008).

8.10. Eukaryotic translation initiation factor 4 gamma 1 (*EIF4G1*)

The protein encoded by this gene is a component of the multi-subunit protein complex *EIF4F*. This complex facilitates the recruitment of mRNA to the ribosome, which is a rate-limiting step during the initiation phase of protein synthesis. In chromosome 3q26-q28 of a northern French family with AD late-onset Parkinsonism, a mutations in the eukaryotic translation initiation factor 4-gamma (*EIF4G1*) as a causal factor was reported. Analysis recognized a heterozygous mutation in *EIF4G1* that was established afterwards in LB disease patients and several other PD subjects (Chartier-Harlin *et al,* 2009). Additionally, in a study on a different multiplex separate family,

a pathogenic mutation was detected among all the affected members along with an unaffected 86-year-old kin indicative of partial penetrance (Nuytemans *et al,* 2013).

8.11. Phospholipase A2 group VI (*PLA2G6*)

The *PLA2G6* gene provides instructions for making a type of enzyme called an A2 phospholipase that is involved in breaking down (metabolizing) fats called phospholipids. In an investigation employing three patients of two inbred Pakistani families, a homozygous mutation was reported in phospholipase A2 gene (*PLA2G6*) found on chromosome 22q13.1. Their phenotype was related to cognition and psychiatric difficulty and dystonic features, pyramidal syndrome (Paisan Ruiz *et al,* 2009). Frontotemporal lobar atrophy and dementia were prominent in compound heterozygous mutations in *PLA2G6* induced early-onset recessive Parkinsonism in a study involving an Asian group (Yoshino *et al,* 2010).

8.12. Parkinson disease 16 (susceptibility) (*PARK16*)

Following genome wide association and a double replication studies, this locus was identified in chromosome 1q32 in Japanese patients (Satake *et al,* 2009). It was replicated in Caucasians and Han population (Chang *et al,* 2013; Simon-Sanchez *et al,* 2009). Evidence of association with PD was found in a case control Scandinavian study for a coding variant that is located around 5' region of RAB7L1 (Pihlstrom *et al,* 2015). In PD patients of United Kingdom other variants were also identified within the *SLC41A1* gene (Tucci *et al,* 2010) although it was not replicated in another study of PD patients from Spain (Mata *et al,* 2011).

8.13. Spinocerebellar ataxias type 2 (*ATXN2*)

ATXN2 gene encodes Ataxin-2 protein involved in epidermal growth factor receptor (EGFR) trafficking, acting as negative regulator of endocytic EGFR internalization at the plasma membrane. L-dopa responsive Parkinsonism identified in a large Asian-American family showed that the cause of disease was due to an expansion mutation in *ATXN2,* the cause of spinocerebellar ataxias type 2 (SCA2) (Gwinn-Hardy *et al*, 2000). Two out of the three affected family members met the PD Society Brain Bank and NINDS (National Institute of Neurological Disorders and Stroke) norms for diagnosis of PD. The other member was also diagnosed under PD by their physician, although the patient did not satisfy the norms. This study was briefly followed by a number of independent results with parallel conclusion (Furtado *et al*, 2004). Patients may present both L-dopa responsive Parkinsonism and also show features like ataxia, disease reminiscent of advancing supranuclear palsy, trembling and dementia. A comprehensive neuropathologic description on patients with *ATXN2* expansion mutation and L-dopa-responsive Parkinsonism is lacking.

9. Animal Models of PD

Animal models allow researchers to recapitulate some of the clinical manifestation of the diseases of humans hence giving an optimal opportunity to better understand the mechanism involve in the disease progression. Though model animal shares several molecular and cellular processes common to humans there exist limitations too. Complex human behaviors such as aggression, circadian rhythms, sleep, learning, memory and mating are observed in other animal as well such as *Drosophila*. Therefore, by investigating these processes and behaviors in animal models, one can gain an understanding of the basic biology underlying them and apply this knowledge to figure out how diseases occur and find the corrective measures.

9.1. Non-Human Primate Models

Non-Human Primates has been successfully modeled to study PD. The clinical and behavioral assessments like abnormal in voluntary movement scale for dyskinesia can be practically applied to the marmoset monkeys free from any adaption (Van Vliet *et al*, 2006). MPTP induced marmoset monkeys show clear and lasting behavioral features of PD and many quantitative motor behavior tests such as full body motor behavior such as locomotor activity, rotational task; specific motor performance such as 'arm reaching' motor behavior is very well described in such model (Marshall and Ridley, 1996). These studies are extensively used in PD diagnosis and moreover treatment in non-human primate model can directly be used for PD patients at the clinical level (Philippens, 2008). However, high cost of production and maintenance limit the wider use of non-human primate model; there is very less genetic data to work with considering the fact that there is a low efficiency of assisted reproductive technology for producing genetically engineered oocytes and embryos necessary for genetics base study for PD (Oliveira *et al*, 2017). Also, there are several technical issues for deriving and cloning embryonic stem cells and long period of gestation of embryo and fewer off spring per year makes it very extended process to make any result conclusive.

9.2. Rodent Models

Mice and rats represent one of the most commonly used animal models in PD. Most of these models have been developed with use of neurotoxins and also genetic models through genomic manipulations. α-synuclein aggregation have been implicated to be main reason for development of PD and mouse prion promoter (mPrP) A53T α-synuclein transgenic mice display complete range of α-synuclein pathology that is seen in humans including α-synuclein aggregation, fibrils and truncation, α-synuclein

phosphorylation and ubiquitination and gradual age-dependent neurodegeneration (Chesselet, 2008; Dawson *et al*, 2003). Several approach to create α-synuclein transgenic mice with progressive loss of DA neurons such as crossing α-synuclein transgenics to *parkin* knockouts (von Coelln *et al*, 2006), conditional expression of α-synuclein (Lin *et al*, 2009), and DJ-1 knockouts (Ramsey *et al*, 2010) and overexpressing α-synuclein with DA specific promoters have not been very productive (Daher *et al*, 2009; Thiruchelvam *et al*, 2004). Rodent's resistance to neurotoxin like MPTP varies from species to species and even strain to strain (Hamre *et al*, 1999). Even though there is a complete penetrance of α-synuclein in presence of mutant *PARKI, PINK1* or *DJ1*, there is no meaningful DA neuron degeneration in mouse model thus leaving a wide gap on the accuracy of this model. Besides, the open field test and swim tests which is employed to study DA deficiency induced motor deficit in mouse models require the animal to learn first. Not all the animals learn these skills even before receiving DA lesions so when failed it is difficult to deduce if it is learning deficit or PD motor symptom, even the specimen which show symptoms are typical of bradykinesia and akinesia (Potashkin *et al*, 2010).

9.3. Zebra Fish Model

The Zebra fish model is efficient because of its higher offspring production rate and possibility of screening toxin induced or genetically engineered traits very rapidly. The transparent embryo develops outside the mother so the neural developmental pattern under induced PD condition is easily traceable. Its high similarity in catecholaminergic system with human makes them an excellent model for PD (Pienaar *et al*, 2010). Moreover, the TH containing neurons (the DA neurons) in the ventral diencephalon of zebrafish brains seems to be homologous to mammalian midbrain SN and ventral tegmental neurons (Son *et al*, 2003). PD-related genes, such as *DJ-1* (Bai

et al, 2006; Bretaud *et al,* 2006), *UCH-L1* (Son *et al,* 2003), *SNCA, PINK1, PARK2* and *LRRK2* (Flinn *et al,* 2008) are evolutionarily preserved between humans and zebrafish, and their protein products are expressed in zebrafish ventral diencephalic DA neurons and the PD symptom in this region affects the spontaneous swimming phenotype hence the genotype to phenotype is highly relatable.

The similarities are limited to lower motor neurons of the fish while, upper motor neurons are partially resembling with the human as there is absence of the corticospinal and rubrospinal tracts (Babin *et al,* 2014). The progression in knockout lines used as most common way of silencing a gene through Antisense Morpholino Oligonucleotides (AMO) works only during few days of development, therefore generating a late disease specimen like PD is difficult (Babin *et al,* 2014). Moreover, AMOs tend to go off target and induce apoptosis in motor neurons (Bill *et al,* 2009; Eisen and Smith, 2008), thus assessing the phenotype with regard to gene activity becomes challenging. Further, loss or gain of function of a gene produces delayed development and body malformation, so when there is a motor neuron deficiency or malfunction it might be secondary effect rather being primary effect of the gene in question (Babin *et al,* 2014).

9.4. *Drosophila* Model

In the Order Insecta, the family Drosophilidiae comprises of more than 3,500 described species including the genus *Drosophila* (Bachli, 1998). It is estimated that there are more than 2240 biologically valid species of *Drosophila* (Wheeler, 1986). In India, reports from the review of literature show more than 200 *Drosophila* species (Hegde *et al*, 2000) some of which are endemic to certain regions of the country and a few are cosmopolitan in distribution.

Drosophila has been an essential model organism to study human diseases. It was initially used in the laboratory by William Castle at Harvard University as a minute, fast generating organism for embryological studies. It was not until 1909 that the fruit fly made its initial application in the field of genetics. A natural transformation in eye color from brick red wild type to white caught the attention of Columbia University professor Thomas Hunt Morgan. He investigated if this mutation would go after the hereditary patterns as predicted earlier by Gregor Mendel. After mating the white-eyed male (*w*) with a wild type female (*w+*), he found that the resulting ratios of progenies followed the predicted patterns. This demonstration brought to light the importance of *Drosophila* to the research community. It has been reported that about 75% of identified human disease genes have orthologs in fly genome (Llyod *et al*, 2010; Reiter *et al*, 2001). Many investigations employ environmental toxins in *Drosophila* models and try to understand the disease pathology for exploring possible therapeutic intervention.

9.4.1. Environmental Toxin Models of PD using *Drosophila*

A number of environmental neurotoxins including herbicides and pesticides are used in *Drosophila* to mimic PD-like symptoms and understand the disease mechanism (Bonini and Fortini, 2003). The fly has well developed CNS and complex functions like walking, climbing, and flying makes *Drosophila* more appropriate model for understanding PD. Such intricate actions are alike among different strains which makes easier to characterize a toxin induced PD model (Feany and Bender, 2000). Widely employed environment neurotoxin PD models are discussed below.

9.4.2. Rotenone Model of PD in *Drosophila*

Rotenone induced slowing down of mitochondrial respiratory complex has been extensively employed for studying the function of mitochondria in apoptosis (Chauvin *et al*, 2001; Barrientos and Moraes, 1999). Mitochondria are not only involved in ATP generation in eukaryotes but also play significant role in apoptosis (Wang, 2001; Kroemer and Reed, 2000; Green and Reed, 1998). Rotenone is lipophilic and therefore can simply cross the blood-brain barrier (BBB) (Coulom and Birman, 2004). In one such model, rotenone exposure led to neuromotor defect and significant inhibition of neurotransmitter enzyme AchE activity. They reported induction of OS among rotenone exposed flies as evident by sharp increase in ROS and malondialdehyde (MDA) levels. Another study subjected fly to rotenone treatment and reported decreased in DA content and impairment in motor activity, increased mRNA expression of antioxidant enzymes (Rao *et al*, 2016; Sudati *et al*, 2013).

9.4.3. PQ Model of PD in *Drosophila*

PQ is commonly employed in the *Drosophila* model to induce phenotype associated with PD system and other animal models to study PD. It has been reported that D1-like DA receptor (DAMB), strongly add to the rapid CNS breakdown as a result of PQ exposure in the fly (Casser, 2015). They have shown that DAMB expression is reduced upon continuous neuronal DA synthesis and confers resistance to the PQ toxicity while age-related diminution of resistance to PQ was assisted by a considerable enhancement in DAMB receptor. The findings suggest that several other aspects remain to study and understand DA pathogenesis in *Drosophila*. The fly display motor impairment including tremor at rest, slowness, restless and unsteadiness which is similar to PD symptoms. When allowed to climb over the vials in testing motor functions, the flies freeze and frequently lose the grip and fall to the bottom (Chaudhuri *et al*, 2007). Degeneration of DAergic neuron upon PQ exposure is time

dependent where it has been shown that PPL1 (protocerebral posterior lateral) cluster is affected by 6 hours, 12 hours for posterior inferiormedial protocerebrum (PPM) 2 and 3 cluster but 20-24 hours for PPM1 and PPL2 clusters. This process is accompanied by alteration in neuronal appearance by aggregation of cell bodies to circular shape, fragmentation and then loss (Chaudhuri *et al*, 2007). Recent study employing PQ toxin model of PD in fly have also reported increased mortality, locomotor impairment, decreased DA levels (Soares *et al*, 2017) suggesting the toxicity associated with PQ exposure and relevance of the model to induce Parkinsonism.

10. Conclusion

Many workers have made extensive effort to understand the pathophysiology associated with PD and there are numerous data that are available on fly PD models. They treat the organism with environmental toxins and study the modulatory effect of prospective therapeutic compounds. However, till date their study is limited to only in young animals model. PD is a late onset disease; it is important to understand the disease progression and therapeutic effectiveness at later stage of the organism, the age equivalent to disease onset in human. Therefore, I decided to develop stage specific *Drosophila* model of sporadic PD and screen the protective efficacy of K at different stages of *Drosophila* adult life span.

CHAPTER II

DEVELOPING ADULT STAGE SPECIFIC *DROSOPHILA* MODEL OF SPORADIC PARKINSON'S DISEASE

1. Introduction

A suitable model for PD must recreate the condition showing progressive loss of DA neurons, accompanied by degeneration of other neurons and considerable diminution in DA level. In addition, since the onset of the PD is late onset, the model organism should also show the phenotype during adulthood. The model animal must also imitate the PD pathology and neuronal dysfunction by showing phenotype such as bradykinesia, rigidity, postural instability and resting tremor, with motor symptoms having positively reactive to L-DOPA (L-dopa, the precursor of DA) or any anti PD drug therapy.

2. *Drosophila* as a Model of PD

2.1. Advantages

The advantage of using *Drosophila* to study human NDD and their essential molecular and cellular pathology are numerous. One of the main factors is its short life cycle which takes about 10-14 days to develop from egg to fully matured adult fly; single female can produce many offspring and there are several established methods to maneuver its genetic expression (Bilen and Bonini, 2005). Besides, as compared to other models, *Drosophila* is commonly employed to study disease for decades; making its anatomy and phenotypes relatively more comprehensive to experimental biologists (Venken, 2007; Matthews *et al*, 2005). With a well developed CNS and prominent number of DA neurons (White *et al*, 2010, Botella *et al*, 2009), the fly has properly distinguished behaviors that are conserved with different strains in 90% of the cases (Lessing and Bonini, 2009) which makes the *Drosophila* an efficient and less expensive model.

Genetically, about 75% of human disease related genes have functional orthologs in the fly (Lloyd and Taylor, 2010, Reiter *et al*, 2001). The whole resemblance at nucleotide or protein level is 40% while in terms of the conserved domain it is 80%-90% or even higher (Pandey and Nichols, 2011).

Although the invertebrate fly have major differences in physiology when compared to human, yet through genetic manipulations it is possible to induce and mimic human PD pathology in the fly. Over the years, researchers using fly model have developed notable range of genetic tools. The binary system expression like UAS/GAL4 system that allows inserting a specific gene of interest in selected set of cells, activate and deactivate them has made the fly more convenient and cost effective model for research. Using such approach researchers can examine role of gene in the region of interest while keeping the other regions intact and also maintaining the fly health, which is significant for replicating human disease conditions targeting particular cell-types. Such approach using transgenic fly can be achieved with precision including observation of age dependent DA neuronal degeneration and convenient visible of LB and Lewy neuritis (LN) which make up the clinical hallmark of PD (Feany and Bender, 2000). Degeneration of DAergic neurons with aging and motor dysfunction remains the two main phenotypes in *Drosophila* PD model. There are discrete clusters of DA neurons distributed all over the fly brain that are functional for its diverse behaviors. Thus *Drosophila* shows very complex motor behavior like climbing, flying, locomotion, conditioning to fear etc. Hence age dependent loss of DA neurons in PD conditions (Feany and Bender 2000) and sporadically induced PD conditions showing DA neuron dysfunction and subsequent phenotypes are easily characterizable and can be related to humans. Thus apart from its use in advancing the understanding of basic biology, *Drosophila* has been effectively employed to investigate a number of human diseases, including PD.

2.2. Disadvantages

Drosophila being an invertebrate animal shares some important dissimilarity in physiology as compared to human such as the brain anatomy, cardio vascular and respiratory system; Less complex and adaptive immune system as in vertebrates, effects

of some drugs on the organism might differ strongly (e.g. conversion of pro-toxins to toxins in liver) (Prubing *et al*, 2013). Discrepancy related to blood-brain permeability has been a matter of concern when it comes to studing disease of CNS (Stork *et al*, 2008). The model cannot accurately predict the effects of drugs on human and the metabolic distinction is another matter to consider in study of drug efficiency and toxin induced disease phenotype.

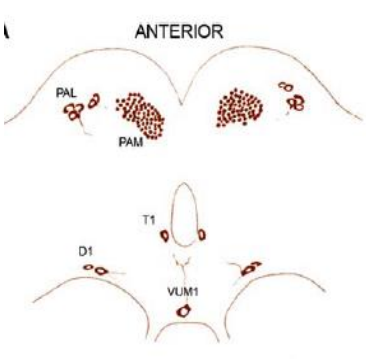
3. *Drosophila* Mimicking Human PD Symptoms

Although the physiological dissimilarity of fly when compared to human are very significant, the central phenotype associated with PD pathology experienced in human are reproduced in a commendable fashion through the method of disease induction using toxins or by employing transgenic modification. The accuracy is so close that brain area specific and age dependent DA neuronal degeneration as commonly observed in PD patients through post mortem studies and hallmark PD biomarker i.e., the LB and LN inclusions are visible in transgenic model system (Feany and Bender, 2000). Behaviors like mating, aggression, conditioning to fear; learning and motor qualities like flying, climbing and walking which are attributed to human are also seen in *Drosophila* (Lessing and Bonini, 2009; Sokolowski, 2001). These multitude triats are affected by the PD onset and progression in human that are reproduced and characterized in the fly model.

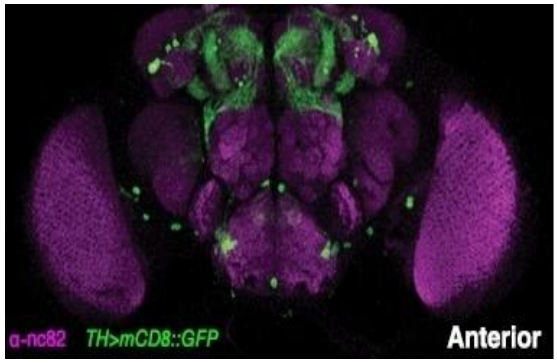
In *Drosophila,* the DA neurons are spread as a group of clusters all over the brain. Specific clusters derive specific functions for various behavioral patterns of the fly. The two areas generally targetted are (i) the mushroom bodies: Associated to memory formation and motivation, (ii) the Central Complex: Centre for motor behavior control. Region specific controlling of diverse behavioral patterns in fly is similar to those seen among the mammalian brain (White *et al*, 2010). Schematic representation of an adult fly brain with the distribution of DA neuron clusters are given in Figure 1.

4. *Drosophila* Genetic Similarities with Human

Genetically, *Drosophila* shares 61% homology with human. All the genes till date reported in human associated with Parkinsonism, be it familial or sporadic are available in *Drosophila* as a homolog except for the *α-synuclein* gene which produces LB and LN at the extracellular brain matrix which is the distinctive hallmark biomarker under PD condition in mammalian brain. This adds up to the advantage for genetic manipulation employing *Drosophila* as a model for PD.



(A)



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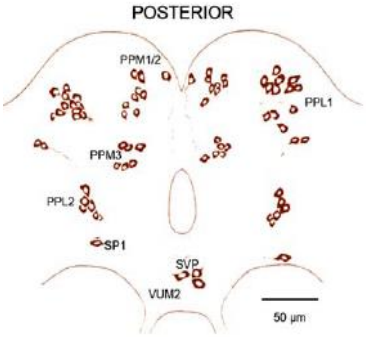


(C)

(D)

Figure.1(A-D) : A and C Schematic representation of an adult fly brain with the distribution of DA neurons grouped in clusters and arranged with bilateral symmetry (modified after Nassel and Elekes, 1992) [Image adapted from Botella *et al*, 2009]. B and D Confocal Z-stack of TH > mCD8::GFP brain; anti-nc82 immuno-reactivity together with GFP labeling reveals DAergic neurons in the anterior and posterior brain (White *et al*, 2010).

List of Parkinsonian genes and their homologues in flies is given in Table 1.

Table 1: Showing Parkinsonian genes and their fly homologues (adapted from Ayajuddin *et al*, 2018)			
Gene/Protein	Inheritance	Fly Homolog	Protein Function
A-synuclein	AD	None	Pre-synaptic protein
Parkin	AR	Parkin/CG10523	E3 ubiquitin ligase
UCH-L1	unclear	Uch/CG4265	E3 ubiquitin hydrolase/ligase
PINK1	AR	Pink1/CG4523	Mitochondrial kinase
DJ-1	AR	DJ-1a/CG6646 DJ-1b/CG1349	Redox sensor/Chaperone
LRRK2	AD	lrrk2/CG5483	Kinase/GTPase
HtrA2	AD	HtrA2/CG8486	Mitochondrial pro-apoptotic protease
GBA	unclear	CG33090	Lysosomal enzyme
POLG	unclear	tamas/CG8987	Mitochondrial DNA polymerase
Tau	unclear	tau/CG31057	Microtubule stabilization

Legends: UCH-L1 = ubiquitin carboxyl-terminal esterase L1; PINK1 = PTEN induced putative kinase 1; LRRK2 = leucine-rich repeat kinase 2; HtrA2 = high temperature requirement protein A2; glucocerebrosidase = GBA; POLG = polymerase gamma; AD = autosomal dominant; AR = autosomal recessive.

5. Scope for Stage Specific studies in *Drosophila* Model of PD

Several laboratory studies have reportedly used *Drosophila* to develop model of PD using young flies of 5-10 days old (Jahromi *et al*, 2015; Cassar *et al*, 2015). Studies regarding susceptibility genes and pathways on PD under PQ treatment have been conducted mostly using young flies (Wang *et al* 2014; 2003). However, it is important to note that NDD such as PD is late onset disease. The adult life span of fly has been reported to comprise of three phases namely health, transition and senescent span (Arking *et al*, 2002). These stages are governed by varied assortment of gene expression and such pattern is comparable to corresponding life stages of human. There is a marked variation of about 23% in genome-wide transcription profiles as the *Drosophila* cross through the adult stages and age. Genes including those associated with stress response and oogenesis showed age dependent transcript representation and this aging process is characterized by changes in expression of many genes depending upon life stage specific factor (Pletcher *et al*, 2002). Since the animal models are indispensable for disease progression studies and therapeutic approach, it is equally important to follow the age specific study while developing animal model for NDD such as PD because the onset of this disease in human is clinically manifested during the later stage of life. Therefore, research comprising the age specific stages will greatly enable the early identification of markers, providing timely interventions in the initial course of disease progression.

Hence, I developed a *Drosophila* model of PD by considering all the different time points in the fly life span i.e., health span, transition span and senescence span.

All these life stages of *Drosophila* are believed to have both common and unique complex process and can be influenced independently by a relatively large number of stages associated pathways. Therefore, adult specific model of PD is important because a particular target may be active at one stages of life while inactive in another stage and

therefore genetic targets of compounds such as curcumin (K) may be present only at one stage but not in all stages of life. As such the compound will be effective in the organism only during those phases when its target molecules are present (Soh *et al*, 2013) which is an important and interesting paradigm.

Until now, there are no reports relating to the effectiveness of K neuroprotection using PD models at later stage of adult life. It is an indispensable aspect while developing animal model for studying the disorder since it is during the later phase where late-onset NDD like the idiopathic PD sets in.

Drosophila has been an excellent organism to study different stages of life span and specific gene expression. Similar to the different human life stages like childhood, adulthood and old age. Various phases of life stages have a distinctive pattern of gene expression (Pletcher *et al*, 2002). There exists considerable variation of about 23% in genome-wide transcript profile according to different age group in *Drosophila*, demonstrating that genetic targets of prospective polyphenol compounds may be absent during some phase in the entire stages of life. Therefore, this aspect has to be considered while developing any disease model for therapeutic approach. Also implicating that polyphenol compound may have distinctive beneficial effect during certain stage of life and later turn out to have neutral or harmful action in another stage in the adult life period of *Drosophila* (Phom *et al*, 2014).

Major changes in gene expression with age occur in the genes related to energy metabolism such as proton transport, energy pathways, oxidative phosphorylation and neuronal function, especially responses to light. Genes involved in protein catabolism and several other metabolic processes also showed age dependent changes. Interestingly, biological processes namely, the caloric restriction and the light reduction are two known

environmental changes that increase life span in *Drosophila*. Decline in reproductive capacity is an age-related phenotype, and the reproductive system seems to play an important role in longevity (Finch *et al*, 1990). There are decreased RNA levels for several genes that are involved in reproduction including two genes that encode for members of the Acyl carrier protein (Acp) family. The Acp from male flies stimulates female egg-laying and facilitates storage of sperm in the female genital tract. The transition span and the senescence span of *Drosophila melanogaster* are accompanied by the reduction of transcript levels for genes involve in reproduction, metabolism, and protein turnover stemming from age regulated transcript level changed in response to OS and accumulation of free radicals.

6. Oxidative Stress in PD

OS is a condition of cellular discrepancy in the levels of reactive oxygen species (ROS) generated and the capacity of a biological system to detoxify the reactive intermediates, which creates an imbalance and lead to harmful state that contributes to cellular injury. OS is classically defined as a redox imbalance with an excess formation of oxidants or a defect in antioxidants (Sies, 2015). In physiological conditions, tissues have a functional anti oxidative system glutathione (GSH) that is depleted due to OS. Excessive generation of free radicals causes alteration in GSH level thus decrease in GSH concentration contributes in OS and consequent brain damage (Shungua *et al*, 2017; Jain *et al*, 1991). Alterations in the physiologic maintenance of neuronal redox balance interfere with several biological processes that eventually lead to cell death. Several machineries are suggested to be responsible for ROS influx that include the normal metabolism of DA itself, injury to mitochondria, iron, neuroinflammatory cells, calcium, and aging (Dias, 2013).

The body in general has developed several defense mechanisms to counteract OS, however, the brain which comprises only 2% of the total body weight, appears to be more susceptible to oxidative damage than any other organ as it consumes about 20% of the resting total body oxygen processing a lot of oxygen per unit tissue mass. Human brain has relatively low levels of antioxidants, high polyunsaturated fatty acids and large amount of iron in regions like the globus pallidus and the SN, thus susceptible to OS. Humans need oxygen to survive, but hyperoxia produces toxicity, including neurotoxicity (Chavko *et al*, 2003). Moreover, being post mitotic, when the neurons in the brain are injured, their functional ability becomes permanently impaired (Calabrese *et al*, 2005).

OS is suggested to be involved in the degeneration of DAergic neurons in PD. Abnormal acculmulation of reactive oxygen and nitrogen species in PD lead to injury in important cellular components such as lipids, proteins, and DNA. Elevated levels of MDA which is an indicator for lipid peroxidation and 4-hydroxynonenal; high protein oxidation as indicated by protein cross-linking, carbonylation and breakdown; high levels of 8-hydroxy-2′-deoxyguanosine, a product of DNA oxidation, causes oxidative injury to brain. Due to all these modifications which are experimentally demonstrated, the "free radical hypothesis" has become a major factor for understanding the etiology and therapeutic approach to PD (Jenner, 2003).

7. Environmental Exposure to PQ

PD progressions have been associated with exposure to environmental stressors like PQ an herbicide widely used in agricultural farming. Ingestion of PQ by accident or for suicidal purposes causes severe pulmonary lesions, which are often fatal. PQ has two positive charges and it undergoes single electron reduction chemistry in those cells that can sequester it, which result in a widespread oxidative and nitrosative stress. The

underlying mechanisms are directly or indirectly related to reactive oxygen species. PQ can induce PD-like lesions in certain mouse strains and rats and number of evidences suggests that the exposure of PQ results in neurotoxicity. Therefore, PQ is generally used in animal models including the fruit fly *Drosophila* to study and understand PD and the degeneration of DAergic neurons which characterizes the disease.

Since PQ is used extensively in agriculture, there has been a concern that gradual exposure to herbicide may increase the risk of developing PD in the human population. Considerable attention was gained when cases of human exposure resulting in extreme toxicity were reported and its use in eradicating marijuana crops. Additionally, the structure of PQ is closely comparable to that of MPP^+, which led to assumption that PQ also might be a potential DA neurotoxin as well (Dawson *et al*, 2003). PQ is accumulated into the brain through the BBB, possibly via a carrier mediated mechanism (Shimizu *et al*, 2001).

8. Epidemiological studies on link between PD and PQ

A number of epidemiological studies have reported a connection between PQ exposure and the development of late onset of PD suggesting the possible role of environmental factors in developing sporadic PD, which make up about 90% of the PD cases. A study conducted in Taiwan between 1993 and 1995 on 120 PD patients and 240 hospital controls was reported (Liou *et al*, 1997), generating more attention on possible association between PQ and PD. In their analysis, they showed that the account of living in rural surroundings, regular exposure to herbicides/pesticides and farming and use of PQ were linked with an increased possibility to PD risk. Reports explaining the interaction between PQ and PD have been published (Hertzman *et al*, 1990). A study conducted on disease risk linked

with occupational exposure to environmental factors like pesticides support a significant association of PD with toxin exposure (Tanner *et al*, 2011).

Laboratory studies have provided contradictory outcome. Early studies suggested that the pharmacokinetic properties of PQ made it an improbable candidate since it has low partition coefficient, restricted absorption and poor accessibility to CNS (Koller, 1986). Besides that, PQ also showed modest penetrative ability in the brain structures of rats with an intact BBB (Widdowson *et al*, 1996). However, some researchers have reported the capacity of PQ to pass through the BBB, probably via the neutral amino acid transporter (Shimizu *et al*, 2001), and to build up in certain brain regions of the mouse (Prasad *et al*, 2014).

9. PQ induced Parkinsonism in *Drosophila* Model

Exposure to environmental toxins like the herbicide PQ over a period of time has been linked to the development of PD. PQ is a nonselective pesticide that was first produced in 1961; a member of a chemical class known as bipyridyl derivatives, which includes diquat (1,10-ethylene-2,20-bipyridylium dibromide) and cyperquat that has the same structure as the MPTP metabolite MPP^+. The main cause of death associated with PQ consumption and poisoning is due to pulmonary accumulation of bipyridyl compound causing respiratory failure due to oxidative induced damage to the alveolar epithelium with subsequent pulmonary fibrosis. PQ exposure also triggers OS by disturbing the redox homeostasis in the body and generates abnormal amount of ROS which can neutralize cellular defense mechanisms (Fig 2).

Laboratory exposure of model animals to PQ leads to reduction in action of neurotransmitters in brain (Mehdi and Qamar, 2013). Also, several other studies have reported neuronal damage and death induced by PQ; cerebellar granule neurons in rats

(Stelmashook *et al*, 2007), spinal motor neurons (Kriscenski-Perry *et al*, 2002). Using rat brain neuroblast cell model it is shown that PQ also triggers rapid activation of intracellular signaling cascades leading to neural cell death (Niso-Santano *et al*, 2006).

To carry out initial discovery and characterization of neurodegenerative genes and the molecular pathways involved, *Drosophila* model offers an optimal research tool. More importantly, the fly model is extensively used to study gene-environment interactions with influence on phenotypic outcomes (Okray and Hassan, 2013). Study of survivability of *Drosophila* exposed to PQ reveals that the fly is vulnerable to PQ in a concentration dependent way. Varying degrees of toxicity has been shown upon PQ exposure. Exposing PQ systemically cause about 30% selective DA neuronal loss in the SN*pc* and augmented expression and aggregation of α-synuclein (Manning-Bog *et al*, 2002). PQ also is reported to cause loss of DAergic neurons from SN and disintegration of striatal terminals accompanied by a reduced locomotor action (Ossowska *et al*, 2005; Brooks *et al*, 1999). These studies support the understanding that loss of DA neurons upon PQ exposure can be experimentally reproduced. A recent study in *Drosophila* has suggested that DA receptor contributes to PQ induced neurotoxicity. Targeted inactivation of D1-like DA receptor, DAMB in glutamatergic neurons (GNs) noticeably improved the survival of *Drosophila* exposed to either PQ or neurotoxic levels of DA, and DAMB overexpression in these cells made the flies more susceptible to both compounds (Cassar *et al*, 2015).

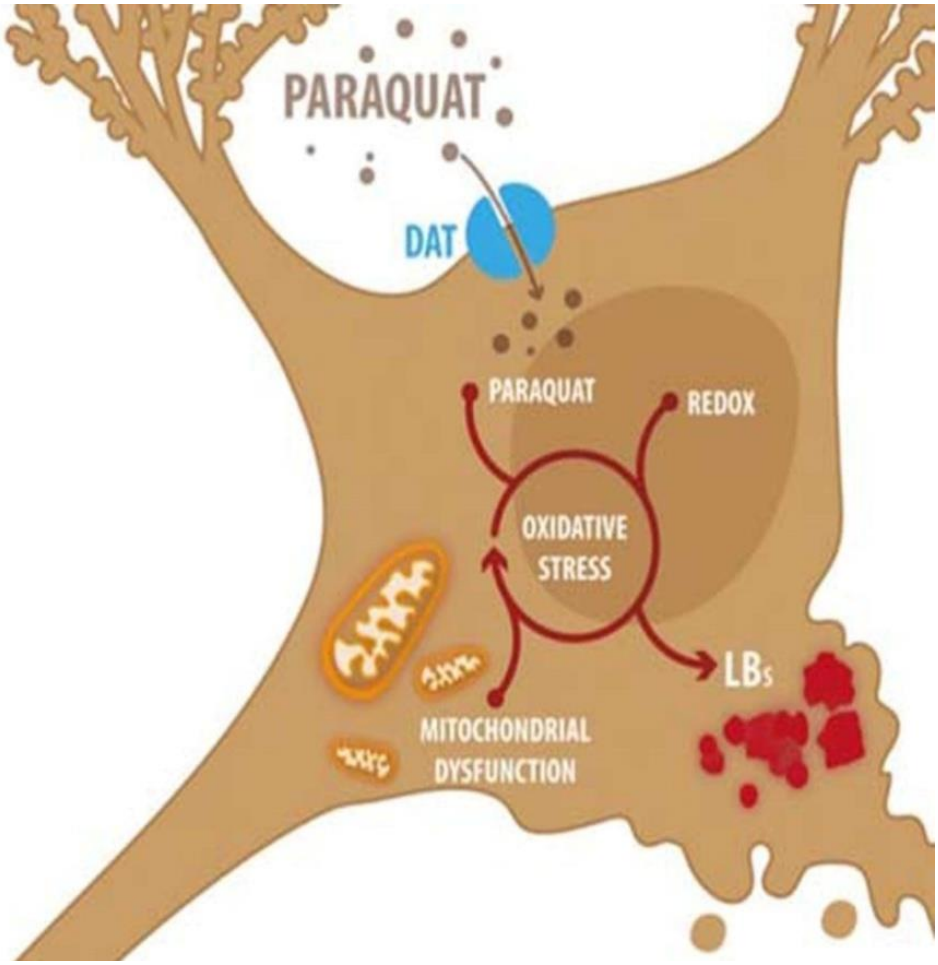


Figure.2. Molecular events related to PQ-induced damage. PQ crosses the BBB, entering DAergic neurons via the DA transporter (DAT). In neurons, PQ triggers a vicious cycle related to OS that involves an imbalance in the redox status, resulting in mitochondrial dysfunction, protein aggregation (the formation of LBs) and further production of pro-oxidant mediators (Adapted from Bastías-Candia *et al*, 2018).

10. Materials and Methods

10.1. Fly Stock

Drosophila melanogaster Oregon K were obtained from National *Drosophila* Stock Centre of University of Mysore, Karnataka, India. The flies were raised at 22°C-24°C in a *Drosophila* environmental chamber (Percival, DR41VL). The flies were fed with regular culture media prepared with sucrose, yeast, agar agar, and propionic acid. Male flies of were used in the present study.

10.2. Chemicals

Methyl viologen dichloride hydrate or Paraquat was purchased from Sigma Aldrich (St.Louis, MO). Sucrose was procured from Sisco Research Laboratory (India). Whatman filter paper no.1 disc was used as a feeding medium in the experiment in 30x100mm glass vial.

10.3. Treatment Protocol

Life span curves were obtained from independent trials with a minimum of 100 flies per experiment and the experimental flies were shifted to freshly prepared culture media every third day until all the flies were recorded dead. In filter disc treatment method volume of 250µL of 5% sucrose was feed to the control flies and 250µL of different concentrations of PQ solution prepared in 5% sucrose were pipette on a filter disc placed in 100x30mm glass vial. Twenty-five flies were transferred on each vial for the treatment and total of 100 flies were used for each concentration in the experiment. At every 24 hours (hrs) duration fresh media solution was prepared and the flies were transferred on it. Mortality was recorded till all flies were dead.

10.4. Negative Geotaxis Assay

Taking advantage of the fly natural tendency to climb towards the light the negative geotaxis assay also called as climbing assay was performed. The assay was employed using a plastic climbing tube where each experimental fly was dropped in the tube using an aspirator and initially left it undisturbed to become accustomed in the new environment for 2 minutes. Each tube was fixed on a sponge and the experimental flies were gently tapped to the base of plastic climbing tube. The distance (measured in centimeter) 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 experimental set (Botella *et al*, 2004).

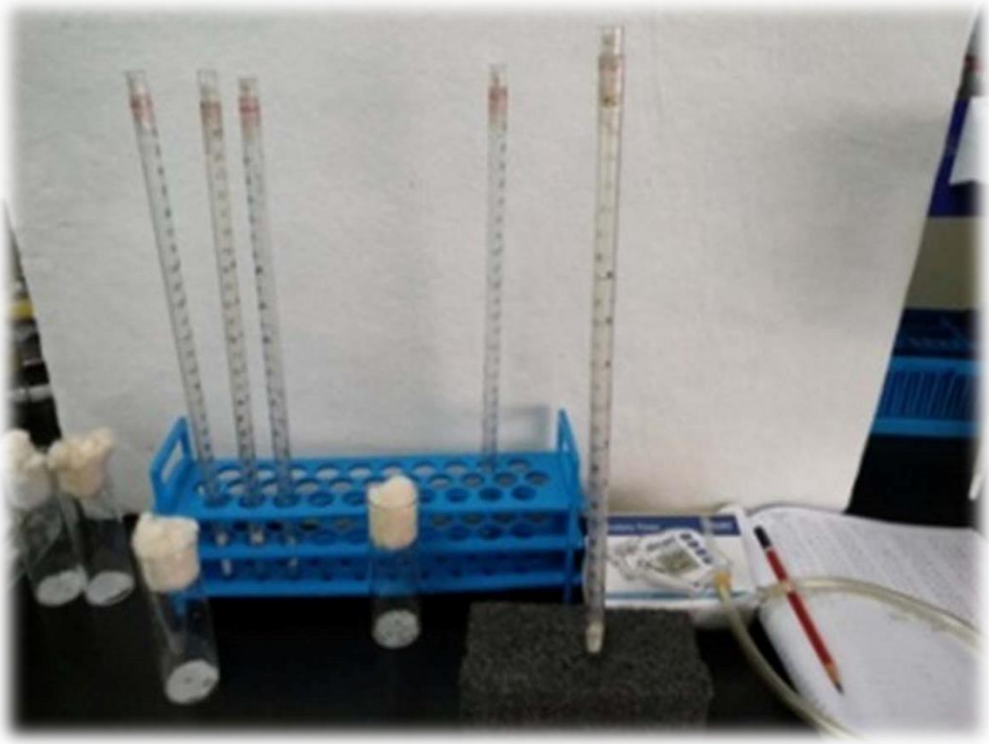


Figure.3. Set up for Negative geotaxis assay. Individual flies were made to climb up the cylindrical tube. The distance travelled by the fly in 12 seconds was recorded.

11. Results

11.1. Characterization of adult Health, Transition and Senescence Life Stage in *Drosophila*

The adult life period of *Drosophila* has three stages namely; health span, transition span and senescent span (Arking *et al*, 2002). The health phase is indicated by a period of life span where no natural deaths occur. The adult transition period is recognized when there is visible but less significant decrease in the survival of adults and described by instance accompanied by about 10% mortality and 90% survival. The adult senescent phase is defined by the slow and stable decrease in the number of live flies as evident by decline in the survival curve due to gradual increase in mortality rate. It is illustrated with gap in between last part of the transition stage and the greatest extent of fly prolonged existence. In animal studies the upper limit of longevity is normally considered as the mean life stages of the longest lived 10% of a particular group. Flies were cultured on the standard culture medium by transferring to freshly prepared culture media on 3rd days. Number of death in each groups were recorded till every flies were dead. Basing on the experiments of longevity assessment using Oregon K strain of *Drosophila*, it was found that the health span extended up to 30 days; the transition stage was recorded from 31-60 days of adult period and the senescent period is from 61-120 days. The greatest extend of the fly longevity was recorded as 121 days, while the median survival duration was 95 days as shown (Fig.4) (Phom *et al*, 2014).

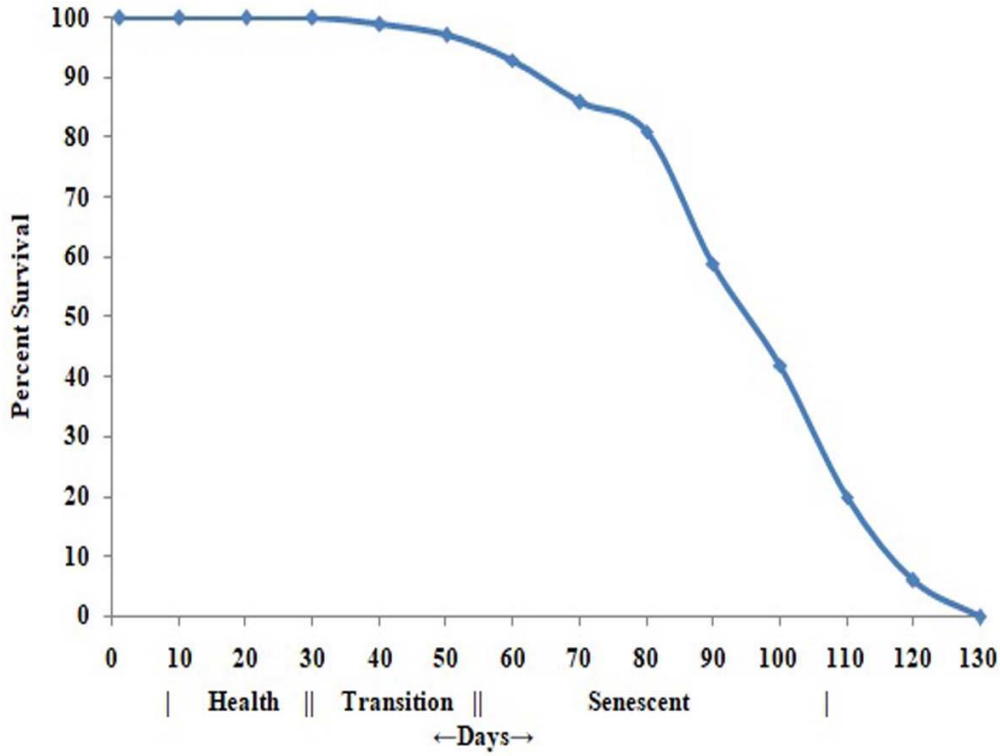


Figure.4. Survival curve of Oregon K male flies in standard culture media. The flies were transferred to fresh media on every 3rd day. The numbers of mortality were recorded until all of the flies died. Health span extends from day 4/5 to 30 days; the transition phase is 31–60 days of adult span, and the senescent span is 61–120 days. Maximum life span is 121 days and median life span is 95 days.

11.2. *Drosophila* is susceptible to PQ in a concentration dependent manner

For exploring the effect of OS in *Drosophila*, the flies on eclosion were collected and fed on normal culture medium till they were age 4-5 days old. Then they were transferred to a glass vials containing several range of PQ concentrations (2.5, 5, 10, 15, 20, and 40 mM). Total of 100 flies with 25 flies per vial were used for each concentration. In a glass vial single filter paper disc was soaked with 250µL of 5% sucrose solution as control and with various concentrations of PQ prepared in 5% sucrose solution and experimental flies were transferred on it. The survival rate of the flies were noted at each 24 hour time point till all the experimental flies were recorded dead in 2.5mM PQ solution which is the most diluted concentration used (Fig.5). After 24 hrs of exposure the mortality in the highest concentration was about 15%, while in 20mM and 15mM, it was about 8% and 5% respectively. In the concentrations of 10mM and below, there was no mortality recorded at 24 hrs of exposure. At 72 hrs of exposure, the survival rates were 95%, 79%, 48%, 15%, 8% and 5% from lowest to highest PQ concentrations respectively. The importance to assess the toxicity is evident from the experimental results in the highest concentration i.e., 40mM, where the toxicity of PQ was so high that, 98% of the flies were dead by 72 hrs of exposure; whereas in the lowest concentration i.e., 2.5mM all the flies were dead by 13th days of exposure. Therefore, it is necessary to circumvent the toxin concentration while developing a model so as to avoid wrong interpretation of the results from false phenotype that may be visible only due to the high concentration of the toxin and possible organismal failure, but not the actual phenotype required from the model organism.

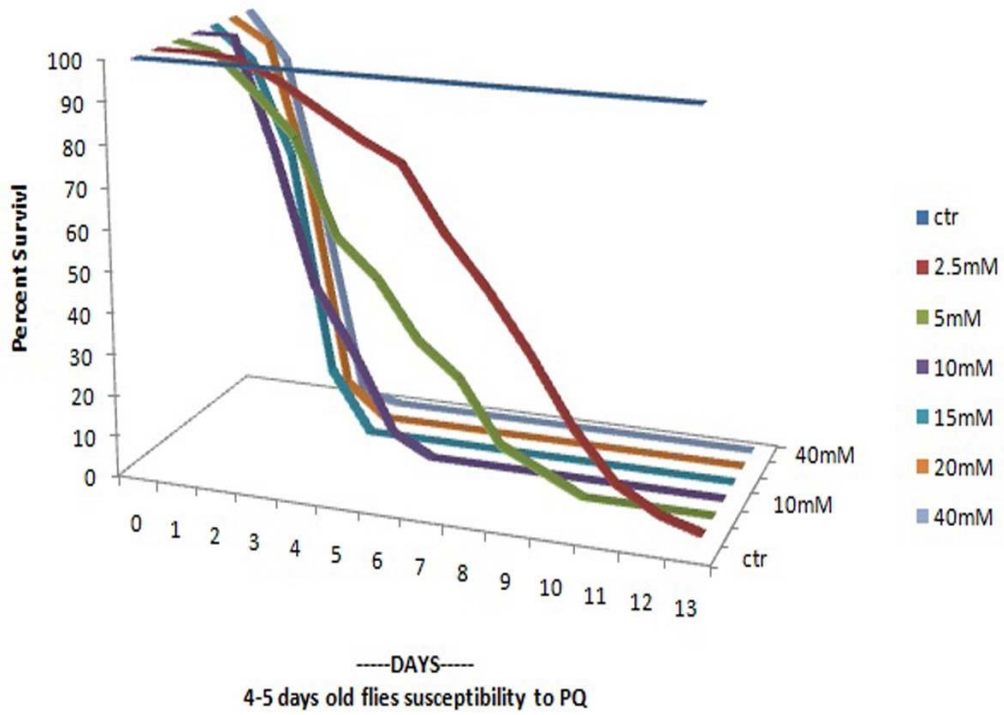
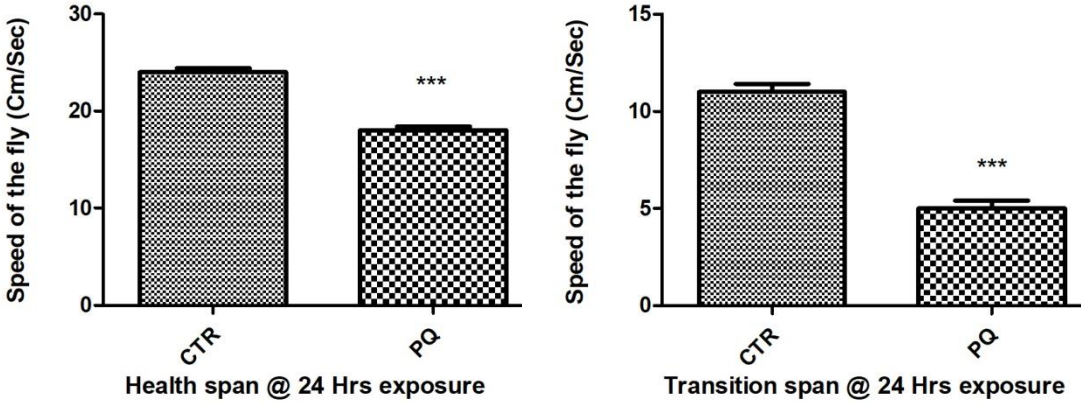


Figure.5. The mortality curves of *Drosophila melanogaster* in various concentrations of PQ. Different concentrations (2.5, 5, 10, 15, 20, 40mM) of PQ were fed to adult 4-5 days old flies. The flies were susceptible to PQ dose and time dependent manner. Comparison of survival curves reveals that the response difference among different tested concentrations was significant (CTR=Control) (log-rank [Mantel–Cox test, $p < 0.0001$]).

11.3. Negative Geotaxis Assay to characterize mobility defects

PQ induced motor dysfunction was assessed by exposing the flies to 10mM PQ and subjecting them to Negative geotaxis assay. After 24 hrs of feeding, the flies were allowed to climb up on the tube to perform climbing assay also called the negative geotaxis assay. The climbing speed of PQ treated flies were decreased by 30% in 4-5 days and 56% among 55 days old flies (Fig.6a,b) when compared to their respective controls. PQ treated flies mimic the symptoms of resting tremor and bradykinesia distinguishing clinical symptoms associated to human PD patients. Some of the flies showed agitation and restlessness as seen from their continuous flipping of wings and some tried to climb up but lose their grip and fall back to the bottom of the climbing tube. This shows a typical motor dysfunction which is caused due to the effect of PQ treatment on DAergic neurons. Thus using the *Drosophila* as the model organism, PQ treatment could replicate the conditions related to motor symptoms that are seen among the human PD patients.



(a) (b)

Figure.6a,b. Negative geotaxis assay performed on (a) Health stage (4-5 days) and (b) Transition stage (55 days) old male fly. Compared to the control flies, the PQ treated flies showed reduced mobility in both the age groups. As compared to controls there was 30% and 56% reduction in climbing ability of PQ treated flies in Health and Transition stages respectively. Suggesting the toxicity of PQ on DAergic neurons affects the motor ability of the flies (***) $p<0.0001$.

12. Discussion

12.1. Developing Fly Model of PD

Epidiomological studies have shown a link between exposure to environmental toxins like PQ and development of PD during later phase of life, which could be an additive factor of sporadic PD that comprise of more than 90% of the PD cases. *Drosophila* shows susceptibility to PQ exposure in a dose dependent manner.

12.1.1. Health Phase Specific Fly Model of PD

The health stage begins when the developmental period ends. It is defined by a low age specific mortality rate and a high survival rate resulting in a significant increase in both mean and maximum life span of *Drosophila*. The adult cohort starts with 100% survival and this high level is maintained for some time in most health cohorts. The HS ends when the survival rate declines to below 90%, or when there is a negative sign in the survival curve whichever comes first (Arking *et al*, 2005; 2002). Survival and longevity studies of *Drosophila* Oregon K strain was done on standard culture medium and on its basis, the survival of flies up to 30 days (adult fly eclosion from pupa to 30 days of culturing) is considered as the phase termed as health life stage of fly. Effect of OS on the health stage of *Drosophila* was studied using varied concentration of PQ. Upon 24 hrs exposure, no mortality was observed at 10mM and below whereas significant death was seen for flies exposed to the highest concentration of PQ. One of the hallmarks of PD is the onset of motor symptoms. Exposure of health stage flies 10mM PQ showed a 30% decline in climbing speed as seen by performing the negative geotaxis assay. The exposed flies also showed PD like symptoms of resting tremor and bradykinesia. This motor symptom is atypical of effect of PQ on DAergic neurons as loss of DAergic neurons in the SN along with associated reduction in motor ability upon PQ administration have been reported (Bastias-Candia *et al*, 2018).

12.1.2. Transition Phase Specific Fly Model of PD

The transition stage is defined as a period of the Life Span that encompassed between the adult survival rates of 89% and 81%. The transition stage shows an increased early survival that leads to a significant increase in mean, but not in maximum life span. The transition stage is variable. Some strains of *Drosophila* display an almost continuous decrease in the survival curve, suggesting that these animals are somewhat weaken such that they have a relative high age specific mortality rate from eclosion onwards (Arking *et al*, 2005; 2002). The transition life stage of *Drosophila* is considered from 31 days to 60 days as seen from the experiments done on survival and longevity. Exposure to PQ induces OS and increases the mortality of flies is a dose-dependent manner. Transition flies when exposed to 10mM PQ showed 36% decrease in climbing ability as performed using the negative geotaxis assay. Some of the flies showed agitation and restlessness and loss of grip which are typical motor dysfunction symptoms.

13. Conclusion

To develop an animal model including *Drosophila* model for disease such as PD and study of its pathophysiology, the need to figure out the adult life stages and prospective model toxin concentration is very essential. Because if the extent of its toxicity is not established, the particular model organism may die of organismal breakdown due to higher concentration of the toxin even before the DAergic neurons gets degenerated and the phenotype that the researchers see may not be relevent to the condition they are trying to understand. Therefore, after establishing the toxicity of PQ, I decided to expose the flies with 10mM PQ and differentiate the behavioral, biochemical, cytological, and molecular markers that are associated with PD at 24 hrs time point where there was no mortality.

CHAPTER III

ASSESSING THE TOXICITY OF CURCUMIN IN *DROSOPHILA* MODEL

1. Introduction

Several researchers have suggested the neuroprotective efficacy of natural products by demonstrating their modulating ability of oxidative stress markers and phenotype associated rescue upon exposure to neurotoxic agents in different disease models. However the pathway of activity of these compounds, in what concentration and biologically active form remains obscure. Sirtuins, an evolutionary conserved family of proteins has a modulatory function on age associated process among higher organisms. Their varied natural role in organisms overlay the basis in which more detailed studies were carried out for their therapeutic prospect in presently not treatable neurodegenerative disorders. Sirtuins inhibitors are found to show protective action on α-synuclein mediated toxicity (Outeiro *et al*, 2007). Resveratrol, (3,5,40-trihydroxystilbene) a natural polyphenol phytoalexin present in red wine and grapes exhibits strong antioxidant activity (Tadolini *et al*, 2000; Belguendouz *et al*, 1997). An aqueous extract of terrestrial herb *Selaginella delicatula* compensate abnormally altered oxidative function and neurotoxicity caused by rotenone in mice (Girish and Muralidhara *et al*, 2012). A shrub *Buddleja cordata* extracts led to considerable reduction of ipsilateral rotations in rat, which was coupled with significant protection of DA levels and high reduction of lepidic fluorescent products in the striatum (Pérez-Barrón *et al*, 2015). It has also been shown that the aqueous solution of *Gastrodia elata* improves the movement dysfunction and exerts anti-inflammatory action following traumatic brain damage in rats (Ng *et al*, 2016).

2. Turmeric

Naturally produced plant products have been used in various categories to meet different needs. The natural products in plants are produced as secondary metabolites which are found to be increased when they are attacked by herbivores and pathogens suggesting their protective role. Most of the natural products consist of active compound that have

biological importance and can be explored to develop pharmaceutical drug for treating disease and infections. Both during ancient and in modern cultures plant products have been an indispensable in developing medicines and thus taking an essential role in health care (Newman and Cragg, 2007). In Indian system of holistic cure, Ayurvedic medicine is practiced and passed on from one generation to another for more than 5000 years. Ayurveda, known as the 'science of life' focus in prevention of disease and restoring body natural systems, prolonging life by means of intervening in the way of life and through application of natural therapies (Garodia *et al*, 2007). In the same way, there is a traditional Chinese medicine which is also popularly practiced all over the East Asian countries. In addition to these, traditional medicine of Unani, Japanese, Egyptian medicine, Korean and traditional Native American medicine have been in practice. In every systems of traditional medicine the ground rules are use of medicinal flora in the management and prevention of any ailment and safeguarding good health (Acharya *et al*, 2008).

Of all the molecules introduced for drug development, some are synthetically produced while most of them are based from natural products. Drug development for human diseases has been based on natural products derived from plants, animals and minerals. Plant based drugs have proven to be suitable for human use and medicines developed from plant and its products keep on holding a vital role disease treatment all over the world.

In pharmacological research, many medicines are directly use from natural products or its derivatives. The knowledge of traditional medicines has enabled to serve as a foundation for several early medicines such as aspirin, digitoxin, morphine, quinine, and pilocarpine which are derived predominantly from plants. These compounds are an important resource for therapeutic areas of drugs, particularly for treatment against disease like cancer,

hypertensive, immunosuppressant, neurological disorder and other infection (Butler, 2004).

2.1. Uses of Turmeric

Besides its use in kitchen as popular spices, turmeric is also an important component in commercially manufactured food products such as beverages, dairy products, cereals, sauces and curry powder. Apart from coloring and adding taste to the food, turmeric has long been considered to possess medicinal value and effectively used in the indigenous system of medicine.

Turmeric (the common name for *Curcuma longa* L. of the Zingiberaceae family) is a rhizomatous herbaceous perennial plant that has a long history of association for medicinal use as a treatment for several ailments. Its use dates back since the Vedic age in Ayurveda in India where it is commonly used as kitchen spice derived from the rhizomes of the plant. In the region of Southeast Asia, apart from its popular use as kitchen spices, turmeric is also used in religious functions. In most of the South Asian countries it is widely cultivated and popularly used as an antiseptic and antibacterial agent.

The initial processing of turmeric includes washing, drying and grinding then sieving the powder to produce a uniform colored product. It is practiced as storing the turmeric powder in shade protected from sunlight which retards its rate of deterioration. In India turmeric crop is produced in large scale as compared to other countries and also consumes about 80% of it. Indian turmeric contains high amount of its bioactive compound K and as such are regard as the best quality available in the world. The turmeric production and popular trade Centre is located at Erode in Tamil Nadu state of India.

The active component of turmeric includes tetrahydro curcuminoid, K, dimethoxy K and bis-methoxy K and nutritional analysis showed that turmeric is rich in omega-3 fatty acid and α-linolenic acid (Goud *et al*, 1993). Traditionally it has been used to treat abnormal health condition such as biliary disorders, anorexia, coughs, diabetic wounds, hepatic disorders, rheumatism and sinusitis. It has been attributed numerous pharmacological properties including treatment for chronic frontal uveitis, conjunctivitis, skin disease, chicken pox, injury curative, urinary tract infections, and liver ailments (Dixit *et al*, 1988). The rhizome of turmeric is used as a calming agent, a diuretic, a hepato protectant and in the treatment of skin diseases such as scabies, leech bites and bruises (Kirtikar and Basu, 1993). Further it is reported as anti-inflammatory, choleretic, antimicrobial, and carminative actions. Turmeric extract has been shown to be effective for improving the condition of type-2 diabetes by restoring increase in blood glucose level (Kuroda *et al*, 2005). The yellow pigments curcuminoids which is a component in turmeric powder has been found to exert protective action against aflatoxin B1 (Gowda *et al*, 2008). It has also been suggested that turmeric and its active compounds have distinct antioxidants, anti-mutagenic, anti-tumorigenic, anti-carcinogenic, anti-inflammatory, anti-arthritic, anti-microbial and hypocholesterolemic properties; including action against diabetic, bacterial and fungal infections, protozoal and viral infections (Abbas *et al*, 2010; Miquel *et al*, 2002). Thus with its effective remedial properties on several diseases as suggested, currently turmeric is investigated for possible benefits in human diseases like neurodegenerative Ad, Cancer, Arthritis and other disorders (Henrotin *et al*, 2010). It improved the pancreatitis condition linked with lung damage in mice (Seo *et al*, 2011) and consists of an active component that is useful to fuel bile secretion and bile surge that will ultimately support and maintain liver in proper conditions (Dono, 2013).

K and curcuminoids account for 2-6% of turmeric. Turmeric as a herb or spice has much more to offer than just K. But for certain health conditions, high concentrations of K are required which can't be provided by the amount of turmeric one consumes on a daily basis. That is one of the prime reasons why K supplements are prepared when considered it in a health perspective.

Also, we cannot discount the fact that it is much easier to consume K supplements rather than thinking of ways to include turmeric in diet and manage the taste.

Following table shows some of the popular K products and their formulation available commercially.

Table 1: Commercial formulation and supplements of Curcumin (Turmeric for Health, 2018).

Commercial Curcumin Formulation	Products
Curcumin C3 complex	Sports Research Turmeric K C3 Complex, Viva Naturals Non-GMO Turmeric K C3, Doctor's Best K C3 Complex.
Curcumin Bioperine	Schwartz Bioresearch Turmeric K Bioperine, Doctor Danielle Turmeric K with Bioperine, Nutravita - Organic Turmeric.
Turmeric Curcumin Meriva Supplement	Source Naturals Turmeric with Meriva, Thorne Research – Meriva.
Longvida	NOW CurcuBrain, Longvida by Nutrivenee.
Curcumin Theracurmin	Natural Factors – K Rich Double Strength Theracurmin
BCM-95 Curcumin	Terry Naturally CuraMed, Progressive Labs - BCM-95.

2.2. Clinical Studies of Turmeric

Clinical studies of turmeric have been done against several diseases. A study on Antimutagenic property of turmeric involving 16 chronic smokers was performed (Polasa *et al*, 1992). In this study, dose of 1.5 g per day were given to the subjects for one month. This gives rise to considerable decrease in urinary flow of mutagens. Among six non-smoker subjects studied, there was no alteration in urinary excretion of mutagens, suggesting that turmeric consumption act as a strong antimutagen that can be explored for chemo preventive studies. Ethanol extracted from turmeric produced significant respite in patients with outside cancerous lesions by reducing smell in 90% of the case in a number of 62 patients studied and itching was reduced in almost all cases and about 10% of the subjects attained reduced in pain and size of lesion (Kuttan *et al*, 1987). Turmeric offered counter action against benzo-a-pyrene induced increase in number of micronuclei (Hastak *et al*, 1997). In a subsequent study, turmeric extract was given to oral submucous fibrosis (OSF) patients at 3g per day for the period of 90 days and the micronuclei amount was found to be reduced in oral exfoliated cells to a large extent comparable to the level of normal healthy individuals. In peptic ulcer patients, when turmeric filled capsules was given orally at dose of 2 capsule of 300mg each five times daily. Following treatment for 4 weeks, the results was significant with the ulcers diminished in 48% of the cases and by 12 weeks of treatment the reduction in ulcers increased to 76% (Prucksunand *et al*. 2001). In another study among patients suffering from irritable bowel syndrome, the occurrence was significantly reduced upon daily consumption of standardized turmeric extract tablet for 8 weeks; including relief from the abdominal pain/discomfort score (Bundy *et al*, 2004). Further, it has been shown that turmeric consumption mixed in curry help to increase bowel movement and activate bacterial flora that generates colon hydrogen thereby raising the hydrogen concentration of breath (Shimouchi *et al*, 2008). A paste of

turmeric is often applied for preventing infection and wound healing. Traditionally, turmeric is also used as healing agent and natural protectant by applying on the cut umbilical cord after delivery (Alam *et al*, 2008).

Now multiple successful clinical trials have been completed using K.

Following table shows the successful trials of K on various diseases and the outcome.

Table 2: Successful trials completed in Curcumin (Gupta *et al*, 2012)			
Disease	No of Patients	Dosage, duration	Outcome
Cancer			
Colorectal cancer	15	0.036-0.18 g/day, 4 months	Reduced glutathione S-transferase activity
	15	0.45-3.6 g/day, 4 months	Reduced PGE_2 production
	12	0.45-3.6 g/day, 7 days	Reduced the levels of M_1G
	5	1.44 g/day; 6 months	Reduced the number and size of polyps without any appreciable toxicity
	44	2 and 4 g/day; 1 month	Reduced ACF formation in smokers
	126	1.08 g/day; 10–30 days	Improved body weight, reduced serum TNF-α, and induced p53 expression
Pancreatic cancer	20	1.5 g/day; 6 weeks	Reduced the lipid peroxidation and increased GSH content in patients
	25	8 g/day	Well-tolerated, limited absorption, and showed activity in some patients
	17	8 g/day; 4 weeks	Not feasible for combination therapy
	21	8 g/day	Safe and well-tolerated in patients
Breast cancer	14	6 g/day; 7 day, every 3 weeks	Safe, well-tolerated, and efficacious
Prostate cancer	85	0.1 g/day; 6 months	Reduced the serum PSA content in combination with isoflavones
Multiple myeloma	26	4 g/day; 6 months	Decreased paraprotein load and urinary N-telopeptide of type I collagen
	29	2–12 g/day; 12 weeks	Safe, bioavailable, and efficacious against multiple myeloma
Lung cancer	16	1.5 g/day; 30 days	Reduced the urinary excretion of mutagens in smokers
Cancer lesions	62	Ointment	Produced remarkable symptomatic relief in patients with external cancerous lesions
	58	3.6 g/day, 3 months	Reduced the number of micronuclei in mucosal cells and in circulating lymphocytes
	25	8 g/day, 3 months	Improved the precancerous lesions
	100	2 g/day; 7 weeks	Well tolerated, but not efficacious
	75	1 g/day, 7 day	Increased vitamins C and E levels, decreased MDA and 8-OHdG contents in the serum and saliva
Head and neck cancer	39	2 tablets	Decreased IKKβ kinase activity and IL-8 levels in the saliva
Inflammatory diseases			
Crohn disease	5	1.08 g/day, 1 month + 1.44 g/day,	Significant reductions in CDAI and inflammatory indices in patients
	2 months		
Ulcerative proctitis	5	1.1 g/day for 1 month + 1.65 g/day for 1 month	Significant reduction in symptoms as well as inflammatory indices in patients
Ulcerative colitis	89	2 g/day; 6 months	Prevented relapse of disease
	1	0.5 g/day; 2–10 months	Associated with clinical and endoscopic remission of the disease
Inflammatory bowel disease	ex vivo	5–20 μM; 0.5–24 h	Suppressed p38 MAPK activation, reduced IL-1β, and enhanced IL-10 levels in mucosal biopsies; suppressed MMP-3 in colonic myofibroblasts
Irritable bowel syndrome	207	0.072 and 0.144 g STE/day; 8 weeks	Produced significant reduction in the prevalence of symptoms
	8	0.5 g in food	Increased bowel motility and activated hydrogen producing bacterial flora in the colon
Rheumatoid arthritis	18	1.2 g/day; 2 weeks	Improved joint swelling, morning stiffness, and walking time
	45	0.5 g/day; 8 weeks	Improved the RA symptoms in patients alone and in combination with diclofenac sodium
Osteoarthritis	50	0.2 g/day; 3 months	Efficacious in the management and treatment of osteoarthritis
	100	1 g/day; 8 months	Efficacious in the long-term management of osteoarthritis
Chronic anterior uveitis	53	1.125 g/day; 12 weeks	Efficacy and recurrence of the disease comparable to that for corticosteroid therapy without any adverse effect
Recurrent anterior uveitis	106	1.2 g/day; 12–18 months	Reduced the eye discomfort after a few weeks of treatment in more than 80% of patients
Postoperative inflammation	46	1.2 g/day; 6 day	Exhibited superior anti-inflammatory property compared with phenylbutazone
Gastric ulcer	60	1 g/day; 6–12 weeks	Reduced ulcer formation after 12 weeks
Peptic ulcer	45	3 g/day; 4 weeks	Reduced ulcer formation
H. pylori infection	25	0.06 g/day; 1 week	Improved dyspeptic symptoms and reduced serologic signs of gastric inflammation
	36	0.12 g/day; 4 weeks	Insignificant effect on H. pylori eradication
	8	1.125 g/day; 6–22 months	Patients recovered from the disease

3. Curcumin

Curcumin (K), 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione, is the main, active, yellow colored natural polyphenolic compound. It is taken out from rhizomes of *Curcuma longa* L. (turmeric), a plant in the ginger family (Zingiberaceae). K is widely used all over the world and extensively used in Southeast Asia (Goel *et al*, 2008; Aggarwal *et al*, 2007). Turmeric contains curcuminoids in several forms (Prasad *et al*, 2014). Various researches on K have showed that it also exhibits anti-inflammatory and anti-oxidant properties. Thus K is one such potential candidate that can be explored for therapeutic approach to several human diseases including neurodegenerative disorder like PD.

3.1. Curcumin Toxicity Studies

Several studies have suggested the concern about K safety under various experimental conditions. Reports suggesting that K may cause toxicity under specific conditions are shown with the studies in mammalian cell lines on treatment with turmeric in which there is a dose and time-dependent induction of chromosome aberrations (Goodpasture and Arrighi, 1976).

NTP Toxicology and Carcinogenesis Studies of Turmeric Oleoresin in 2-year feed studies on rats found that turmeric oleoresin consumption was linked with higher incidences of ulcers, hyperplasia, swelling of the forestomach, cecum, and colon in male rats and of the cecum in female rats (NTP, 1993). Further studies on reports of K concentration having beneficial effect have also demonstrated that it induce DNA damage and chromosomal alterations (Urbina-Cano *et al*, 2006; Cao *et al*, 2006). An experiment testing if the chelator action of K is adequate to cause iron deficiency in vivo, it was shown that K suppressed the production of hepcidin, a peptide which plays an essential role in regulation

and balancing of systemic iron. It causes iron insufficiency leading to anemia in mice that are fed with poor iron diets (Jiao *et al*, 2009). K therefore has ability to influence systemic iron metabolism among individuals with suboptimal iron status (Means, 2009).

K also confer negative action activity of enzymes such as glutathione-S-transferase and UDP-glucuronosyl transferase apart from its hampering the action of drug metabolizing enzyme such as cytochrome P450 (Appiah-Opong *et al*, 2007; Thapliyal and Maru, 2001; Oetari *et al*, 1996). Such action of K may result in abnormal plasma flow of certain drugs that may lead to toxic action among the individuals taking K (Mancuso and Barone, 2009). Contradicting to the reports that suggest beneficial properties of K, a study suggested both advantage and disadvantage of K in alcoholic liver injury and concentration-dependent K toxicity was reported in animal models in which K was found to accelerate liver injury and liver cellular edema (Zhao *et al*, 2012).

In a study on human oral administration of K among the individuals with chronic anterior uveitis, all the patients who received K alone improved but those who additionally received antitubercular therapy had developed complications in their eyes and lost vision over time (Lal *et al*, 1999) suggesting that K may react with certain compounds resulting in detrimental effect. In another randomly assigned 8 week crossover study consisting eleven healthy subjects in the age group of 21-38 years old, it was reported that consuming turmeric as additional drugs markedly enhanced the levels of urinary oxalate which among the vulnerable subjects increases the probability of developing kidney stone (Tang *et al*, 2008). In a clinical trial of K for the prevention of colorectal neoplasia it was found that, overall, 61% of participants showed toxicity, mainly the gastrointestinal disturbances, most prevalently diarrhea, as well as swollenness, gastroesophageal reflux (Caroll *et al*, 2011). Another experiment aimed to establish the linkage between exposures of mixed

metals with child health outcomes due to association of lead with environmental sources such as spices, it was found that most of the turmeric samples had high level of lead and upon further study showed its increased bio-accessibility. Suggesting that turmeric powder upon contamination through environmental lead exposure can have potential lead toxicity and poisoning among some population (Gleason *et al*, 2014).

Therefore while developing therapeutic approach for a disease, it is important to thoroughly validate the toxicity as well as beneficial activity of prospective compound in the model organism. Taking this into consideration, I used wide range of K concentrations and assess its toxicity. In order to understand this paradigm I made an effort to assess the toxicity of K in fly model at different age groups.

4. Materials and Methods

4.1. Fly Stock

Drosophila melanogaster Oregon K strain were obtained from National *Drosophila* Stock Centre of University of Mysore, Karnataka, India. The flies were raised at 22°C–24°C in a *Drosophila* environmental chamber (Percival, DR41VL) and fed on a regular culture media prepared with sucrose, yeast, agar agar, and propionic acid. Male flies of were used in the present study.

4.2. Chemicals

Curcumin was procured from Sigma Aldrich (St. Louis, MO). Sucrose and Dimethyl Sulfoxide, were procured from Sisco Research Laboratory (India). Whatman filter paper no.1 disc was used as a feeding medium in the experiment.

4.3. Treatment Protocol

In filter disc treatment method volume of 250µL of 5% sucrose was fed to the control flies and 250µL of different concentrations of K solution were pipetted on a filter disc placed in 100x30mm glass vial. Twenty-five flies were put on each vial for the treatment and total of 100 flies were used for each concentration in the experiment. At every 24 hrs duration fresh media solution was prepared and the flies were transferred on it. Longevity curves were obtained from independent trials with a minimum of 100 flies per experiment until all the flies were recorded dead.

5. Results

5.1. Assessing Curcumin toxicity during Health stage and Transition stage of adult *Drosophila*

It has been shown that K act against acute concentration (20mM) of PQ to improve the survival rate and climbing defects (Park *et al*, 2012). Also contrasting to some findings suggesting the effectiveness of K, it has been reported that K exhibit a concentration-dependent toxicity in animal models (Zhao *et al*, 2012). Hence, I initially tested a range of K concentrations from 25µM to 50mM using 4-5 days old male *Drosophila* to assess potential deleterious effects. It was found that K exerts concentration dependent effect on the fly. The toxicity of highest concentration (50mM) was so high that all the flies were dead by 3 days of feeding. At 5mM concentration the files lived for 25 days; 35 days at 500µM; 40 days at 100µM. The toxicity effect of lowest concentration (25µM) was negligible and the flies lived up to 45 days under the experimental conditions. While K concentration of 2.5mM and above show toxicity effect on flies, concentrations below 2.5mM reveal no visible toxicity and there was no toxicity related death up to 10 days of feeding (Fig.1). Data was collected every 24 hrs for each group.

K toxicity experiment was also performed on the Transition span (55 days old) male flies with selected concentrations of 100 µM, 1mM and 1.5mM. It was found that there was no toxicity associated mortality up to 10 days of K feeding with all the selected concentrations in this age group (Fig.2). Data were collected every 24 hrs for each group. To assess the effectiveness of therapeutic molecules, several laboratories employ young animal models. They treat the animals for few days and estimate if the therapeutic molecule is efficient to shield the DA neuronal degeneration through assessment of behavioral assay like motor defect and biochemical markers like estimating the brain DA levels or cytological markers like DAergic neuron degeneration. Subsequently, all the experiments employed

performed in this study used K concentrations of 2mM or less to avoid drug related toxicity.

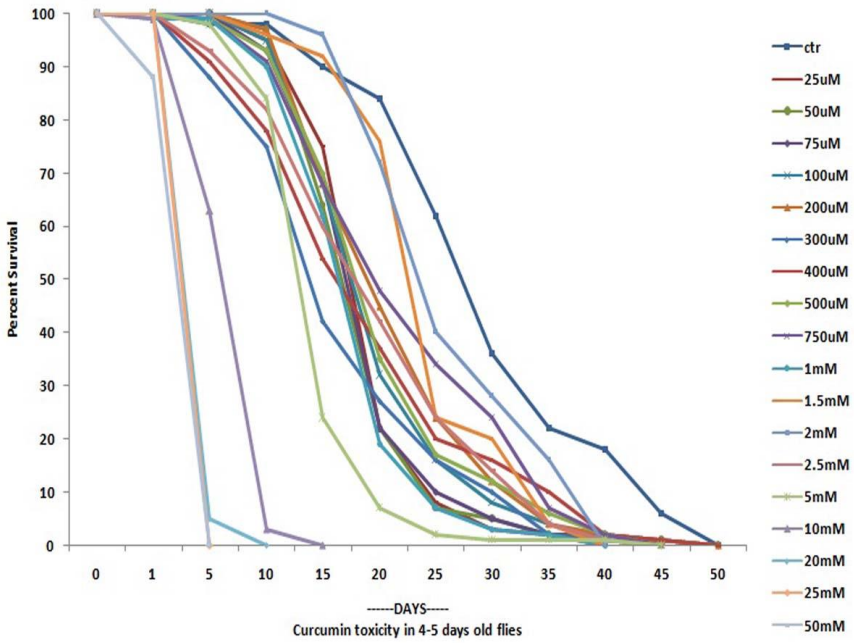


Figure.1. Curcumin toxicity in health stage (4-5 days old) of fly. Effect of increasing concentrations of K (25 µM to 50mM) on survival of 4–5 days old male flies was concentration dependent toxicity. 4-5 days old flies were fed on fresh experimental medium of filter disc every 24 hrs and data were collected every 24 hrs for each group. Feeding of K concentration of 2.5mM or above has deleterious effect on viability of the fly, whereas concentrations lower to 2.5mM showed no observable toxicity (CTR=Control; K=Curcumin).

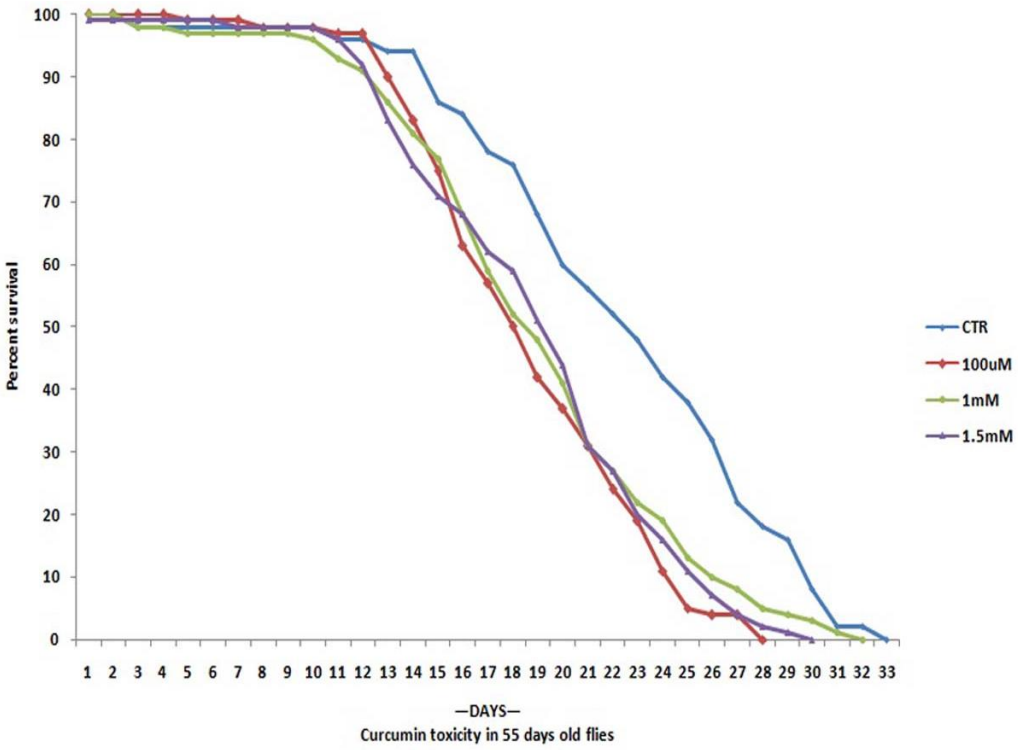


Figure.2. Curcumin toxicity curve in transition stage (55 days old) of fly. Effects of K concentrations (100 µM, 1mM and 1.5mM) on survival of 55 days old male flies were investigated. The flies were aged in standard culture medium transferring to fresh media every 3rd day. At 55th day old, they were fed on fresh experimental medium of filter disc method every 24 hrs and data were collected every 24 hrs for each group (CTR=Control; K=Curcumin).

6. Discussion

Studies have suggested K toxicity under various conditions. K may react with certain compounds resulting in detrimental effect on certain individuals; enhance the levels of urinary oxalate which among the vulnerable subjects increases the probability of developing kidney stone (Tang *et al*, 2008). Turmeric powder may be environmentally contaminated by lead causing potential lead toxicity and poisoning among some population. K toxicity has also been studied suggesting that it primarily causes gastrointestinal disturbances, diarrhea, as well as distension and gastroesophageal reflux disease (Caroll *et al*, 2011).

In the present study, the first experimental approach employing K was to expose the model animal to a range of concentrations. I selected 18 concentrations ranging from 25µM to 50mM of K in 4-5 days old male *Drosophila* and 100µM, 1mM and 1.5mM on 55 days old male *Drosophila* to assess possible detrimental effects of K.

It was found that K concentration of 2.5mM or above has deleterious effect on viability of the fly, whereas concentrations lower to 2.5mM showed no observable toxicity (Fig.1). In the highest concentration (50mM) all the flies employed in the experiment were dead by 5th day of K treatment, whereas 20% mortality was recorded in 2.5mM concentration on the 10th day of treatment. The concentration below 2.5mM did not show any mortality up to 10 days of exposure with K, indicating that there was no toxicity associated fly mortality in all the lower concentrations. K toxicity experiment was also performed on the 55 days old male flies (Fig.2). It was also found that there was no toxicity up to 10 days of K feeding on the fly which also indicates there was no toxicity associated mortality when treated with the selected concentrations of K.

Reports suggesting that K may cause toxicity under specific conditions are shown with the studies in mammalian cell lines on treatment with turmeric in which there is a dose and time-dependent induction of chromosome aberrations (Goodpasture and Arrighi, 1976). Further studies on reports of K concentration having beneficial effect have also demonstrated that it induce DNA damage and chromosomal alterations (Urbina-Cano *et al*, 2006; Cao *et al*, 2006). K has also been reported to cause anemia due to lack of iron content from low iron consumption in mice (Jiao *et al*, 2009) suggesting its ability to influence systemic iron metabolism among people with suboptimal iron status (Means, 2009). Further concentration-dependent K toxicity was reported in mice in which K was found to accelerate liver injury and liver cellular edema (Zhao *et al*, 2012).

7. Conclusion

From the present experimental results it is evident that increased in mortality rate among flies exposed to higher concentrations is due to K related toxicity. Therefore, after figuring out the concentration that is harmful to the fly, I wanted to use K as a neuroprotective agent in *Drosophila* model of sporadic PD.

This finding is important because for understanding any disease in animal model when the concentration of compound under investigation is not optimized, the phenotype or markers that the researchers find may be associated with its toxicity which can be wrongly interpreted. Therefore, in the subsequent experiments all the K concentrations that are used in rescue studies are sub lethal in all the age groups as shown in the result section.

CHAPTER IV

DECIPHERING THE NEUROPROTECTIVE EFFICACY OF CURCUMIN IN *DROSOPHILA* MODEL OF PARKINSON'S DISEASE

1. Introduction

A number of natural products with neuroprotective effects are assessed and reported by many researchers. Among them about 6000 research articles have suggested the multi therapeutic properties of K including potent antioxidant (Alhusaini *et al*, 2018; Trujillo *et al*, 2013; Dutta *et al*, 2005), anti inflammatory (Tizabi *et al*, 2014; Biswas *et al*, 2005), anticancer (Murray-Stewart *et al*, 2018; Singh and Khar, 2006), iron chelating (Yang *et al*, 2017; Du *et al*, 2012; Daniel *et al*, 2004) and neuroprotective activities (Huang *et al*, 2018; Ataie *et al*, 2010) in cell cultures and animal models (Yang *et al*, 2014). In addition, more than 100 clinical investigations have been done using K (Prasad *et al*, 2014). Apart from these properties, many other studies have reported the capability of K to slow down the key distinguishing features of PD like ROS accumulation, apoptosis, platelet assembly, cytokine production, cyclooxygenase lipoxygenase isoenzymes activities, repress oxidative injury, cognitive deficits in cell cultures and animal models (Yang *et al*, 2005). Therefore, it remains one of the natural products containing exceedingly promising curative properties that have been widely investigated by researchers for therapeutic approach to PD.

2. Antioxidant Properties of Curcumin

K exhibit potent antioxidant action that is similar to vitamins C and E (Toda *et al*, 1985). It has been reported to be a strong forager of a range of reactive oxygen species including superoxide anion radicals, hydroxyl radicals and protection of oxidative damage of kidney cells (LLC-PK1) by suppressing lipid degradation, lipid peroxidation and cellular breakdown (Cohly *et al*, 1998). K is also shown to inhibit the injury which is caused by hydrogen peroxide in human keratinocytes and fibroblasts (Phan *et al*, 2001) showing its antioxidant role in enhanced wound repair. Direct detoxification of reactive nitrogen species such as peroxynitrite by K has been demonstrated in vitro (Iwunze *et al*, 2004).

Treatment of DAergic neuronal cells and mice with K reinstate reduction of GSH levels, counter act protein oxidation and maintain mitochondrial complex I action that are generally affected because of low GSH activity (Jagatha *et al*, 2008). It is also suggested that iron induced primary cortical neurons toxicity was improved by K action through attenuating necroptosis (Dai *et al*, 2013). Out of 87 genes found to be differentially expressed in K treated *Drosophila melanogaster*, 50 genes were markedly up regulated and the rest 37 were down regulated (Zhang *et al*, 2015). They also suggested that K can be explored for an alternative therapeutic approach to developing drugs for age related disorder. Antioxidative properties of melatonin and K were tested in another experiment using liver of aging mice which showed that there was inhibition of protein carbonyls (PC) formation suggesting the health benefits of the compounds (Dkhar and Sharma, 2010). Also when K was fed on developing larva, it triggers pathways that help to extend the health span accompanied by marked increase in median and maximum longevities in adult fly (Soh *et al*, 2013). Recently it has also been reported in *PINK1* knock down SH-SY5Y cell model that K rescue mitochondrial dysfunction and cell death (van der Merve *et al*, 2017).

3. Neuroprotective Role of Curcumin

3.1. Studies in Mice

Studies in mice showed that K attenuates loss of striatal DA axons triggered by 6-hydroxyDA (6-OHDA) and reduces glial response and maintains SOD1 level in the 6-OHDA lesioned striatum (Tripanichkul *et al*, 2013). K treatment was also shown to diminish the Aβ40, Aβ42 and clumping of Aβ-derived diffusible ligands in the mouse hippocampal Cronu Ammonis-1area; reduced the expression of the γ-secretase component presenilin-2; upregulate the expression of enzymes that digest β-amyloid, insulin and neprilysin (Wang *et al*, 2014). Study on mice with liver lymphoma have shown the

anticarcinogenic properties through initiation of antioxidant defense mechanisms and also hamper the process of angiogenesis by repressing the stress active genes and glycolytic pathway (Das *et al*, 2014). K also markedly diminishes the quantity of apoptotic cells and repressed the upregulation of cytochrome-c, caspase-9, and caspase-3, in an experiment to understand mitochondrial injury and oligodendrocyte apoptosis. They suggested the ability of K to inhibit apoptosis and thereby confer mitochondria protection (Feng *et al*, 2014). Study conducted on the effect of fluoride in 30 days old mice found elevated lipid peroxidation that was accompanied by high amount neurodegenerative cells in the hippocampal sub-regions. When K was co-treated with fluoride, lipid peroxidation was markedly decreased with simultaneous decrease in neurodegenerative cells, suggesting K is useful in ameliorating the effect of fluoride in mice brain (Sharma *et al*, 2014). In another investigation in adult and D-galactose induced old mice, K markedly decreased the escape latency and improved D-galactose induced decline of cellular proliferation and neuroblast differentiation in the sub granular zone of hippocampal dentate gyrus, amplified the levels of phosphorylated CREB (cAMP response element binding protein) and brain-derived neurotrophic factor in the sub granular zone of dentate gyrus suggesting the efficacy of K to alleviate cognitive impairment through action on CREB signaling in the hippocampal dentate gyrus (Nam *et al*, 2014).

3.2. Studies in *Caenorhabditis elegans*

Studies in *Caenorhabditis elegans* (*C.elegans*) model also provide several advantages to investigate the link between OS and DAergic neurodegeneration in PD. Human α-synuclein over-expression in DAergic neurons in *C.elegans* showed age-dependent neuronal loss (Cao *et al*, 2005). Study on the effects of K has reported lifespan extension and reduction in intracellular ROS and lipofuscin during aging in C. *elegans*. This extension in longevity is credited to antioxidative properties of K but not its antimicrobial

activity (Liao *et al*, 2011). K feeding also improves egg generation, offspring size and survival of the worm which was positively associated with DAergic neuroprotection and attenuation of acetylcholine esterase activity (Satapathy *et al*, 2016).

3.3. Studies in *Drosophila*

Using *Drosophila*, it is reported that K have gender and genotype specific life span extension and sequester OS mediated free radicals, enhance locomotor ability and show chemo preventive property, suggesting its potential treatment applicability in higher organisms (Lee *et al*, 2010). K decreases death of SH-SY5Y human neuroblastoma cells induced by rotenone; improve characteristic symptoms associated with PD in *Drosophila* via reduction in intracellular and mitochondrial ROS levels and acting against the caspase-3/caspase-9 activity (Liu *et al*, 2013). Transgenic fly expressing human α-synuclein was exposed to different concentrations of K and found considerable delay in the loss of activity pattern, decrease in the level of OS and apoptosis, and extended life span (Siddique *et al*, 2014) suggesting potential role of K in neuroprotection.

4. Use of Curcumin in PD Studies

Several other laboratories have studied the effect of K in PD models. K has been reported to chelate iron, copper and other metals consequently hampering the α-synuclein or LB accumulation (Perez *et al*, 2008). In a study using the fly and two-dimensional nuclear magnetic resonance, it was found that K reduce toxic action via interaction with α-synuclein oligomers and fibrils, adjusting the morphology and enhancing their separation (Singh *et al*, 2013) clearly suggesting that K act against α-synuclein clustering and stopping LB formation and attenuate α-syn oligomer toxicity. K also attenuates reduction in DA levels and degeneration of DA neurons (Mythri *et al*, 2012) and assist improvement of macro-autophagy through action on transcription factor EB, consequently controlling

cell loss and toxicity to neurons (Jiang *et al*, 2013). In another study it is reported that K slow down activity of monoamine oxidase like that of the monoamine oxidase (MAO) inhibitor, thereby reinstating the diminish DA levels (Khatri *et al*, 2016) and decreasing depression (Nam *et al*, 2014). It is also shown to modulate OS, memory deficits, motor impairments (Cole *et al*, 2007) and decrease ROS levels, preserve mitochondrial integrity and attenuate neuroinflammation which consequently help to shield DA neurons in brain. K also impairs the c-Jun N-terminal kinases (JNK) pathway and thus avoids DAergic neuronal loss by the mechanism of apoptosis (Jayaraj *et al*, 2014). K is also reported to ameliorate oxidative injury to DAergic neuronal by activation of the Akt/Nrf2 pathway (Cui *et al*, 2016) suggesting its protective properties.

All these findings have shown the neuroprotective effectiveness of K by employing health stages of animal models. It may be noted that the investigation of different phases of life in *Drosophila* have shown that each life stages are distinguished by diverse pattern of gene expression. This pattern is comparable to equivalent life phase in human. It is also known that in *Drosophila* there is a marked variation of about 23% in genome-wide transcript patterns with age (Pletcher *et al*, 2002).

Therefore, it is important to follow the age specific study for late onset NDD such as PD. Although K has been shown to have protective effect in numerous studies as discussed above, there is an indispensable need to further decipher its action because all the model organisms employed in the studies are in health span stage.

Considering this fact, I try to further understand the action of K in both health and transition stage of *Drosophila*.

5. Materials and Method:

5.1. Fly Stock

Male *Drosophila melanogaster* Oregon K strain was used in the present study. The fly was obtained from National *Drosophila* Stock Centre of University of Mysore, Mysore, Karnataka, India. The flies were raised at 22°C-24°C in a *Drosophila* environmental chamber (Percival, DR41VL) and fed on a standard culture medium with composition of sucrose, yeast, agar agar, and propionic acid.

5.2. Chemicals

Curcumin and Methyl viologen dichloride hydrate or paraquat was purchased from Sigma Aldrich (St. Louis, MO). Dimethly Sulfoxide (DMSO), Sucrose was procured from Sisco Research Laboratory (India). Whatman filter paper no.1 disc was used as a feeding medium in the experiment.

5.3. Methods of Treatment

Two different methods of treatments are used for this experiment viz-

1. Co-feeding regime
2. Pre-feeding regime

5.3.1. Co-feeding Regime

For this regime, the flies are exposed to PQ and K simultaneously. That is one group of flies are treated with PQ while other groups are treated with PQ along with K at the same time. 10mM PQ was prepared by dissolving the PQ in 5% sucrose. Different concentrations of K (100µM, 500µM, 1mM, 1.5mM and 2mM) are prepared by dissolving the K in PQ solution. A volume of 250µL of the same solution is pipetted into each vial containing filter disc at the bottom. The control flies remain in 5% sucrose while K *per se*

group was fed with 5% sucrose along with K. The flies were transferred to fresh vial every 24 hrs. The mortality and climbing ability were noted for every 24 hrs.

5.3.2. Pre-feeding Regime

In this regime, the flies are first fed with multiple concentrations of K (100µM, 500µM, 1mM, 1.5mM and 2mM) for 5 days. After feeding the flies with K, the flies are then exposed to 10mM PQ. The control and K *per se* group remain in 5% sucrose only. A volume of 250µL of the same solution is pipetted into each vial containing filter disc at the bottom. The flies were transferred to fresh vial every 24 hrs. The mortality and climbing ability were noted for every 24 hrs.

5.4. Negative Geotaxis Assay

The Assay was discussed in detail in Chapter 2, page no. 53.

5.5. Quantification of Dopamine Levels

The HPLC apparatus (Thermo Scientific Dionex Ultimate 3000) consisted of a XPG 3000 series pump feeding MD-TM mobile phase containing Acetonitrile, phosphate buffer and an ion pairing agent (Thermo Scientific, 70-1332) to the stationary phase built in BDS Hypersil C-18 column with a dimension of 150x3mm and a particle allowance size of 3µ (Thermo Scientific, 13741) connected to an ultra analytical electrochemical detector cell (ECDRS 1) followed by a Omni coulometric cell (ECDRS 2) to reduce noise during catecholamine detection. The sample was injected to the Column/ECDRS compartment by a temperature regulated Auto-sampler and the reduction and oxidation potential for ECDRS 1 was kept at -175 mV and 225 mV while ECDRS 2 was kept at oxidation potential of 500 mV. The acquisition of data was performed in Chromaleon-7 within a

gain range of 1μA and was collected at a rate of 5Hz while keeping the data acquisition filter at 2.0 for the analytical cell.

For the sample, 15 heads were homogenized in 150μl ice cold phosphate buffer (0.1M, pH=7.4) and centrifuged for 10 minutes in 6000rpm at 4^{o}C and supernatant was collected. 1:1 ratio of the supernatant and 5% TCA was again centrifuge in 5000rpm for 10 minutes 4^{o}C. 50μl of the resulting supernatant was injected into HPLC column/ECDRS compartment. Data processing was accomplished using Thermo Scientific Chromaleon-7. Quantification was performed by integration of peak areas to pure catecholamine standards (Sigma-Aldrich) as obtained by processing through the HPLC.

6. Results

6.1. Curcumin rescues the mobility defects induced by PQ under Co-treatment and Pre-treatment regime during Health stage (4 days old) of *Drosophila* model of PD

To understand the efficacies of K to rescue the motory defects induce by PQ, as some of the studies have already reported (Inamdar *et al*, 2012), negative geotaxis assay also called as the climbing assay was employed. This assay will show the motor impairment due to action of PQ that serves as a perceptive display of degeneration of DAergic neuron and consequently the onset of movement dysfunction. As expected, PQ feeding made the flies to exhibit tremor at rest and bradykinesia when the motor ability was assessed after 24 hrs. These motor symptoms are the distinguishing features linked to PD in human. It was noticed that a number of flies tried to climb up the wall of climbing tube but they could not hold on the tube firmly and fall on the bottom of tube. Some flies showed restlessness with continuous wing flipping. The flies treated with PQ exhibited reduced motor function as seen by 30% decreased in their ability to climb at 12 seconds when compared to controls. Where as on those flies pre-treated with K or co-treated with K and PQ, the motor function were significantly rescued, as seen from the flies climbing higher distance when compared to PQ flies (Fig.2; 4). Comparision between the K only fed flies and controls showed that the distance flies travelled were comparable, indicating that K *per se* has no negative action on fly mobility. In the co-feeding regime where the motor function was assessed even at 72 hrs of exposure to PQ, the protective effect of K was consistent even at this time point (Fig. 2) which clearly affirms the effectiveness of K. Apart from co-treatment regime, inorder to undersrtand if the neuroprotective efficiency is conferred through antagonistic interaction with PQ, the pre-treatment regime was employed. The flies were first fed with selected concentrations of K for 5 days followed by treatment with

PQ. When the flies were subjected to negative geotaxis assay, it was found that K attenuated the PQ-induced motor dysfunction even in this method of feeding, which is similar to the one observed in co-treatment regime (Fig. 4).

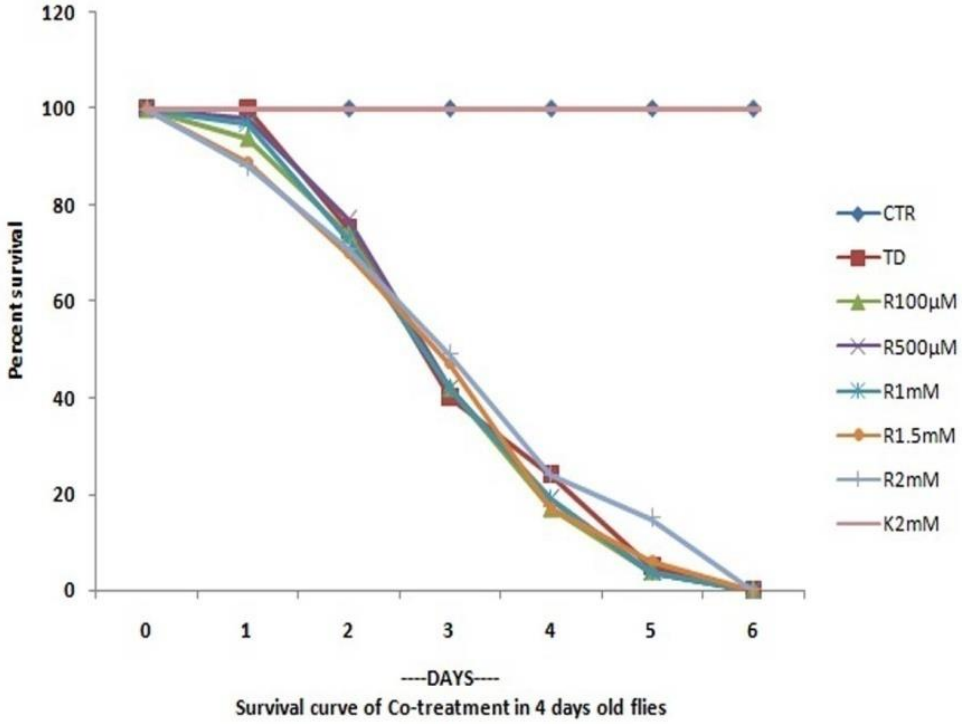
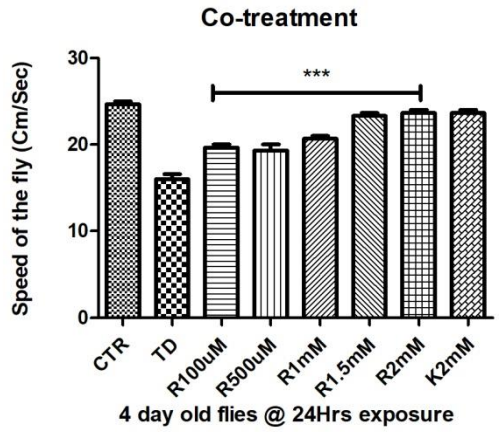


Figure.1: The survival curve of co-treatment in 4 days old flies (health stage). (CTR=control, TD=treated with PQ, R(rescue)=K+PQ, K=Curcumin).



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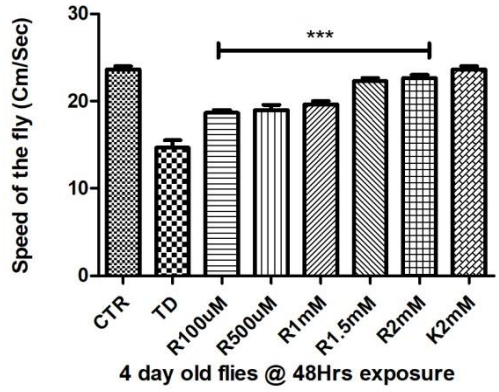

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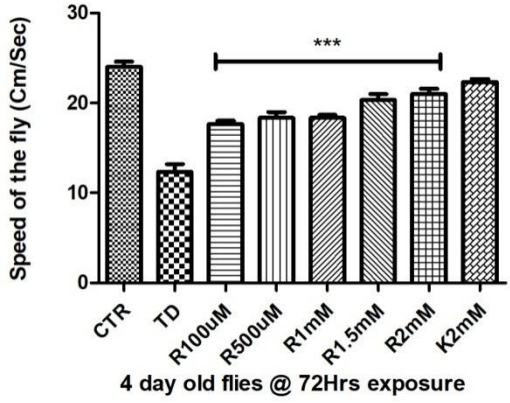


(c)

Figure.2: Negative geotaxis assay for co-treatment regime in 4 days old flies (health stage). K rescues the mobility defects induced by PQ under co-treatment regime during health span. 10mM PQ induces mobility defect in 4 days adult flies at different time points. The mobility defect is significantly altered when the flies are co-fed along with K (100 µM, 500 µM, 1, 1.5 and 2mM for 24hrs (a), 48hrs (b) and 72hrs (c). The ingestion of K alone does not make any difference in climbing ability of the fly when compared to control (***$p<0.0001$). (CTR=control, TD=treated with PQ, R (rescue)=K+PQ, K=Curcumin).

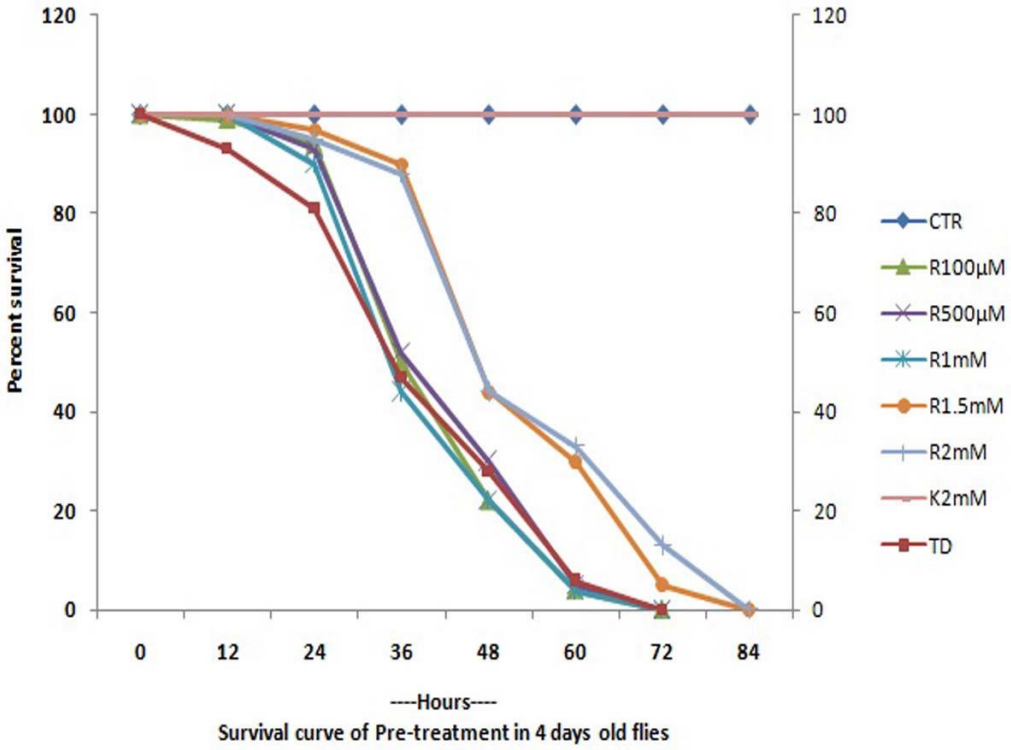
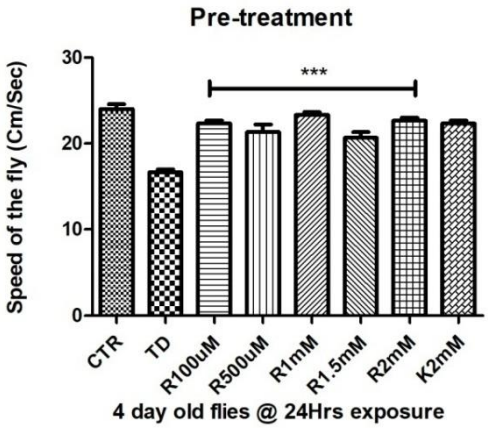


Figure.3: The survival curve of pre-treatment in 4 days old flies (health stage). (CTR=control, TD=treated with PQ, R (rescue) = K + PQ, K = Curcumin).



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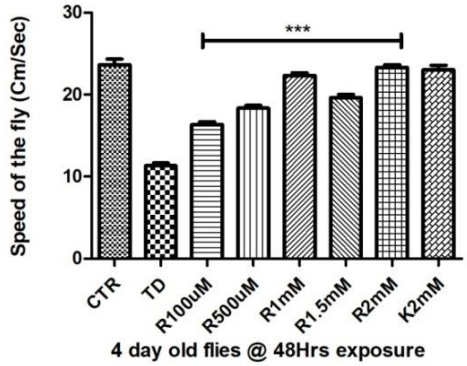

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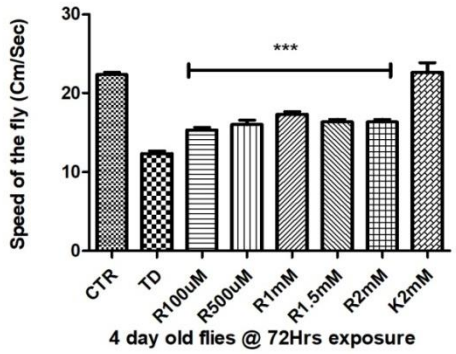


(c)

Figure 4: Negative geotaxis assay for pre-treatment regime in 4 days old flies (health stage). K rescues the mobility defects induced by PQ under pre-treatment regime during health span. 10mM PQ induces mobility defects in 4day adult flies at different time points. The mobility defect is significantly altered when the flies are pre-fed with K (100µM, 500µM, 1mM, 1.5mM and 2mM for 24hrs (a), 48hrs (b) and 72hrs (c). The ingestion of K alone does not make any difference in climbing ability of the fly when compared to control. (***$p<0.0001$). (CTR=control, TD=treated with PQ, R (rescue)=K+PQ, K=Curcumin).

6.2. Curcumin rescues the mobility defects induced by PQ under Co-treatment and Pre-treatment regime during Health stage (30 days old) of *Drosophila* model of PD

The health span ranges from adult eclosion from pupa to 30 days old. To proceed with assessment for K neuroprotection in transition phase, it is necessary to also check the negative geotaxis assay even in later part of health stage which will serve as study on wide window of health phase. Therefore, in order to have this confirmative knowledge, K was also tested in 30 days old (health stage) *Drosophila* and the flies were subjected to negative geotaxis assay after treatment of flies with both the feeding method. K could significantly modulate the motor dysfunction cuased by PQ even during this stage of health span in fly (Fig.6; 8). PQ exposure reduced the fly's motor ability by 30% when compared to control. The mobility defect is significantly altered when the flies are co-fed with K (100µM, 500µM, 1mM and 2mM) for 24hrs, 48hrs and 72hrs (Fig.6) respectively. Also in the pre-treatment method the PQ induced motor defect was rescued by all the concentrations of K employed (100µM, 500µM, 1mM and 2mM for 24hrs and 48hrs (Fig.8) respectively. It was found that ingestion of K alone does not affect any difference in climbing ability of the fly when compared to controls in both the treatment methods employed. Therefore it is clear from the result that protective efficacy of K was not limited only to 4-5 days old but also efficient even at 30 days old flies in the health stage. The results confirm the neuroprotective efficacy of K during the health stage in *Drosophila* model of PD.

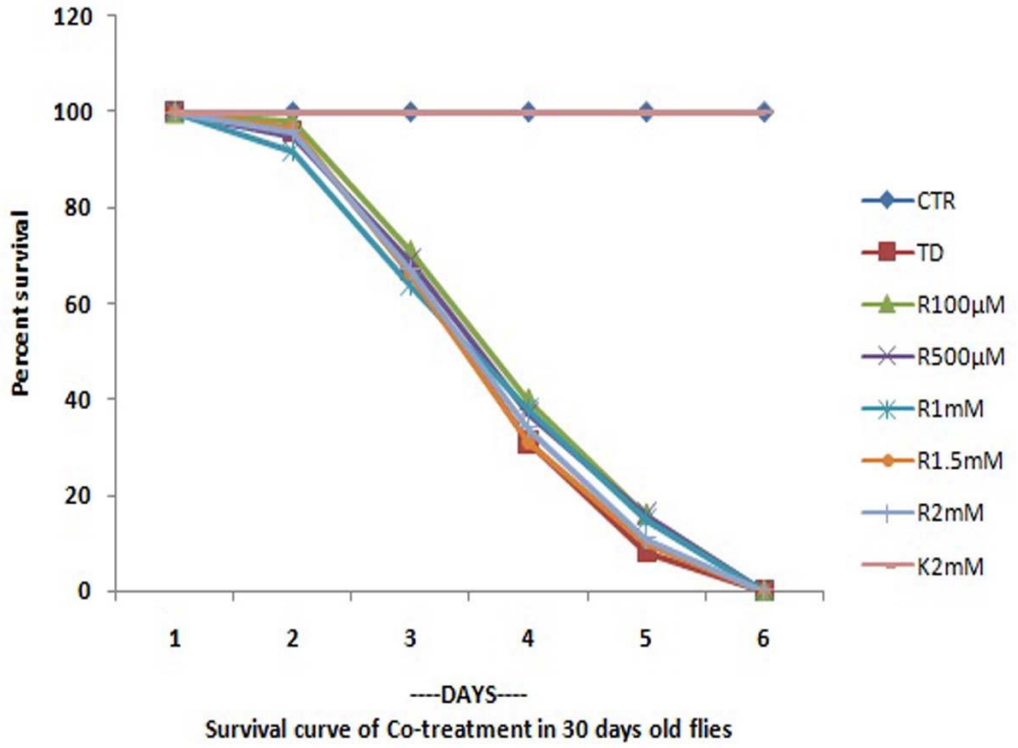
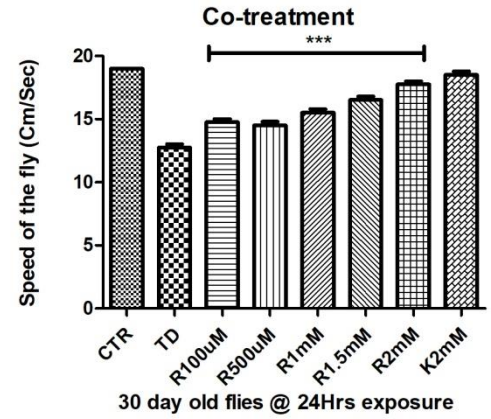


Figure.5: The survival curve of co-treatment in 30 days old flies (health stage). (CTR=control, TD=treated with PQ, R (rescue) = K + PQ, K= Curcumin)



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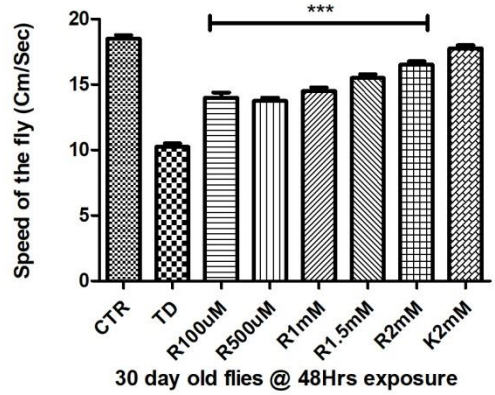

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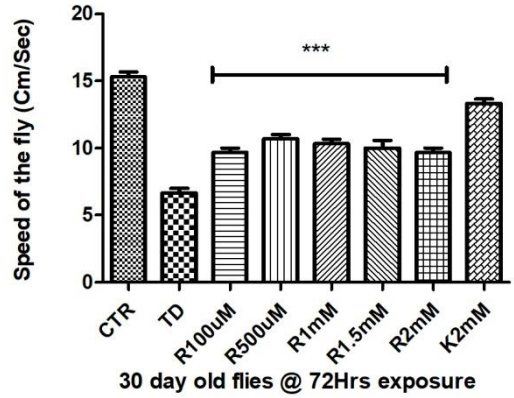


(c)

Figure 6: Negative geotaxis assay for co-treatment regime in 30 days old flies (health stage). Co-treatment regime for 30days adult flies for 24hrs (a), 48hrs (b) and 72hrs (c) indicates a similar pattern as that of the 5 day adult flies. The mobility defect induced by 10mM PQ is significantly altered when the flies are treated with PQ along with K concentrations of 100µM, 500µM, 1mM, 1.5mM and 2mM. Feeding K alone does not alter the mobility defect (***$p<0.0001$). (CTR=control, TD=treated with PQ, R (rescue)=K+PQ, K=Curcumin).

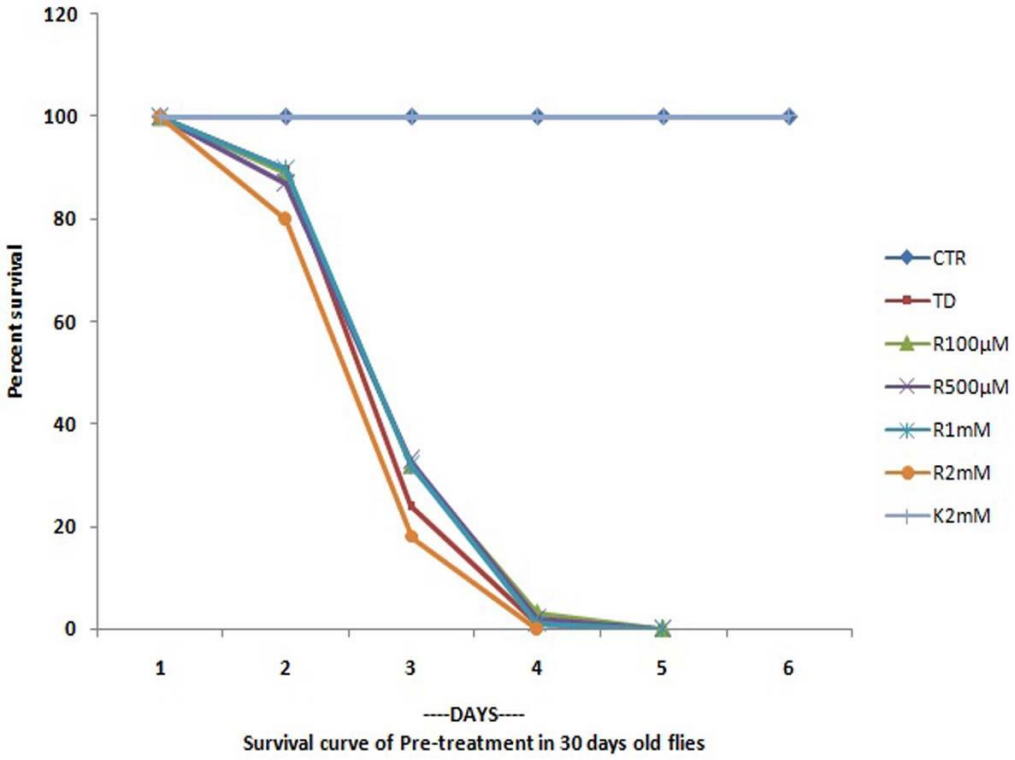
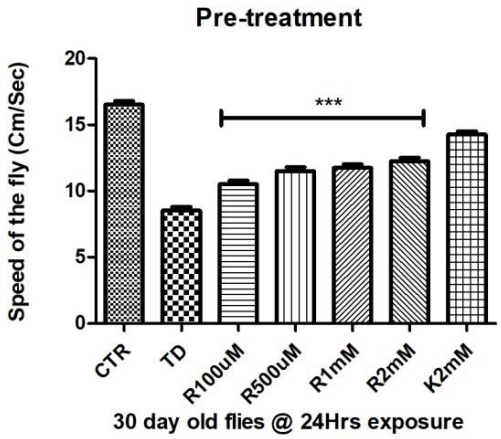
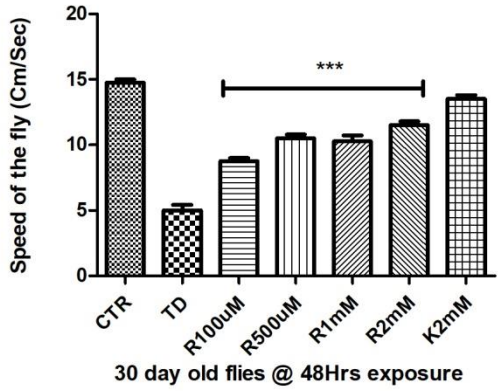


Figure.7: The survival curve of pre-treatment in 30 days old flies (health stage). (CTR=control, TD=treated with PQ, R (rescue) = K + PQ, K= Curcumin).



(a)



(b)

Figure.8: Negative geotaxis assay for pre-treatment regime in 30 day old flies (health stage). Pre-treatment regime for 30days adult flies for 24hrs (a) and 48hrs (b) indicates a similar pattern as that of the 4 day adult flies. The mobility defect induced by 10mM for is significantly altered when the flies are treated with PQ along with K concentrations of 100µM, 500µM, 1mM, and 2mM. Feeding K alone does not alter the mobility defect. (***$p<0.0001$). (CTR=control, TD=treated with PQ, R (rescue)=K+PQ, K=Curcumin).

6.3. Curcumin fails to rescue the mobility defects induced by PQ under Co-treatment and Pre-treatment regime during Transition stage of *Drosophila* model of PD

K efficacy was then tested in the age group of the transition phase (55 days old) with the same feeding methods employed for health stages (4-5 days and 30 days old). Interestingly, all the concentrations of K employed in the experiment could not improve the speed of the flies and the motor activity was similar between PQ fed flies and PQ+k groups in both co-treatment and pre-treatment regimen (Fig 10; 12). Importantly it suggests broad implications with regard to the neuroprotective effect of K and its therapeutic applications in NDD such as late onset PD that is manifested during the age period of 60 years in humans. Thus it is pertinent to properly screen and understand the efficacy of therapeutic molecules at the time point where the disease sets in.

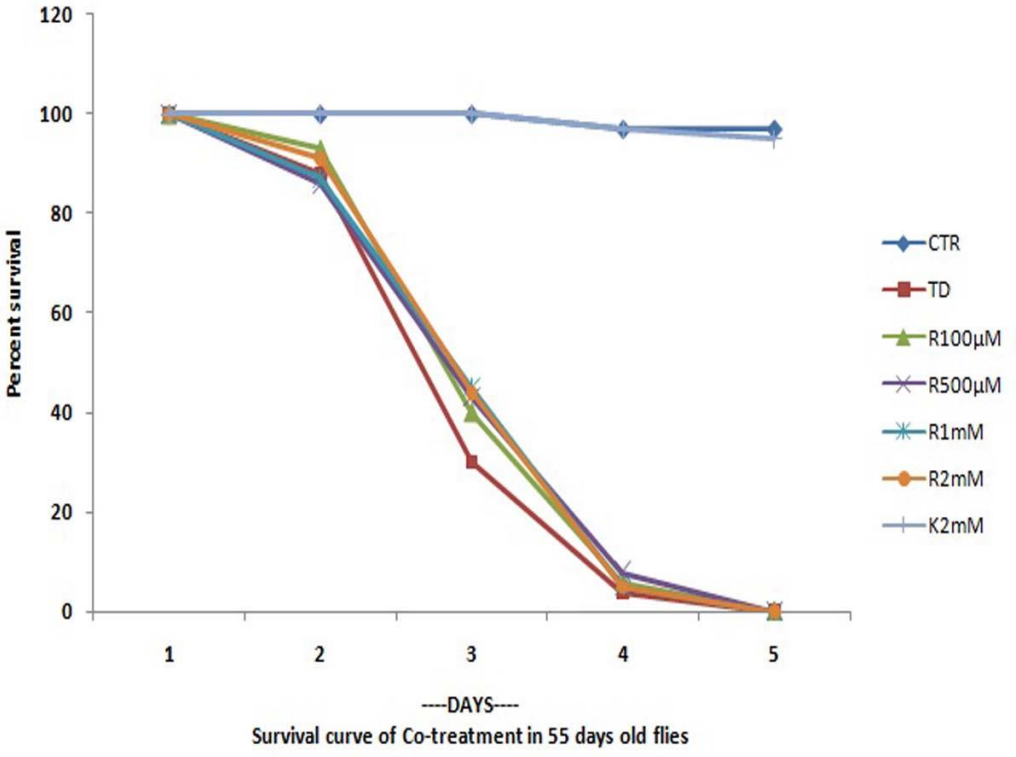
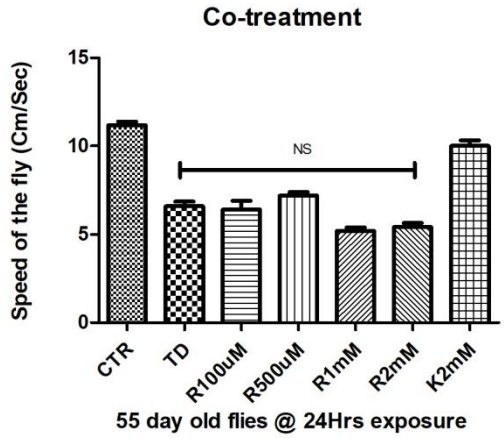
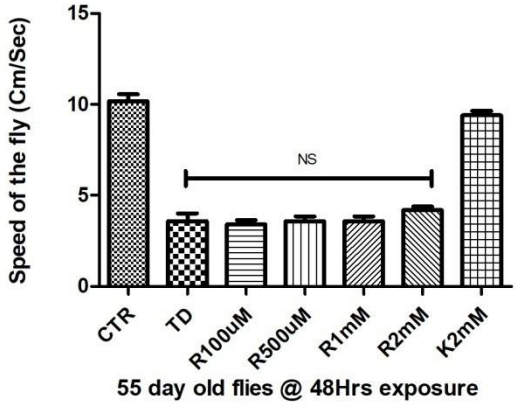


Figure.9: The survival curve of co-treatment in transition stage (55 days old flies). (CTR=control, TD=treated with PQ, R (rescue)=K+PQ, K=Curcumin).



(a)



(b)

Figure.10: Negative geotaxis assay for co-treatment regime in transition stage (55 days old flies). Co-treatment regime for 55 days old adult flies for 24hrs (a) and 48hrs (b) indicates a different pattern than that of the 4 day and 30 days adult flies. All the K concentrations used (100µM, 500µM, 1mM, and 2mM) fails to rescue the mobility defect induced by 10mM PQ. Feeding K alone does not alter the mobility defect (NS-Not Significant). (CTR=control, TD=treated with PQ, R (rescue)=K+PQ, K=Curcumin).

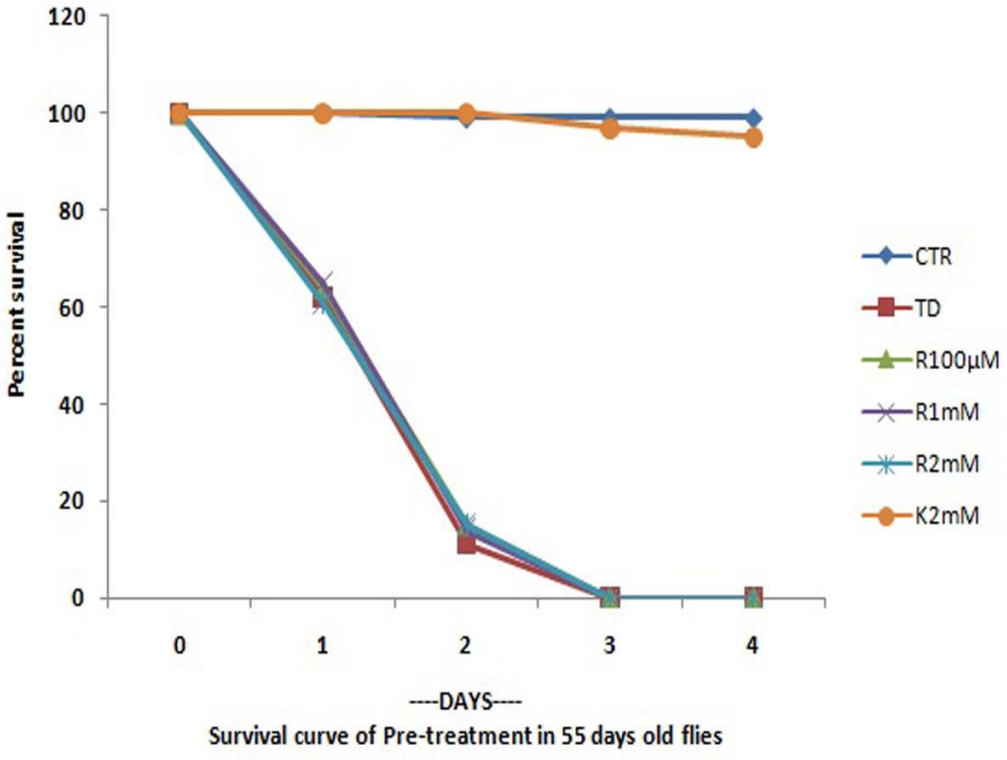
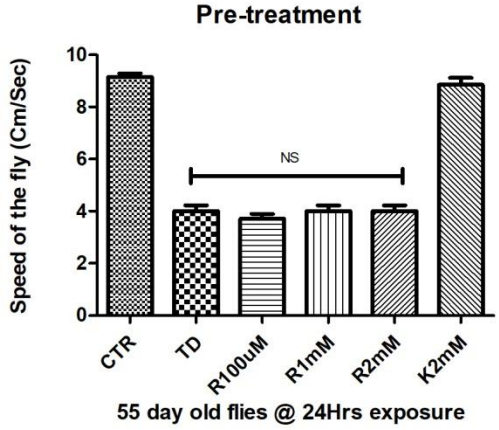


Figure.11: The survival curve of pre-treatment in transition stage (55 days old flies). (CTR=control, TD=treated with PQ, R (rescue)=K+PQ, K=Curcumin).



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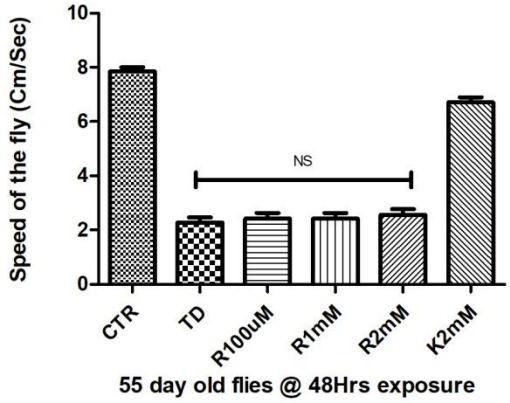


(b)

Figure.12: Negative geotaxis assay for pre-treatment regime in transition phase (55 days old flies). Pre-treatment regime for 55 days old adult flies for 24hrs (a) and 48hrs (b) indicates a different pattern than that of the 5 day and 30 days adult flies. All the K concentrations used (100µM, 1mM, and 2mM) fails to rescue the mobility defect induced by 10mM PQ. Feeding K alone does not alter the mobility defect (NS- Not Significant). (CTR=control, TD=treated with PQ, R (rescue)=K+PQ, K=Curcumin).

6.4. Curcumin could replenish decreased DA levels caused by PQ exposure in *Drosophila* during Health stage but not in Transition stage

It was found that although K clearly rescues mobility defect during health stages, it did not rescue the motor dysfunction during the transition stage. This raised the questions of whether the inability of K to rescue the observed climbing defect in transition stage is related to its failure to replenish the brain DA levels during that particular stage. Thus it was necessary to test the K effectiveness in DA activity at this phase. To confirm this phenomenon, employing the co-treatment method, I estimated DA levels in head tissue extracts of control flies and flies treated with PQ, PQ and K, and K alone in both the life stages. It was found that diminished brain DA levels were clearly replenished on K co-treatment during the health stage but it failed to replenish the decreased DA levels in transition stage (Fig.14). This result indicates the neuroprotective effectiveness of K could be in stage specific pattern where it may exert positive action at one stage but neutral or negative action during another stage of *Drososphila* life span. The present findings also observation suggests that K has limitation of its therapeutic efficacy in late onset NDD disease such as PD.

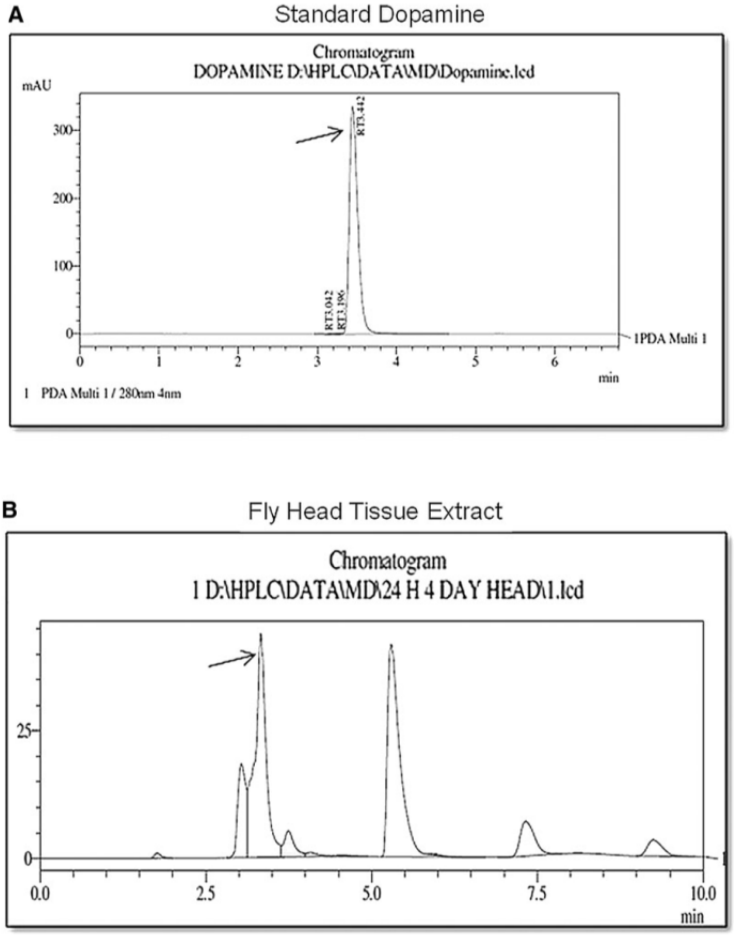


Figure.13: Quantification of brain DA level with high-performance liquid chromatography (HPLC). Chromatogram of standard DA showing a retention time between 3.196 and 3.442min (A) and chromatogram for *Drosophila* head tissue extract, showing a peak during the observed time window for standard DA (B) (peak for DA is pointed with an arrow in both the panels). Peaks can be seen (at 5min and 7.5min) much away from retention time window observed for standard DA that amount to artifact.

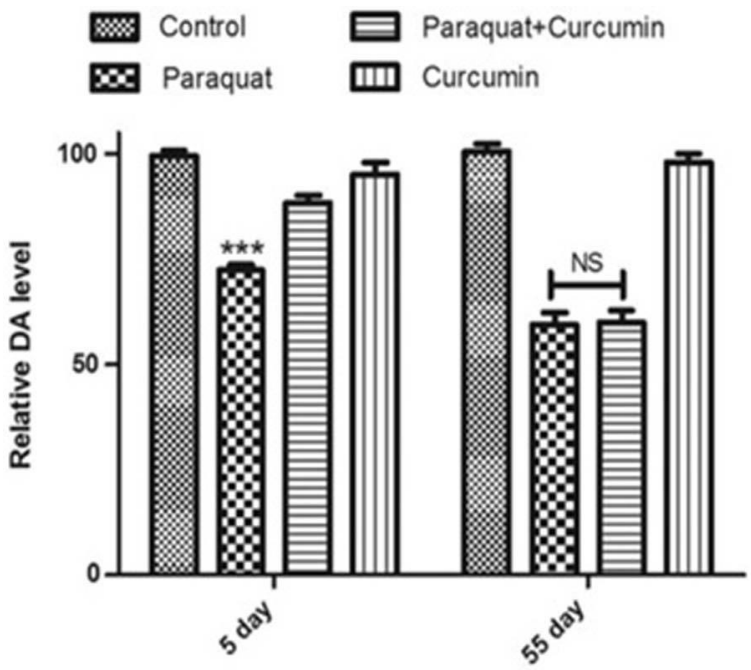


Figure.14: Relative DA levels in health stage (5 days old) and transition stage (55 days old) fly brains exposed to 10mM paraquat (PQ) for 24hrs. (***) $p<0.0001$ compared to control and K fed alone. NS, lack of significance between toxin treated group (PQ) and rescue group, i.e., PQ+K).

7. Discussion

In this chapter, multiple concentrations of K (100µM, 500µM, 1mM, 1.5mM and 2mM) along with 10mM PQ was employed to decipher the efficacy of K in mitigating the neurodegeneration against PQ induced mobility defects by employing co-feeding and pre-feeding regime.

I evaluated the PQ-induced locomotory deficits through negative geotaxis assay at 24 hrs, 48 hrs and 72 hrs time point after treatment. The experimental flies were exposed to 5% sucrose, 10mM PQ prepared in 5% sucrose, 10mM PQ with various K concentrations and K *per se*. Each experimental fly was allowed to climb up the tube towards the light source for 12 seconds. The total distance each fly travelled in 12 seconds was recorded both in co-treatment (Fig 2 and 6) and pre-treatment regime (Fig 4 and 8) in health stages (4 days and 30 days old flies). While control flies could climb up the climbing tube to over 20cm distance in 12 seconds, the patterns of movement of the flies treated with PQ was entirely different and their motor ability was reduced by 30% when compared with the controls. Some flies tend to remain at the bottom of the tube with signs of restlessness and others tried to climb up but would fall back to the bottom of tube implying that PQ caused neurotoxic effects in the fly, which was clearly manifested as impairment of locomotion ability. This observation is consistent with the previous report that PQ affect the behavior patterns in flies including altered climbing activity, continuous wing flipping, rotation and unsteady body and postural, that are identified characters as the human PD symptoms (Chaudhuri *et al*, 2007). The behavioral changes induced by PQ may be due to impairment of DAergic neuronal function and manifested through reduced climbing ability (Brooks *et al*, 1999). It has also been shown that PQ cause considerable death of DAergic neurons in flies (Chaudhuri *et al*, 2007).

Interestingly, there was no effect on the motor function of fly among the group to which K alone was fed, whereas ingestion of PQ alone negatively affected mobility as evident from inability of the flies to normally climb up the wall of climbing tube. K co-treatment with PQ showed recovery of the motor deficit as seen from its improved climbing speed (Fig 2 and 6). Even in the pre-treatment regime (Fig 4 and 8), it is clear that K effectively rescue the motor defects caused by PQ during all the tested time period of exposure. I observed the similar pattern of recovering the defect associated with PQ exposure in both the K pre-feeding and co-feeding regime, indicating that the efficacy of K is not due to its antagonistic interaction with PQ but by activation of molecular targets in the organism that confers protection to DAergic neurons.

However, in the case of transition stage, the phenomenon is completely different. All the concentrations of K used for the experiment could not rescue the mobility defect induced by PQ in both the co-treatment (Fig 10) and pre-treatment regimens (Fig 12) which is an interesting phenomenon.

It has been reported that there exist dissimilar patterns of gene expression at different life stages. About 23% in genome-wide transcript patterns variation are in *Drosophila* (Pletcher *et al*, 2002) has been shown indicating that natural compounds like K which has a genotropic action may not have its targets during the whole life period.

This finding was well substantiated by the results obtained from estimation of DA levels in health stage and transition stage (Fig.14). HPLC quantification of brain DA levels in health stage reveal that K co-treatment efficiently replenishes the PQ induced decreased level of DA. Whereas in the transition span there is no replenishment clearly indicating that targets of K is absent at this stage of *Drosophila* life span.

The property of K to alleviate expression intensity of age associated genes in young flies suggests the action of K on such genes is a cause of its longevity extending effects. These results signify that compounds like K may have definite positive effect at one stage while having neutral or detrimental action in another stage of adult life span. As it has been demonstrated that developmental or health phase feeding of K extends life span, however it shows harmful effect if fed for whole adult stages or during later stage such as the transition and senescent phases (Soh *et al*, 2013). They have demonstrated from the expression analysis that when flies are fed in stage specific manner pathways such as target of rapamycin (TOR) are affected, clearly signifying that K is a genotropic nutraceutical that confer its action by means of developmental stage-specific target pathways. Therefore, it also unravels the fact that K is an early-acting stage-specific inducer of extended functional longevity in *Drosophila*. The genetic targets of K may be absent in some point of life span, implying its limitations with reference to a long-life phenotype.

8. Conclusion

Several studies employ co-treatment or pre-treatment method in young model animals. They treat the prospective molecules for a short duration and try to understand the effectiveness of therapeutic efficacy in PD models. They investigate if the toxin mediated DA neuronal toxicity is protected by the prospective therapeutic molecule through assessment of behavioral markers like motor defect, and biochemical indicator like the quantification of DA levels or cytological pointers like the degeneration of DAergic neurons.

The above findings demonstrate that K improves motor dysfunction caused due to PQ exposure in *Drosophila* during the health stages (4-5 days old and 30 days old flies) in both the co- and pre-treatment regimens. This motor dysfunction assay was assessed at 24

hrs time point after treatment where there was no apparent mortality seen in longevity experiment. K also recovers the motor defects associated with PQ exposure even at 48 hrs and 72 hrs of treatment, suggesting its underlying neuroprotective effectiveness. On the contrary, K could not improve the motor dysfunction in transition phase (55 days old flies) clearly signifying its constraint as a therapeutic agent in late onset NDD such as PD.

Considering the present experimental findings, I further try to understand the mechanism of K neuroprotection by looking into the OS markers, antioxidant and neurotransmitter enzyme activities employing both the health and transition span of *Drosophila*.

CHAPTER V

MECHANISTIC INSIGHTS INTO THE THERAPEUTIC PROPENSITY OF CURCUMIN IN *DROSOPHILA* MODEL OF PARKINSON'S DISEASE

1. Introduction

Oxidative Stress (OS) is classically defined as a state of redox imbalance caused by an excess formation of oxidants or a defect in antioxidants (Sies, 2015). It has been proposed that OS act as one of the factors that possibly play a role in the pathogenesis of neurodegenerative disorders. Clinical and preclinical studies point out that neurodegenerative diseases like PD are characterized by increased level of OS biomarkers and by decreased level of antioxidant defense biomarkers in the brain and peripheral tissues (Niedzielska *et al*, 2016).

In normal physiological state the tissues have a functional antioxidative system, GSH that is depleted due to OS. Unwarranted increase and accumulation of free radicals lead to imbalance of cellular oxidative homeostasis and decrease of GSH concentration contributes in OS and consequent brain damage (Jain *et al*, 1991). Physiologic maintenance of the redox potential is critical for normal functioning of neurons and disruptions on this balance interfere with a number of biological processes, which eventually lead to cell death. A number of machinery that are responsible for ROS production are known which include the metabolism of DA itself, mitochondrial impairement, iron, neuroinflammatory cells, calcium, and aging (Dias, 2013).

The mechanism leading to OS due to the action of ROS can be via the intracellular change in redox potential and oxidative modification in the protein molecules. Such alteration in redox potential of intracellular cystol of the cells take place under reduced conditions due to redox buffering capacity of intracellular thiol groups like GSH and thioredoxin (TRX) (Ravindran *et al*, 2012). The oxidized form of GSH and TRX are maintained in stable ratio by their reductase enzyme activities. Both of these enzymes are responsible for the reduction of hydrogen peroxide and lipid peroxidase that are carried out by the peroxidase

enzymes. They have the ability to act as the antioxidant enzymes and are involved in the cell signaling mechanism (Sesti *et al*, 2010). GSH also perform redox signaling process by transporting out the differentiation in both the total level of GSH and also in the ratio of its oxidized to reduced forms. Under physiological condition the reduced form of GSH is higher than its entire oxidized species (Aquilano *et al*, 2014).

Further, the ROS have been accounted for the structural modification in protein molecules by causing protein dimerization and also by altering the molecular structure in amino acid residues during the second stage of oxidase modification in protein molecules (Ravindran *et al*, 2012). The protein molecules are altered oxidatively in ways that include the cysteine residue. The sulfhydryl group (-SH) gets oxidized to produce the sulfenic (-SOH), sulfinic ($-SO_2H$), sulfonic (SO_3H) moieties. Such modification results in alteration of enzyme biological functions, when cysteine is located inside the catalytic domain (Wu *et al*, 2008).

2. Parkinson's Disease and Oxidative Stress

Oxidative modifications of enzymes and structural proteins play a significant role in the etiology and progression of several human neurodegenerative diseases. Accumulation of free radicals and subsequent neurodegeneration in specific brain regions have been proposed as the underlying factors in neurodegenerative diseases such as Alzheimer's and PD (Halliwell, 2006).

Studies on post-mortem PD brains link the role of oxidative injure in the progression of PD (Yuan *et al*, 2016; Zeevalk *et al*, 2008; Bosco *et al*, 2006).

3. Oxidative Stress and its Markers

3.1. Non Enzymatic Markers

3.1. 1. Reactive Oxygen Species (ROS)

ROS are molecular entities that react to cellular components which result in detrimental effects on their function. Neurons and glias are prone to produce ROS as an inevitable outcome of oxygen dependent respiration (Dumont *et al*, 2011). ROS can be generated via a number of pathways which may include direct interactions between redox active metals and reactive oxygen species through the Fenton and Haber-Weiss reactions; through indirect pathway via activation of enzymes such as nitric oxide synthase (NOS) or nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidases). As a common principle, the chemical origin of the majority of free radicals requires the activation of molecular oxygen (Smith *et al*, 2007). Examples of ROS include the superoxide (O_2^{2-}), hydroxyl (OH.), peroxyl (RO_2.), alkoxyl (RO.), hydroperoxyl (HO_2.), and nonradical species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($1O_2$), and peroxynitrite (ONOO-).

Several investigations have suggested the ROS to be the major factor contributing to DAergic neuronal loss in the PD brain, as a consequence of DA metabolism, decreased GSH activity, and elevated levels of iron and calcium in the SN*pc* (Jenner and Olanow, 2006).

The problem with ROS arises when free radical concentration is more than the antioxidant concentration and results in programmed cell death (Brieger *et al*, 2012). Thus in order to prevent the subsequent OS damage of cell, the inhibition of ROS must be established. Apart from estimation of general ROS level, several enzymes and non enzymatic function are studied as biomarkers to understand their activity variation under normal and disease conditions.

3.1.2. Lipid Peroxidation (LP)

LP is another common illustration of oxidative damage in cell membranes, lipoproteins, and other lipid-containing molecules. Peroxidative modification of unsaturated phospholipids, glycolipids, and cholesterol can take place in reactions triggered by free radical species such as oxyl radicals, peroxyl radicals and hydroxyl radicals derived from iron-mediated reduction of hydrogen peroxide or by non-radical species like singlet oxygen, ozone, and peroxynitrite generated by the reaction of superoxide with nitric oxide. Lipid hydroperoxides are well known non-radical intermediates of lipid peroxidation and its identification can often give essential mechanistic details such as whether a primary reaction is mediated by singlet oxygen or oxyradicals. Unsaturated phospholipids, glycolipids, and cholesterol in cell membranes and other organized systems are major targets of oxidant attack. This can result in lipid peroxidation, a degenerative process that disturbs structure and function in the target systems resulting in cytopathological consequences (Halliwell and Gutteridge, 1990; Girotti, 1985). Several products from lipid peroxidation are chemically reactive and are thought to be the major effectors of tissue damage (Mattson, 1998). Lipid peroxidation is measured by levels of MDA which is an intermediate compound and a major pointer of lipid peroxidation. The brain has high concentrations of polyunsaturated fatty acids that cause peroxidation of lipids and in the process accumulates toxic products when there is a condition of OS.

3.1.3. Protein Carbonyl (PC)

PC content is the most general and well used biomarker of severe oxidative protein damage. Carbonyl groups (aldehydes and ketones) are produced on protein sidechains especially of Proline, Argenine, Lysine, Threonine, when they are oxidized (Fig.1). These moieties are chemically stable, and important for both their detection and storage.

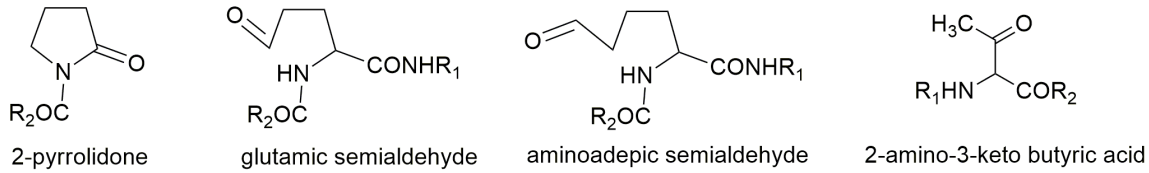


Fig.1. The structure of carbonyl derivatives produced by direct oxidation of amino acid side chains: 2-pyrrolidone from prolyl residue, glutamicsemialdehyde from arginyl and prolyl residue, a-aminoadipicsemialdehyde from lysyl residue, and 2-amino-3-ketobutyric acid from threonyl residue (Dalle-Donne *et al*, 2003).

Carbonylation of proteins is a permanent oxidative injury resulting in functional loss often forming a clumping of damaged unfolded proteins. Such aggregates can inhibit proteasomal function which is directly linked to the increase in aggregates of carbonylated proteins in tissues that are resistant to proteolytic process. Uneven protein structures and folding that arise due to functional impairment are closely associated with several human diseases (Bossy-wetzel *et al*, 2004). Bioinformatics Analyses on pesticide treated mice showed that protein carbonylation affects cellular junctions, cytoskeleton, and the proteasome (Coughlan *et al*, 2015). Elevated levels of protein carbonylation associated with PD have been reported (Floor *et al*, 1998). In some case, this elevations correlate well with the progression or severity of the disease.

3.1.4. Hydroperoxides (HP)

HP is one of the major modifications of organic compounds under the influence of well-recognized reactive oxygen species. HP are the intermediate to produce more reactive oxygen species and are well established biomarkers for OS. Commonly used HP bio markers are protein HP and lipid HP. Studies indicate that proteins are most likely to be among the first targets of ROS cells and protein HP is the main products of this interaction. Formation of Protein HP has an important biological implication since it results in

modification of the structure and properties of amino acid residues. Moreover, HP groups are powerful oxidants that can induce secondary damage through inactivation of thiol-dependent enzymes essential to cell functions. Hydroxyl radical (•OH), superoxide radical anion ($O^{2\bullet}$), and singlet oxygen ($1O_2$) are the major contributors to the overall amount of protein HP formed in living organisms. This ROS modify the amino acid residues with an addition of HP group (-OOH). The protein HP (PrOOH) has a tendency to react with the metals and proteins and induces DNA damage, deactivation of enzyme, free radical formation (Fig.2).

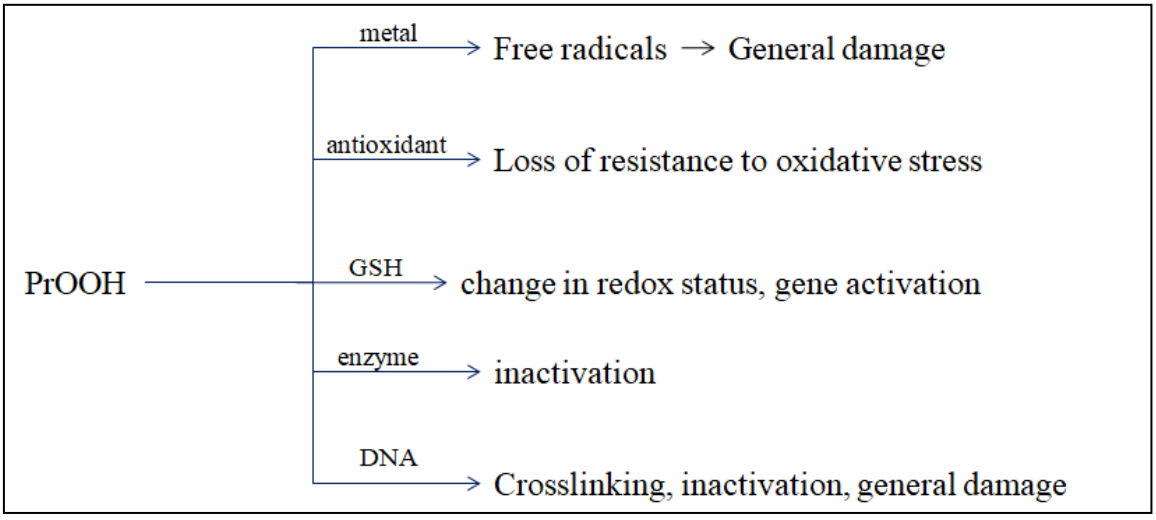


Fig.2. A summary of the reactions of protein hydroperoxides and their potential consequences in vivo (Gebicki, 1997).

3.2. Antioxidant Enzymes

3.2.1. Superoxide Dismutase Enzyme (SOD)

SOD which convert superoxide to hydrogen peroxide (H_2O_2) in the cytoplasm and mitochondria are often regarded as the first line of defense against ROS (Zhou *et al*, 2008) among the ROS-scavenging enzymes. They convert naturally occurring superoxide radicals to molecular oxygen and hydrogen peroxide. Three different SOD isoenzymes, that are well compartmentalized, have been characterized in humans (Zelko *et al*, 2002).

Study on PD brain showed an increased superoxide dismutase like activity most prominent in SN and basal nucleus (Marttila *et al*, 1998). Post mortem studies in PD showed increased activity of encephalic superoxide dismutase (Taylor *et al*, 2012). Also recent study on patient's blood has reported that PD is positively associated with increased levels of SOD activity (de Farias *et al*, 2016).

3.2.2. Catalase (CAT)

CAT is known as a major antioxidant enzyme that neutralizes the harmful effects of ROS and as such, is considered beneficial in the treatment of many diseases. CAT protects cells by separation of hydrogen peroxide into water and oxygen and by prevention of hydroxyl radical generation. Thus it helps to remove the oxygen free radicals, which is essential for maintaining a stabilized redox status of cells (Halliwell and Gutteridge, 1990). Study on CAT-loaded, poly (lactic co-glycolic acid) nanoparticles (NP) in human neuronal protection against oxidative damage showed very efficient CAT encapsulation capable of retaining ~99% enzymatic activity. The NP-mediated CAT delivery effectively protected cultured neurons from H_2O_2-induced OS. CAT loaded nanoparticles significantly reduced H_2O_2-induced protein oxidation, DNA damage, mitochondrial membrane transition pore opening and loss of cell membrane integrity and restored neuronal morphology, neurite network and microtubule-associated protein-2 levels (Singhal *et al*, 2013).

3.2.3. Glutathione S-Transferase (GST)

GST is a group of enzymes important in the detoxification of many xenobiotics in animals. They guard cells against toxicants by conjugating the thiol group of the GSH to electrophilic xenobiotics, and thereby protect cells against the mutagenic, carcinogenic, and toxic effects of both exogenously and endogenously derived compounds (Hayes and

Strange, 2000). GST activity is present in plants, insects, yeast, bacteria, and most mammalian tissues particularly in the liver, which plays a key role in detoxification.

Human GSTs are a functionally diverse family of soluble enzymes of detoxification that use GSH in conjugation and reduction reactions. Toxic electrophiles, including a variety of carcinogens, are substrates for the GSTs and after conjugation or reduction they are more easily excreted into bile or urine. There are at least 42 GST-like genes in *Drosophila melanogaster* belonging to six putative families including genes from the Delta, Epsilon and Sigma classes (Rubin *et al*, 2000).

3.2.4. Reduced Glutathione (GSH)

GSH is an important antioxidant enzyme capable of preventing the ROS mediated cellular damage. GSH activity loss is linked with incidental LB disease and it may represent the initial biochemical marker of nigral cell loss. GSH depletion alone may not result in damage to nigral neurons but may increase susceptibility to subsequent toxic or free radical exposure. In a study to access the alterations in GSH levels in PD and other neurodegenerative disorders affecting basal ganglia, GSH and oxidized GSH activity were monitored in various brain areas from patients dying with PD. GSH levels were found to be reduced by 40% as compared to control subjects in SN in PD patients and oxidized GSH levels were insignificantly elevated while there were no changes in other brain areas. In spite of severe nigral cell loss in the SN in PD, multiple-system atrophy, and progressive supranuclear palsy, significant reduction in GSH level was seen only in PD. The result suggests that nigral cell death alone is not responsible for alteration in GSH level (Sian *et al*, 1994). Decreased synthesis due to inhibition of GSH reductase or from increased levels of GSH disulfide (GSSG) and change the GSH: GSSG ratio could be the cause for the drop down in GSH level (Genestra, 2007).

3.2.5. Total Thiols

Thiol redox balance regulation is crucial for multiple cellular metabolic, signaling and transcriptional processes. Thiol groups are highly reactive and susceptible to oxidation that may cause significant loss of biological activity. In proteins, oxidation of free thiol groups produces modifications that may impact on the structure, catalytic activity or ability to engage in protein-protein interactions depending on their location. An important function of cell based thiol redox buffering systems is to protect thiol groups from oxidation and to repair those that may have become oxidized as a result of normal or aberrant cellular metabolism. The key components of the thiol redox buffering system are the cysteine/cystine and GSH/ GSH disulphide redox pairs, and the thiol disulphide oxidoreductases that include thioredoxin (Trx), glutaredoxins (Grx) and peroxiredoxins (Prx). Grx1 plays an important role in maintaining cell viability in model DAergic neurons in culture (Rodriguez *et al*, 2012). In vitro and in vivo study of potential impact of Grx1 upregulation in the CNS on DAergic viability suggests that the Grx1 upregulation promotes toxic neuroinflammation, potentially contributing to PD (Miller *et al*, 2016).

3.3. Neurotoxicity Marker

3.3.1. Acetylcholinesterase (AChE)

AChE is one of the most efficient enzymes which is concentrated at the cholinergic synapses throughout the CNS and at neuromuscular synapses where it rapidly hydrolyses the neurotransmitter acetylcholine (Ach) into choline and acetate thus playing an essential role in cholinergic neurotransmission (Tripathi and Srivastava, 2008). AChE enzyme is present in high concentration in all types of conducting tissue, nerve and muscle, central and peripheral tissues, motor and sensory fibers, sympathetic and parasympathetic so called cholinergic and noncholinergic fibers and all the regions where cell bodies and junctions are located (Nachmensohn, 1959). AChE plays an essential role in

acetylcholine-mediated neurotransmission. It is neurotransmission activity, rather than re-uptake by transporters as with other neurotransmitter systems, that terminates cholinergic neurotransmission (Massoulie *et al*, 1993). The importance of AChE in mammals is illustrated by the effect of abrupt blockade of AChE catalytic activity, such as by exposure to the nerve gas sarin. Within minutes, inhibition of AChE leads to excess acetylcholine at neuromuscular synapses, continued activation of acetylcholine receptors, subsequent receptor inactivation, respiratory and/or cardiac dysfunction and death (Karalliedde, 1999). Therapeutically, controlled application of AChE inhibitors is used to increase synaptic levels of acetylcholine in diseases that impair acetylcholine neurotransmission.

4. Aging as a factor for Oxidative Stress

Protein, lipids and nucleic acids are biological macromolecules which are oxidatively altered due to ROS, resulting in genetic mutations and cellular senescence (Luceri *et al*, 2017). In complications and pathologies associated with aging, increasing evidence for causal role of cell senescence have been experimentally shown (Correia-Melo *et al*, 2015). Due to stress during aging there is increase in cell senescent. This phenomenon is controlled by a number of counting mechanisms including telomeres shortening, gradual increase in damage of DNA, deviation in normal oncogenes behavior, change in metabolic action and increased ROS generation (Kuilman *et al*, 2010). Such processes lead to impairment of cellular function and thus produce and accumulate ROS which in turn disturb the homeostasis, a vital factor for safeguarding the senescent phenotype. Apart from injuring DNA and damage mitochondrial activity, OS trigger the activity of p53, a gene coding for cell cycle regulator protein, which in turn induces prooxidant genes. Disturbances in redox homeostasis by generating ROS lead to deterioration of cell and impair its function.

To limit over-accumulation of ROS in the body, there exist both enzymatic and non-enzymatic systems that maintain ROS balance. Reports have also shown that natural antioxidant defense systems have limited capacity in the brain as compared to peripheral tissues (Somani *et al*, 1995; Del *et al*, 1987) and buildup of molecular oxidative damage, initiated by ROS is the main causal factor underlying senescence-associated losses in physiological functions (Sohal, 2002).

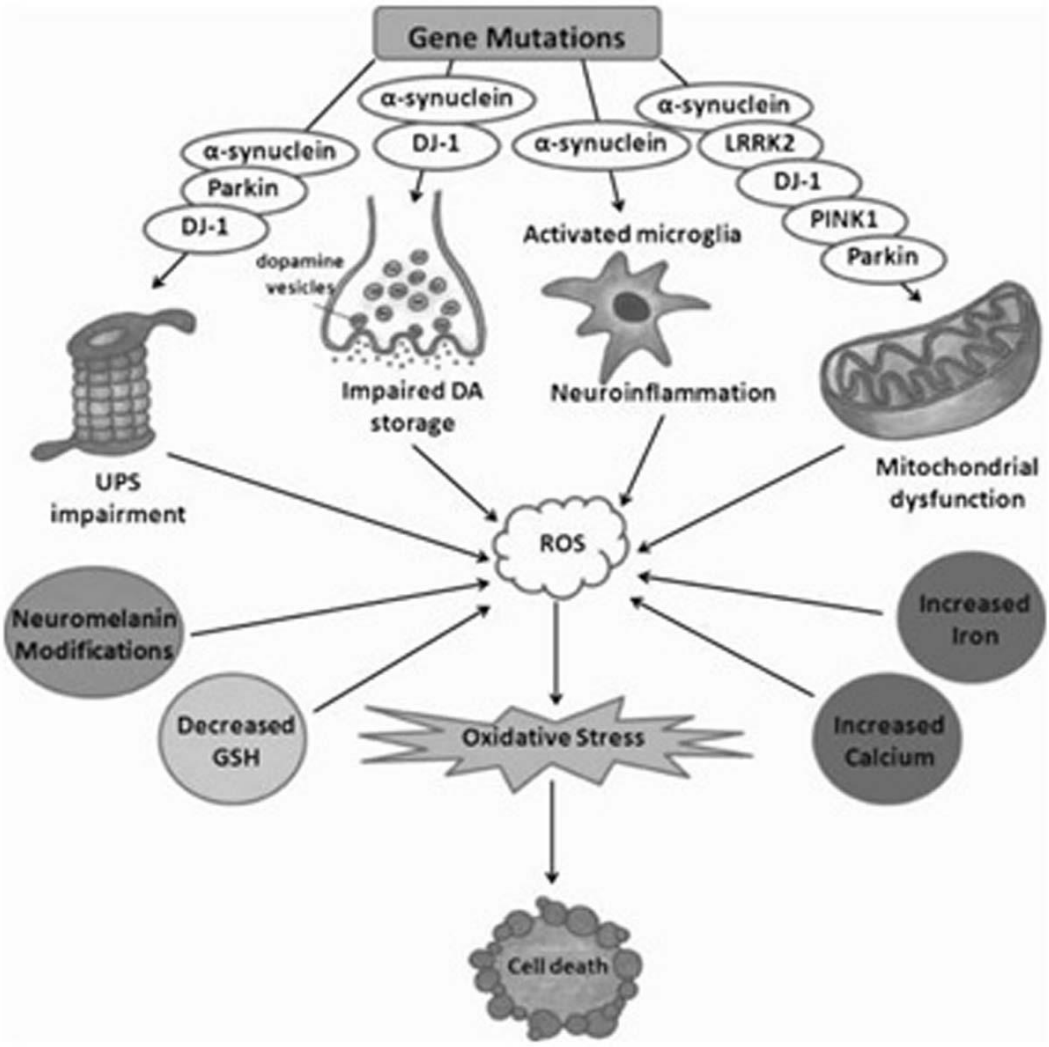


Figure.3. Mechanisms leading to Oxidative Stress in PD and the role of PD-related gene products in this process. DA = dopamine; ROS = reactive oxygen species; UPS = ubiquitin-proteasome system; GSH = glutathione (Adapted from Dias *et al*, 2013).

5. Studies on Efficacy of Neuroprotective Compounds using Fly Model

Using OS induced depletion as a gold standard, many laboratories have studied neuroprotective efficacy of multiple compounds showing modulation of biochemical markers, antioxidant enzymes and phenotype associated rescue on exposure to neurotoxic agents and suggested for therapeutic approach (Jia *et al*, 2018; González-Burgos *et al*, 2017; Anandhan *et al*, 2012; Khan *et al*, 2010; Rajasankar *et al*, 2009; Rojas *et al*, 2008; White *et al*, 1996).

Studies have also shown a number of natural compounds and molecules derived from them have the ability to protect cells from OS and ameliorate various OS-related diseases. Some of the list of pathways through which neuroprotective compounds confer neuroprotection are given in table 1.

Table 1: Listing of pathways through which neuroprotective compounds confer neuroprotection: Lessons from *Drosophila* model of PD (*All treatments were administered as dietary complement) (Ayajuddin *et al*, 2018)

Pathway/ Process	Compound treatment	*Drosophila* model	Modified phenotype/s
Oxidative Stress	Sulforaphane and allyldisulfide	Parkin	DA neuron number
	S-methyl-Lcysteine	α-synuclein	Locomotor activity
	Polyphenols	α-synuclein	Lifespan, locomotor acitvity
	α-tocopherol	PQ and iron	Locomotor activity
	SOD	DJ-1β	Lifespan
	Melatonin	PINK1	Lifespan
	Bacopa monieri	PINK1	Ommatidial degeneration
	leaf extract	DJ-1β	Ommatidial degeneration
		PQ	Lifespan, locomotor activity
		Rotenone	Locomotor activity, DA neurons
		PQ	Oxidative marker levels
Oxidative Stress / Inflammatory process	Miocycline Celastrol	DJ-1α	DA neuron numbers, DA levels, locomotor activity and survival under OS condition
TOR signalling	Rapamycin	Parkin/PINK1	Thoracic indentations, locomotor activity, DA neuron number and muscle integrity
Removal of excess or toxic protein forms	Geldanamycin	α-synuclein	DA neuron number
Zinc homeostasis	Zinc chloride	parkin	Lifespan, locomotor activity, and percentage of adulthood survivors
Chaperone therapies (HSF-1 modulators) Trigger HSF-1 activation Induces downstream Hsp70 expression	Celastrol Carbenoxolone	α-synuclein α-synuclein	DAergic neuroprotection
Hsp90 inhibitors Inhibits the interaction between Hsp90 and HSF-1, leading to increased Hsp70 expression and activity	Geldanamycin 17-AAG 17-DMAG SNX-2112	α-synuclein	decrease α- synuclein aggregation and reduce cell toxicity
mTOR-dependent pathways/AMPK	Metformin AICAR	*Drosophila melanogaster* mutated for LRRK2	Reduced cell death
mTORC1	Rapamycin and Rp analogues (CCI-779, RAD001 and AP23573)	*Drosophila melanogaster* mutated for PINK-1 and Parkin	Reduced mitochondrial dysfunction
mTor-independent pathways/unknown	Spermidine	α-synuclein	Reduced motor dysfunction, increased lifespan; Reduced neuronal cell loss
LRKK2 kinase inhibitors	GW5074, and sorafenib	α-synuclein	Protect against DA neuron degeneration locomotor activity
Histone Deacetylase inhibitors		α-synuclein	Protect against DA locomotor activity
Antitumor agents	Geldanamycin	α-synuclein	Protect against DA Mobilized the stress response and increase levels of chaperon HSP70

6. Necessity of Stage Specific studies in *Drosophila* Model of PD

For understanding the pathology of late onset neurodegenerative diseases, it is important to employ organisms belonging to adult stages/phases where the disease sets in, such as the transition stage of *Drosophila* PD model. Because different stages of the life stage have different patterns of gene expression and the cellular, physiological and molecular phenomenon at old stage may be completely different from young stage. Studies in *Drosophila* using expression analysis has demonstrated that there are about 23% genome-wide transcript patterns variations (Pletcher *et al*, 2002) indicating that the genetic targets of a particular therapeutic molecule may be present at one stage but lacking in another stage of life span. Therefore it may exert positive action at one stage and neutral or negative action in another stage. Genotropic drugs will only exhibit its efficacy in the stage of life cycle where their target molecules are present (Soh *et al*, 2013). Hence, it is possible that the targets of K may be absent in some stages of life, which is a vital and interesting paradigm. But there are no reports showing the effectiveness of K during the later stages of adult fly life. Therefore, it is necessary to understand the neuroprotective efficacy of compounds at transition phase because this is the period during which late-onset neurodegenerative diseases such as idiopathic PD sets in.

7. Neuroprotective Studies of Curcumin employing adult Health Stages in Fly Model

Many laboratories have studied the efficacy of K in fly model. They estimate biochemical markers, antioxidant enzymes and phenotype associated rescue on exposure to neurotoxic agents and suggested K for therapeutic approach.

One such study using 8-10 days old fly has reported that upon treatment with K the neurotoxin acrylamide induced levels of reduced GSH, total thiols and acetylcholine esterase activity was also restored and mitochondrial dysfunctions was alleviated and also

restored the decreased DA levels (Prasad and Muralidhara, 2014). Pre-treatment with synthesized K bioconjugate K monoglucoside also protected against rotenone neurotoxicity and exerted antioxidant effects by replenishing cellular GSH levels and considerably decreasing reactive species, restored mitochondrial complex I and IV activities, restored nuclear damage and induced anti-apoptotic effects. Q-PCR (quantitative polymerase chain reaction) analysis of redox genes showed up-regulation of Nitric Oxide Synthase-2 (NOS2) and down-regulation of NAD(P)H Quinone Dehydrogenase 1 (NQO1) when flies were exposed to rotenone which was attenuated by CMG pre-treatment (Paandaresh *et al*, 2016). Employing newly eclosed adult flies it is also reported that DAergic neuron-specific knockdown of dUCH (a homolog of human UCH-L1, Ubiquitin terminal-hydroxylase-L1) led to locomotor deficit and DAergic neuronal loss and induction of OS. K could improve motor function, decrease the elevated ROS level and minimize the extent of neurodegeneration (Nguyen *et al*, 2018). Another investigation has shown K treatment improve the motor impairment caused by rotenone toxicity in 7-8 days old fly (Khatri *et al*, 2016).

However, it is imperative to note that all the above studies were performed only in young flies in the age group of 1-10 days old. Basing on this result it may be lacking to say that neuroprotective efficiency of K is active in all the life stages of adult fly because it is known that there is 23% variation in genome-wide transcript pattern with respect to age in *Drosophila* (Pletcher *et al*, 2002) signifying that targets of genotropic compounds under investigation may be absent at some life stage; they would be active only during those stages where their target molecules are present (Soh *et al*, 2013). Thus, I decided to understand neuroprotective efficacy of K by studying the stress markers and enzyme activities in both the young and old stage groups of flies through the model developed.

8. Materials and Methods

8.1. Fly Stock

Drosophila melanogaster of Oregon K strain was obtained from National *Drosophila* Stock Centre of University of Mysore, Mysore, Karnataka, India. Male flies were used in the present study. They were kept at 22°C-24°C and fed on a standard culture medium made of sucrose, yeast, agar agar, and propionic acid.

8.2. Chemicals

Bovine Serum Albumin (A-2153), Curcumin (C-1386), Paraquat (methyl viologen dichloride hydrate, 856177), 2,7-dichlorofluorescin diacetate (D-6883), Thiobarbituric acid (T5500), Hydrogen peroxide (323381), Acetylthiocholine iodide (A5751), Quercetin (fluka, 200595), N'N'N'N-Tetramethylethylenediamine (T9281), Glutathione (G-4251), were purchased from Sigma Aldrich St. Louis, USA. Dimethyl sulfoxide (DMSO), 2,4-Dinitro phenyl hydrazine (DNPH), 1,1-dithio nitro-bi-benzoicacid (DTNB), 1-Chloro-2,4-dinitrobenzene (CDNB), Ethyldiaminetera acetic acid (EDTA), Dimethlysulfoxide (DMF), Sodiumdodecyl sulphate (SDS), Ortho-pthaldehyde (OPA), Xylenol orange, Acetone, Glacial acetic acid, Trichloroacetic acid and all other analytical grade chemicals were procured from Sisco Research Laboratory Chemicals and Merck, India. Whatman filter paper no.1 disc was used as a feeding medium in the experiment.

8.3. Treatment Protocol

Male flies were aged for 4 days and 55 days feeding in sucrose-agar media. The flies were transferred to freshly prepared media every 3rd days while aging. The flies were then transferred to vials (30mm x100mm) containing single disc of Whatman filter paper no. 1 saturated with 275µl of 5% sucrose, 10mM PQ in 5% sucrose, Curcumin (500µM, 1mM) with PQ (10mM) and Curcumin in DMSO. At 24hrs of exposure, flies were freezed @-

80˚C. For dissection of head, an aluminium tray which was positioned on the ice block and flies were placed on it. Using Carl Zeiss stereozoom (Stemi 305) microscope the flies were then dissected separating head from body with a sharp razor.

8.4. Preparation of Homogenate

100 heads were homogenized with pestle motor mixer (Argos technologies) in 175µl 0.1M Phosphate Buffer 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.

8.5. 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. The measurement was performed using NanoDrop 2000 (Thermo Scientific).

9. Biochemical Assays

9.1. Reactive Oxygen Species (ROS)

Generation of ROS was determined using 2',7'-dichlorofluorescein diacetate (DCFDA), a non polar compound that can quickly react with ROS to form the highly fluorescent compound, 2',7'-dichlorofluorescein (DCF) after conversion to a polar derivative by intracellular esterases. DCF can be detected by fluorescence spectroscopy.

50µgP of brain sample was incubated in Locke's buffer (NaCl-154, KCl-5.6, $NaHCO_3$-3.6, HEPES-5, $CaCl_2$-2, glucose-10 in mM concentrations, pH 7.4) containing 5µM DCFDA for 45 minutes at room temperature and the fluorescence was measured with excitation 480nm,emission 530nm using Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies). The ROS generation was expressed as pmol DCF/min/mg protein.

9.2. Lipid Peroxide (LP)

Lipid peroxidation was assessed by measured by estimation of MDA by thiobarbituric acid (TBA) reaction method. MDA a product of lipid peroxidation react with TBA to form 1:2 adduct (MDA-TBA2) measured spectrophotometrically at 532nm.

200µgP of 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 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).

9.3. Protein Carbonyls (PC)

This technique involves detecting and quantifying oxidative modification of proteins. 2,4-dinitrophenylhyrdazine (DNPH) reacts with protein carbonyls to form a stable dinitrophenyl (DNP) hydrazone adducts propotional to carbonyls present which can be detected spectrophotometrically at an absorbance of 360 nm.

Protein carbonyl (PC) levels in the samples were quantified following reaction of 100µgP test samples with 2,4-dinitrophenyl hydrazine (DNPH) for 1hr. The protein is precipitated by adding 20% trichloroacetic acid, kept on ice for 5 minutes and it is then centrifuged at 3000rpm for 10 minutes at 4°C. The pellets were washed with 1ml ice cold acetone, followed by dissolving in 20mM Tris-HCl buffer (pH 7.4, containing 2% SDS, 10mM EDTA). The optical density was measured at 360 nm using NanoDrop 2000 (Thermo Scientific) and expressed as nmol carbonyl/mg protein (MEC=22,000/M/cm) (Levine *et al*, 1990).

9.4. Hydroperoxides (HP)

The H_2O_2 assay was based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) wherein the ferric ions (Fe^{3+}) react with the indicator dye xylenol orange to produce a purple colored complex measurable at 560nm. Sorbitol was included in the assay to amplify the color intensity.

HP levels were measured according to a previously described method using Ferrous ion oxidation xylenol orange (FOX1) reagent (100µM xylenol orange, 250µM ammonium ferrous sulphate, 100µM sorbitol, 25mM H_2SO_4). 100µgP of test sample was added to 1ml FOX reagent and incubated in dark at room temperature for 30 minutes. The color developed was read at 560nm in a NanoDrop 2000 (Thermo Scientific). The concentration

of HP was calculated using molar extinction coefficient of 2.2 x 105 M^{-1} cm^{-1} and expressed as nmol HP/mg protein (Wolff, 1994).

9.5. Superoxide Dismutase (SOD)

Superoxide dismutase activity was measured indirectly by monitoring the inhibition of quercetin auto-oxidation. Quercetin is oxidized by O_{2-} 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 test sample was mixed with phosphate buffer (0.016 M, pH 7.8, containing TEMED- 0.8mM and EDTA-0.08 mM) followed by 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 seconds interval 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 and Potapovich, 1989).

9.6. Catalase (CAT)

Catalase is involved in the detoxification of H_2O_2, a toxic product of both normal aerobic metabolism and pathogenic ROS production. This enzyme catalyze the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water and the rate of decomposition of H_2O_2 is measured at 240nm.

CAT activity was estimated by adding 25µgP of samples to phosphate buffer (0.1M, pH 7.4, containing 10 mM H_2O_2). H_2O_2 degradation was monitored at 240nm for 2 minutes with 15 seconds interval in NanoDrop 2000 (Thermo Scientific) and expressed as nmol substrate/min/mg protein (MEC=44/mM/cm) (Aebi, 1984).

9.7. Glutathione S-Transferase (GST)

GST represents a family of enzymes that play important role in detoxification of xenobiotics. GST catalyzes the conjugation of the thiol group of the GSH to electrophilic xenobiotics to protect cell against toxicants. It utilizes GSH to scavenge toxic compounds including those produced due to OS and is part of the defense mechanism against the mutagenic, carcinogenic and toxic compounds (Boyland and Booth, 2003). GST activity was estimated by monitoring the conjugation of glutathione to CDNB.

The reaction was initiated by addition of 40µgP test sample to phosphate buffer (0.1M, pH 6.5) containing 0.5mM EDTA, 0.075mM CDNB, 0.05mM GSH. The increase in optical density was observed for 5 minutes at 340nm using NanoDrop 2000 (Thermo Scientific). The activity was expressed as nmol conjugate formed/min/mg protein using molar extinction coefficient 9.6 mM^{-1} cm^{-1} (Guthenberg *et al*, 1985).

9.8. Reduced Glutathione (GSH)

GSH activity was determined based on fluorimetric method using Orthopaldehyde (OPA) following the standard procedure. GSH react with OPA to form a stable highly fluorescent tricyclic derivate at pH 8.0 which is measured spectrophotometrically.

50µgP of sample protein was mixed with 5% tricarboxylic acid (TCA) and centrifuge at 3000rpm for 5 minutes at 4°C. The supernatant was added to a reaction mixture of buffered formaldehyde (1:4 (v/v) 37% formalin: 0.1M Na_2HPO_4). 900µl of Sodium phosphate buffer (0.1M, 5mM EDTA, pH 8.0) was added to each tube followed by 100µl OPA. Then after dark incubation for 30 minutes at room temperature, the fluorescence was measured at excitation and emission wavelengths of 345 and 425 nm respectively using Fluorimeter (Agile Life technologies) (Cohn *et al*, 1966).

9.9. Total Thiols

The procedure is based on the reaction of the thiol with 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) to give the mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) which is quantified by the absorbance of the anion (TNB^{2-}) at 412 nm. The reagent has been widely used for the quantitation of thiols in peptides and proteins.

Total thiols activity was determined by taking 25µgP of test sample containing Tris buffer (pH 8.0) reacted by adding 25µl DTNB and 1775µl methanol. The reaction was incubated in dark for 30minutes with occasional mixing, followed by centrifugation at 3000rpm for 5 minutes at 4°C. The supernatant was used to measure the ability of thiols to oxidize DTNB using spectrophotometer at 412 nm in NanoDrop 2000 (Thermo Scientific). Total thiols activity was then expressed as nmol/mg protein (Ellmann, 1959).

9.10. Acetylcholinesterase (AChE)

Acetylcholinesterase efficiently catalyze the hydrolysis of acetyl-thiocholine sulfur analogs of its natural substrate, acetylcholine. Upon hydrolysis, these substrate analogs produce acetate and thiocholine. Thiocholine in the presence of the highly reactive dithiobisnitro-benzoate (DTNB) ion reacts to generate the yellow of 5-thio-2-nitrobenzoate anion. The yellow color product can be measured by its absorbance at 405 nm (Ellman *et al*, 1961).

Acetylcholinesterase (AChE) activity was determined by taking 25µgP test sample containing in phosphate buffer (0.1M, pH 8.0) and DTNB (10mM). To the mixture, 20µl of acetylthiocholine iodide (ATCI, 78 mM) was added. The reaction absorbance was monitored at 412 nm for 5 minutes with 30 seconds interval in a NanoDrop 2000 (Thermo Scientific). AchE activity was then expressed as nmol substrate hydrolyzed/min/mg protein (Ellmann *et al*, 1961).

11. Results

11.1. Curcumin diminishes PQ induced ROS level during Health and Transition phase of adult life stage in fly model of PD

The modulatory activity of K on PQ induced elevation in ROS levels was determined. It was found that in 4 day old flies 34% enhanced level of ROS by PQ exposure was effectively decreased by 20% and 60% upon $R_{500\mu M}$ and R_{1mM} K co-treatment. Also, in 55 day old flies there was 5 fold increases in ROS level by PQ treatment when compared to control, suggesting that at later stage of life, the organism becomes more susceptible to OS conditions. K co-treatment could decrease the elevated ROS levels by 60% and 35% in both the concentrations ($R_{500\mu M}$ and R_{1mM}). PQ elevated the ROS levels in both the age group of flies and K *per se* did not have any detrimental effects on 4-day flies. However, in the 55 day old flies it was found that concentration of K *per se* ($K_{500\mu M}$ and K_{1mM}) increased the ROS level when compared to control (Fig.4) suggesting that K itself may act as potential pro-oxidant and therefore the necessity to carefully screen the compound before prescribing them for therapeutic agents.

11.2. Curcumin diminishes PQ induced Lipidperoxide level during Health and Transition phase of adult life stage in fly model of PD

To study the neuroprotective action of K by estimating Lipid Peroxidation, the levels of MDA an end product of LP was analyzed as one of the stress markers. PQ exposure upregulated LP by 60% and K co-treatment attenuated the level by 23% and 31% in $R_{500\mu M}$ and R_{1mM} respectively in health stage. In the transition stage, PQ treatment upregulated LP by 24% when compared to control. The increase in LP level was attenuated by 30% with both the K concentration used ($R_{500\mu M}$ and R_{1mM}) which indicate the effective free radical sequestration properties of K (Fig.5). K *per se* did not show any toxic effect in

transition stage, but in health stage it was evident that the K *per se* exerts toxicity at this stage with the selected concentration.

11.3. Curcumin diminishes PQ induced Protein carbonyl level during Health and Transition phase of adult life stage in fly model of PD

K neuroprotective efficacy was also studied by estimating the PC level. While there was marked increase in PC upon PQ exposure (44% during health stage and 27% increase in transition phase), K co-treatment could effectively attenuate the PC level by all the concentrations used in health stage. There was 18%, 28% and 20% reduction in PC levels with all the tested concentrations of K ($R_{100\mu M}$, $R_{500\mu M}$ and R_{1mM} respectively). At transition stage there was reduction by 25% ($R_{100\mu M}$,), 24% ($R_{500\mu M}$) and 20% (R_{1mM}). At highest concentration of K co-treatment (R_{1mM}) there was relatively least rescue than the lower K concentrations used suggesting K itself may be toxic at this concentration stage and can have detrimental effect which is also evident from K *per se* in higher concentrations used (K_{1mM}) in both stages (Fig.6).

11.4. Curcumin diminishes PQ induced Hydroperoxide level during Health and Transition phase of adult life stage in fly model of PD

Hydroperoxidation was increased by 30% in health stage and 13% in transition stage on PQ exposure. K co-treatment decreased the HP level by 20% and 30% in health stage and 15% and 18% in transition stage with both $R_{500\mu M}$ and R_{1mM} concentrations respectively (Figure.7). There was no toxicity associated with K *per se* treatment. This result further confirms the potent free radical scavenging activity of K. It is evident that K can actively

sequester the OS level in both the health and transition stage of adult life stages of *Drosophila*.

11.5. Curcumin diminishes PQ induced SOD level during Health and Transition phase of adult life stage in fly model of PD

SOD, the first line of antioxidant defense system was elevated by 20% in health stage and 27% in transition stage (Fig.8) by PQ treatment. K co-exposure significantly reduced the antioxidant enzyme activity. It was found that R_{1mM} concentration could bring down the elevated SOD activity by 40% in health stage and 20% in transition stage. It suggests lowered level of OS in the fly brain with K co-treatment. In transition stage, it was found that K *per se* was also found to actively sequester OS as indicated by decreased SOD activity when compared to control.

11.6. Curcumin diminishes PQ induced CAT level during Health and Transition phase of adult life stage in fly model of PD

K effectively decreased the CAT activity which was elevated by PQ. Exposure of PQ increased the CAT level by 43% in health stage and 30% in transition stage (fig.9). Co-treatment of K decreased the level by 44% and 35% in health stage and 40% and 15% in transition stage by both $R_{500\mu M}$ and R_{1mM} respectively. While K *per se* was also found to increase the CAT activity by 30% in health stage, there was no significance in transition stage. The result shows antioxidant activity of K which could reduce the elevated antioxidant enzyme activity suggesting lowered level of OS by K action.

11.7. Curcumin diminishes PQ induced GST level during Health and Transition phase of adult life stage in fly model of PD

The antioxidant property of K was evident even by the measurement of GST activity. It was found that PQ elevated GST level by 20% during health stage and 30% in transition phase when compared to controls (Fig.10). The elevated level was decreased by 18% and 22% in health stage and 15% and 19% in transition stage in both the concentrations used ($R_{500\mu M}$ and R_{1mM}) respectively. This result further reaffirms the antioxidant property of K and its modulatory effect against PQ induced OS in fly model of PD.

11.8. Curcumin replenishes PQ induced Glutathione activities during Health and Transition phase of adult life stage in fly model of PD

PQ exposure led to significant reduction in GSH activities in both the age groups of flies. There was reduction of 30% during health stage and 27% during transition stage (Fig.11) suggesting enhanced levels of neuronal OS. K significantly rescues the diminished level of GSH in both the age groups. It was found that 20% and 15% rescue in health stage and 23% and 27% in transition stage with both R500µM and R1mM concentration respectively. These results further suggest the efficacy of K as an antioxidant therapeutic agent in PD condition.

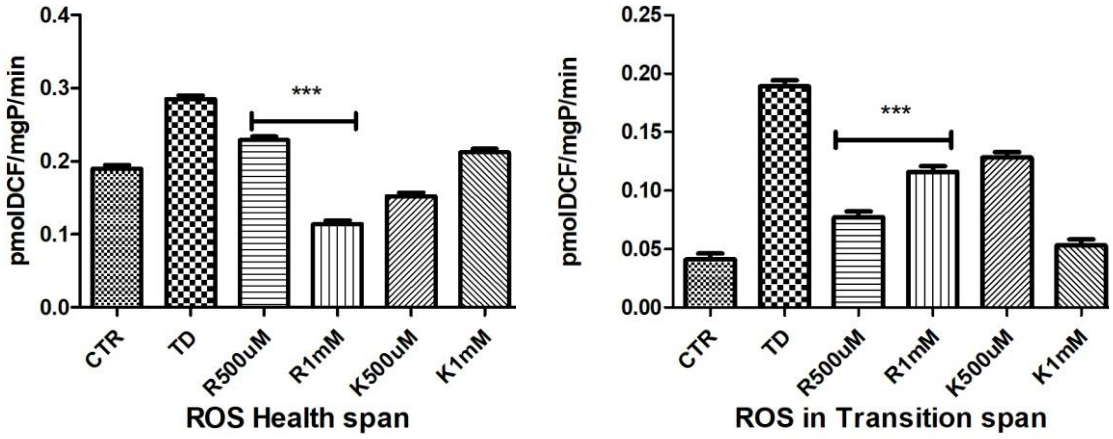
11.9. Curcumin replenishes PQ induced Total Thiols activity during Health and Transition phase of adult life stage in fly model of PD

K could also rescue the reduced thiols levels caused by PQ treatment. In health stage 18% inhibition due to PQ was rescued by 14% and 12% with $R_{500\mu M}$ and R_{1mM} concentration

whereas in transition stage 27% inhibition in thiols levels was rescued by 17% and 14% in both the $R_{500\mu M}$ and R_{1mM} concentration respectively (Fig.12). It further supports the findings in other markers that K has anti-oxidative property and potential for therapeutic agent in PD.

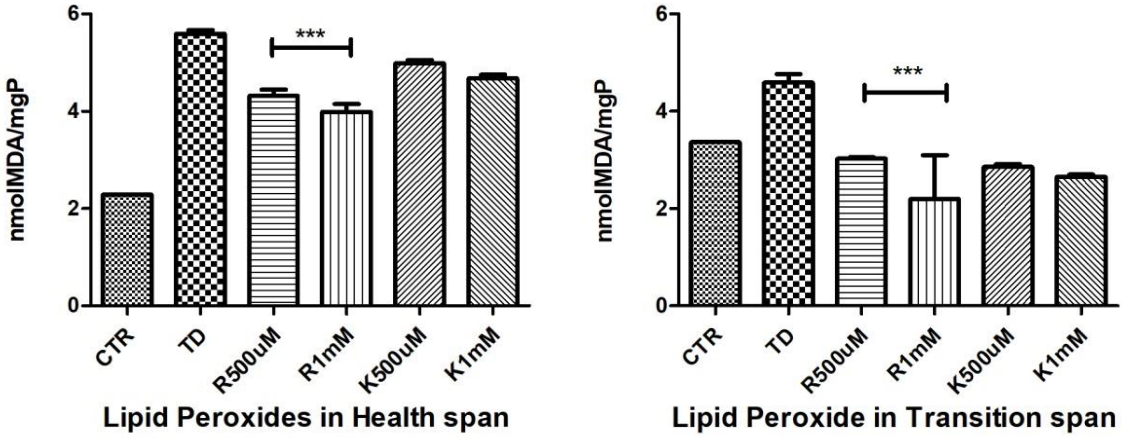
11.10. Curcumin replenishes PQ inhibited AChE level during Health and Transition phase of adult life stage in fly model of PD

K also rescued the PQ induced marginal inhibition of AChE activity in both the age groups. In health stage 20% inhibition in AChE activity was rescued by 13% and 16% and in transition stage 16% inhibition was rescued by 15% and 8% in both the $R_{500\mu M}$ and R_{1mM} concentrations respectively (Figure.13). This result further suggests the neuromodulatory properties of K and its potential application as therapeutic agent in PD.



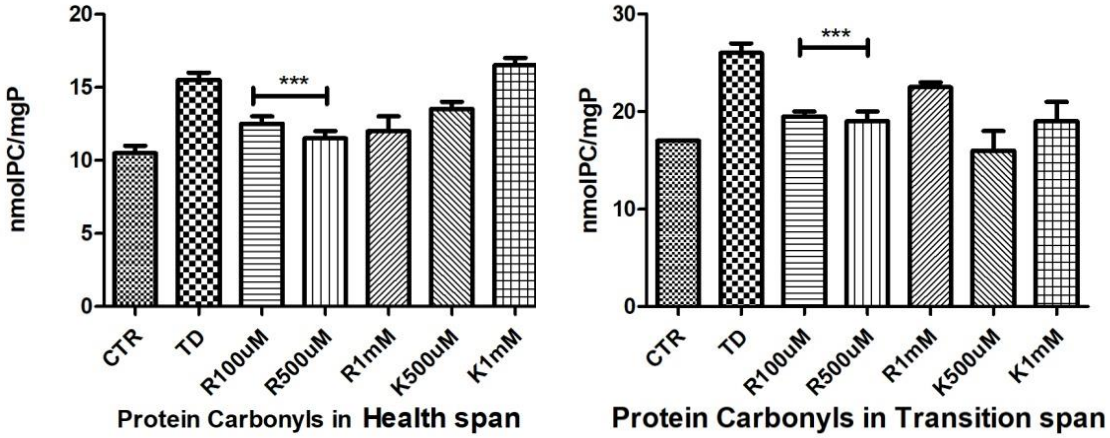
(a) (b)

Figure.4: Measurement of ROS levels in (a) Health stage and (b) Transition stage. PQ enhances ROS significantly up to 34% during health stage (4a) and 5 fold increase in transition phase (4b) of adult *Drosophila* model. On co-treatment with K the enhanced levels of ROS were significantly diminished during both the adult life stages of fly. It was found that 34% enhanced level of ROS in PQ treated sample were brought down by 20% and 60% in $R_{500\mu M}$ and R_{1mM} in health stage respectively (fig.4a); and PQ induced 5 fold ROS increase was brought down by 60% and 35% in $R_{500\mu M}$ and R_{1mM} in transition stage respectively (fig.4b) suggesting K acting as an antioxidant in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).



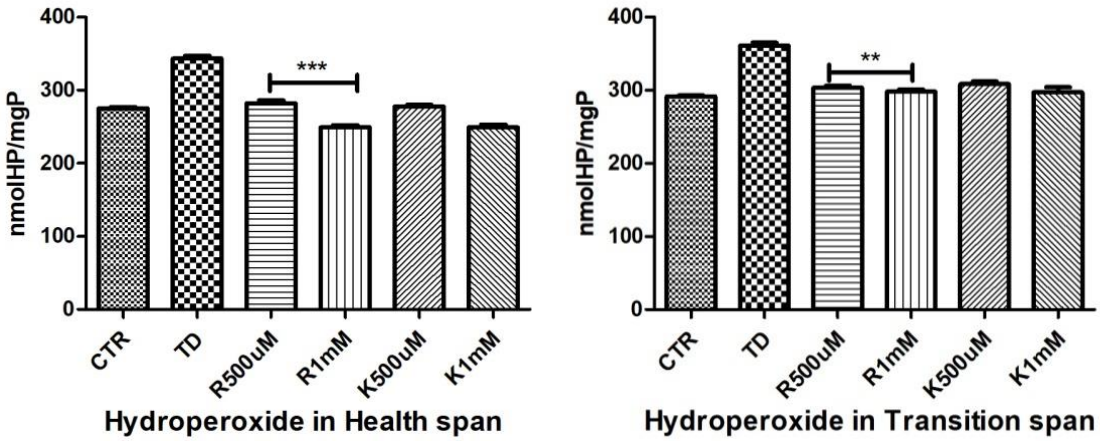
(a) (b)

Figure.5: Measurement of MDA levels in (a) Health stage and (b) Transition stage. PQ induces lipid peroxidation as indicated by measurement of Malondialdehyde (MDA) an end product of LP. It was found that there was up to 60% increase in MDA levels during health stage (5a) and 24% increase in transition phase (5b) of adult *Drosophila* model. On co-treatment with K the enhanced levels of MDA were significantly diminished during both the adult life stages of fly. It was found that 60% enhanced level of MDA in PQ treated sample were brought down by 23% and 31% in $R_{500\mu M}$ and R_{1mM} in health stage respectively (fig.5a); and PQ induced 24% PC increase was brought down by 30% in both the $R_{500\mu M}$ and R_{1mM} concentrations in transition stage (fig.5b) suggesting the effective modulatory action of K against PQ induced OS in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).



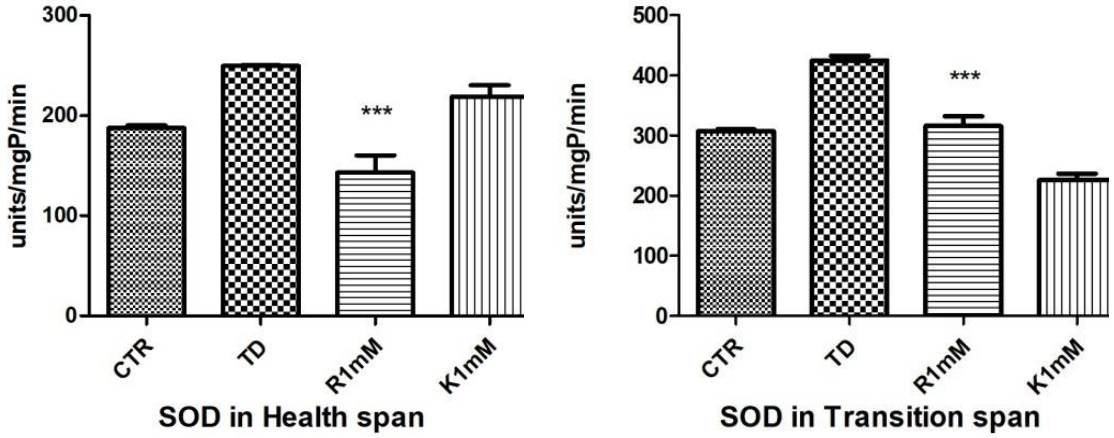
(a) (b)

Figure.6: Measure of Protein Carbonyl levels in (a) Health stage and (b) Transition stage. PQ enhances protein carbonylation up to 44% during health stage (6a) and 27% increase in transition phase (6b) of adult *Drosophila* model. On co-treatment with K the enhanced levels of PC were significantly diminished during both the adult life stages of fly. It was found that 44% enhanced level of PC in PQ treated sample were brought down by 18%, 28% and 20% in $R_{100\mu M}$, $R_{500\mu M}$ and R_{1mM} in health stage respectively (fig.6a); and PQ induced 27% PC increase was brought down by 25%, 24% and 20% in $R_{100\mu M}$, $R_{500\mu M}$ and R_{1mM} in transition stage respectively (fig.6b) suggesting the modulatory effect of K against PQ induced OS in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).



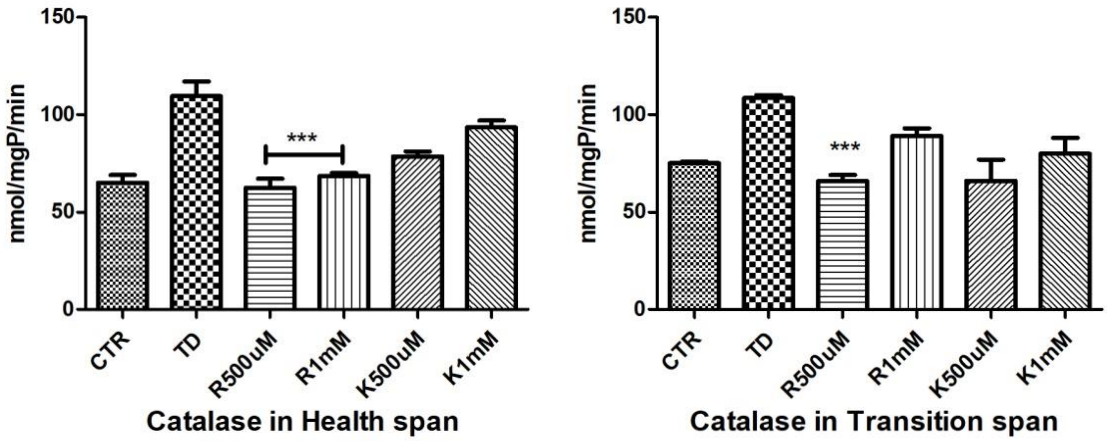
(a) (b)

Figure.7: Measurement of HP levels in (a) Health stage and (b) Transition stage. Treatment of PQ enhanced the hyderperoxidation by 20% and 13% during health stage and transition phase (fig.7) of adult *Drosophila* model. On co-treatment with K the enhanced levels of HP were significantly diminished during both the adult life stages of fly. It was found that 20% increased levels of HP in PQ treated sample were brought down by 20% and 30% in $R_{500\mu M}$ and R_{1mM} in health stage respectively (fig.7a); and PQ induced 13% HP increase was brought down by 15% and 18% in both the $R_{500\mu M}$ and R_{1mM} concentrations in transition stage (fig.7b) suggesting that K has modulatory effect against PQ induced OS in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).



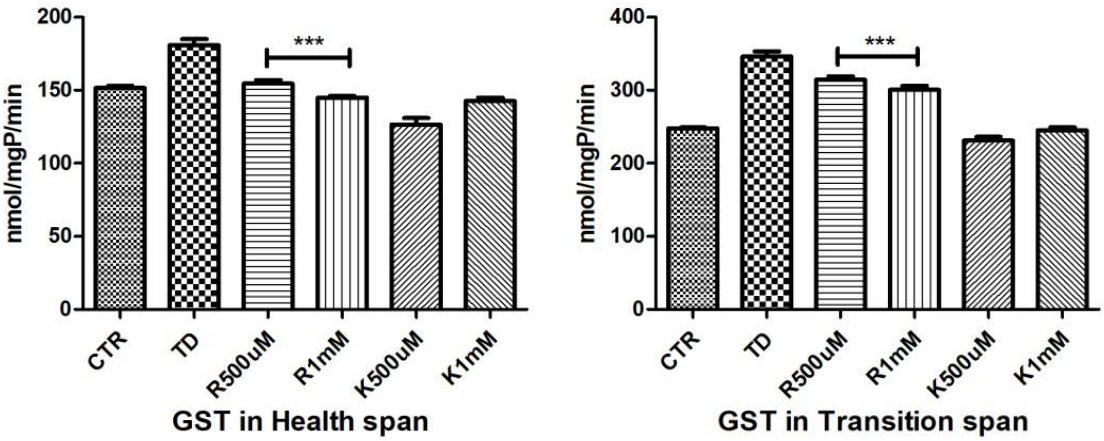
(a) (b)

Figure.8: Measurement of SOD activity in (a) Health stage and (b) Transition stage. PQ increases the SOD enzyme activity by 20% and 27% during health stage and transition phase (Fig.8) of adult *Drosophila* model. On co-treatment with K the increased activity of SOD was significantly diminished in both the adult life stages of fly. It was found that the increased levels of SOD in PQ treated sample were brought down by 40% and 20% with R_{1mM} concentration during both the health stage and transition stage respectively (fig.8a,b) suggesting that K has a potent anti-oxidative effect against PQ induced OS in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).



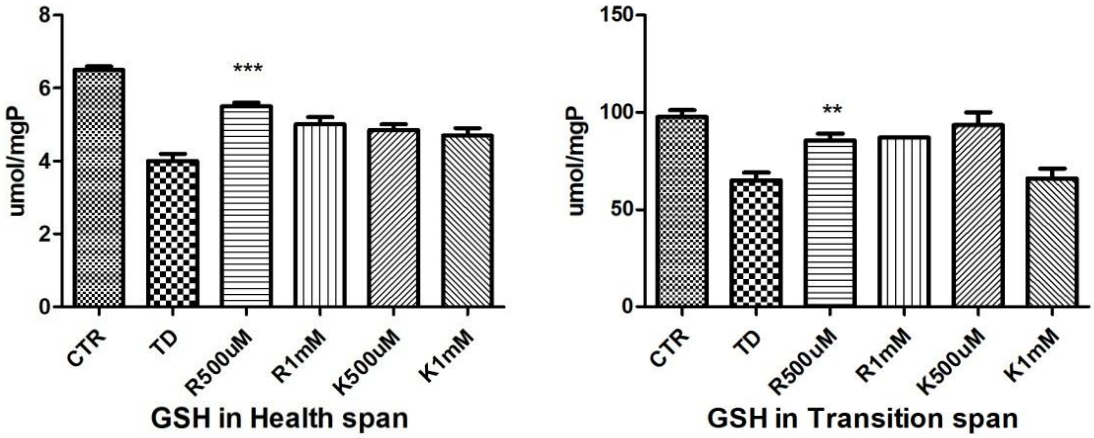
(a) (b)

Figure.9: Measurement of Catalase activity in (a) Health stage and (b) Transition stage. PQ increases the CAT enzyme activity by 43% and 30% during health stage and transition phase (fig.9a, b) of adult *Drosophila* model. On co-treatment with K the increased activity of CAT was significantly diminished during both the adult life stages of fly. It was found that 43% increased levels of CAT in PQ treated sample were brought down by 44% and 35% in $R_{500\mu M}$ and R_{1mM} in health stage respectively (fig.9a); and PQ induced 30% CAT activity increase was brought down by 40% and 15% in both the $R_{500\mu M}$ and R_{1mM} concentrations in transition stage (fig.9b) suggesting that K has a potent anti-oxidative effect against PQ induced OS in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).



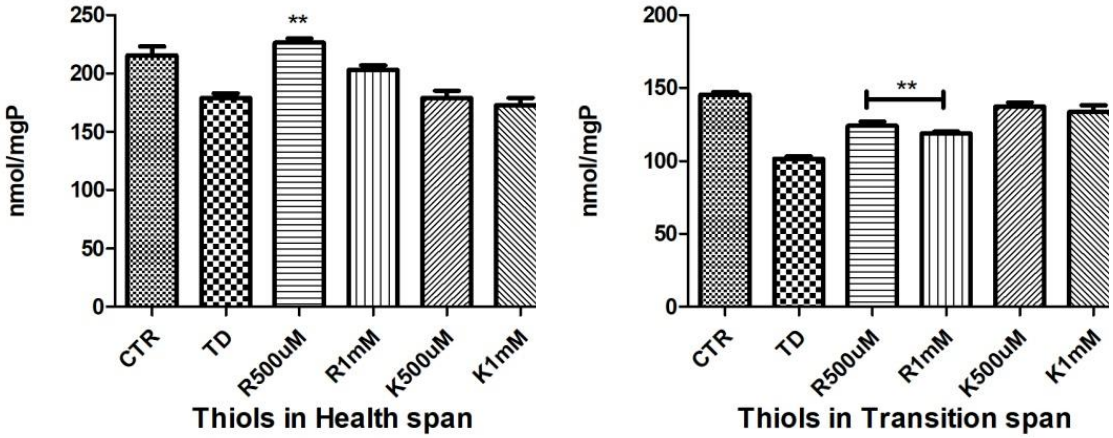
(a) (b)

Figure.10: Measurement of GST level in (a) Health stage and (b) Transition stage. PQ enhances the GST enzyme activity by 20% and 30% during health stage and transition phase (Fig.10a,b) of adult *Drosophila* model. On co-treatment with K the activity of GST was significantly decreased in both the adult life stages of fly. It was found that 20% increased levels of GST in PQ treated sample were brought down by 18% and 22% in $R_{500\mu M}$ and R_{1mM} concentrations in health stage respectively (fig.10a); and PQ induced 30% increase GST activity was brought down by 15% and 19% in both the $R_{500\mu M}$ and R_{1mM} concentrations in transition stage respectively (fig.10b) suggesting that K has modulatory effect against PQ induced OS in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).



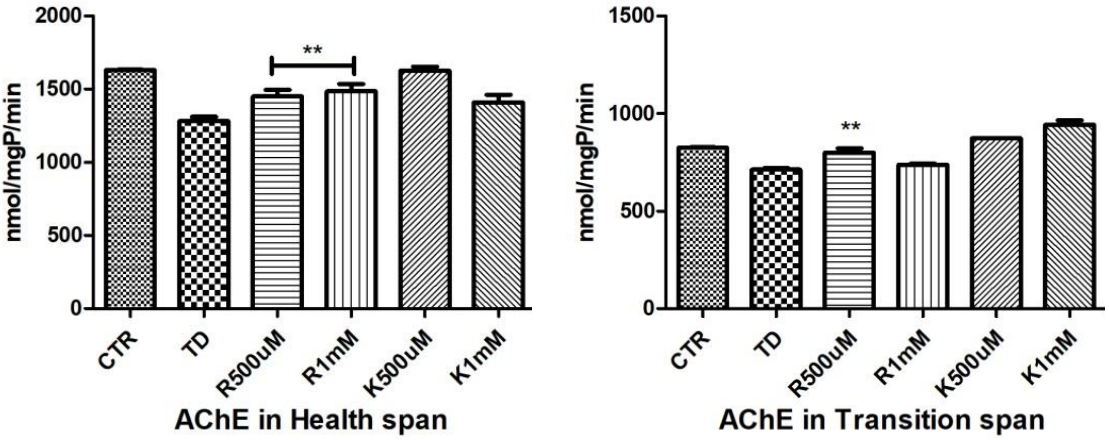
(a) (b)

Figure.11: Measurement of GSH levels in (a) Health stage and (b) Transition stage. PQ treatment inhibits the GSH level by 30% during health stage and 27% during transition phase (Fig.11a, b) in the adult *Drosophila* model. On co-treatment with K the activity of GSH were significantly increased in both the adult life stages of fly. It was found that 30% GSH activity inhibition upon PQ exposure were rescued by 20% and 15% in $R_{500\mu M}$ and $R1_{mM}$ concentrationsin health stage respectively (fig.11a); and 27% GSH activity inhibition was rescued by 23% and 27% in both the $R_{500\mu M}$ and R_{1mM} concentrations in transition stage respectively (fig.11b) suggesting the efficacy of K as a modulant against the PQ induced OS in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).



(a) (b)

Figure.12: Measurement of total Thiols level in (a) Health stage and (b) Transition stage. PQ treatment also inhibits thiols level by 18% during health stage and 27% during transition phase (Fig.12a, b) in the adult *Drosophila* model. On co-treatment with K the activity of thiols was significantly increased in both the adult life stages of fly. It was found that K co-exposure could rescue the inhibited thiols activity by 14% and 12% in $R_{500\mu M}$ and R_{1mM} concentrationsin health stage respectively (fig.12a); and rescued by 17% and 14% in both the $R_{500\mu M}$ and R_{1mM} concentrations in transition stage respectively (fig.12b) further suggesting the efficacy of K as a modulant against the PQ induced OS in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).



(a) (b)

Figure.13: Measurement of AChE activity in (a) Health stage and (b) Transition stage. PQ treatment inhibits AChE level by 20% during health stage and 16% during transition phase (Fig.13a, b) in the adult *Drosophila* model. On co-treatment with K the AChE level was significantly increased in both the adult life stages of fly. It was found that K co-exposure could rescue the inhibited AChE activity by 13% and 16% in $R_{500\mu M}$ and R_{1mM} concentrations in health stage respectively (fig.13a); and rescued by 15% and 8% in both the $R_{500\mu M}$ and R_{1mM} concentrations in transition stage respectively (fig.13b) suggesting the efficacy of K as a modulant against the impairment of neurotransmitter enzyme activity induced by PQ in fly model of PD. (CTR=Control, TD=Treated with PQ, R (Rescue) =PQ+K, K= Curcumin *per se*).

12. Discussion

Evidence suggesting the involvement of OS mechanism arises from both in vitro and in vivo models relevant to PD. It is considered to be the common underlying mechanism that leads to cellular dysfunction and demise (Kurtishi *et al*, 2018). Studies on post-mortem brains from PD patients have implicated the role of oxidative damage in the pathogenesis of PD (Zeevalk *et al*, 2008; Nakabeppu *et al*, 2007; Bosco *et al*, 2006). This illustrates the role of OS in DA degeneration is linked to PD.

12.1. Susceptibility to Oxidative Stress with Age

Protein, lipids and nucleic acids are biological macromolecules which are oxidatively altered due to ROS, resulting in genetic mutations and cellular senescence (Luceri *et al*, 2018). In complications and pathologies associated with aging, increasing evidence for causal role of cell senescence have been experimentally shown (Correia-Melo *et al*, 2015). Due to stress during aging there is increase in cell senescent. This phenomenon is controlled by a number of counting mechanisms including telomeres shortening, gradual increase in damage of DNA, deviation in normal oncogenes behavior, change in metabolic action and increased ROS generation (Kuilman *et al*, 2010). Such processes lead to impairment of cellular function and thus produce and accumulate ROS which in turn disturb the homeostasis, a vital factor for safeguarding the senescent phenotype. Apart from injuring DNA and damage mitochondrial activity, OS trigger the activity of p53, a gene coding for cell cycle regulator protein, which in turn induces prooxidant genes. Disturbances in redox homeostasis by generating ROS lead to deterioration of cell and impair its function and as a response to increased ROS levels, mechanisms for cellular protection are excessively activated including up-regulated expression of antioxidant

enzymes (Ren and Zhang, 2017; Zarse *et al*, 2012) suggesting OS is intricately linked to aging and neurodegeneration.

12.2. Insights from Quantification of Non Enzymatic Markers

In the present study in *Drosophila* model of sporadic PD condition the levels of non enzymatic OS markers such as ROS, PC, MDA, HP were increased significantly during both the phases (health and transition) of adult life. Sequestration of OS is evident from the significant decreased levels of these markers (fig.4-7).

While there was about 35% increase in ROS level in health stage of PQ treated flies when compared to control, in transition stage the level was increased by 5 fold suggesting stress vulnerability of the fly with age.

Increased in ROS levels and MDA an intermediate in the lipid peroxidation process upon PQ exposure has also been shown by other laboratories (Soares *et al*, 2017). Lipid peroxidation is a well-known example of oxidative damage in cell membranes, lipoproteins, and other lipid-containing structures. Study on postmortem PD brain have shown Lipid peroxidation was increased in Parkinsonian nigra compared with other brain regions and control tissue (Dexter *et al*, 1994). MDA levels are also found to be 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). PQ induced increase in the MDA level was effectively reduced with K co-treatment in both the age groups (Fig.5), as also shown by other reports of K ability to attenuate elevated MDA levels (Wang *et al*, 2017).

There was significant protein damage in flies as evident by the high level of PC which indicate the increased rate of protein oxidation (fig.6). Protein carbonylation is an irreversible oxidative damage which leads to loss of protein function and it is considered

as a widespread indicator of severe oxidative damage and disease-derived protein dysfunction. Elevated levels of PC associated with PD have been reported in postmortem PD brains tissues (Alam *et al*, 1997). The efficacy of K to decrease the PC levels has been reported (Kolodziejczyk *et al*, 2011; Dekhar *et al*, 2010).

HP groups are powerful oxidants that can induce secondary damage through inactivation of thiol-dependent enzymes essential to cell functions. Study on α-synuclein implicated in PD catalyzing the formation of hydrogen peroxide has been reported (Turnbull *et al*, 2001). Protein hydroperoxides results in modification of the structure and properties of amino acid residues. Lipid hydroperoxides are also prominent non-radical intermediates of lipid peroxidation that can often provide important mechanistic information. Increased levels of lipid hydroperoxides in Parkinsonian SN has been demonstrated (Dexter *et al*, 1994).

12.3. Estimation of Antioxidant Enzymes Activity

In *Drosophila,* SOD/CAT system is one of the intracellular protective mechanisms against ROS induced damage. Studies have demonstrated that in response to the OS generated by PQ, levels of SOD, CAT and GST are upregulated. K treatment results in the significant reduction of these antioxidant enzymes which suggest lowered level of OS in the brain of K co-treated flies. Post mortem studies have shown an increased SOD activity in PD brains (de Farias *et al*, 2016) where SOD activity was significantly associated with late PD stage. The ability of CAT coated nanoparticles to reduce protein oxidation, DNA damage, mitochondrial, restored neuronal morphology has also been shown (Singhal *et al*, 2013). 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 in adult mice in vivo has also been reported (Peng *et*

al, 2005). There was significant increase in the activity of antioxidant enzymes such as SOD (fig.8), Catalase (fig.9) and GST (fig.10). These findings substantiate well with the increased in level of ROS and other stress marker measured. K therapeutic treatment could attenuate the altered activities of these biomarkers measured in both the age groups.

The GST activity was also elevated under PD condition and K treatment markedly decreased its activity in the present study (fig.10). Antioxidant role of GST in protection against oxidant toxicity and regulation of stress-mediated apoptosis has been carefully reviewed (Sharma *et al*, 2004). GSTs are a functionally diverse family of soluble enzymes of detoxification that use reduced GSH in conjugation and reduction reactions. Depletion in GSH content and an increase in GST activity have been reported in the brains of human PD patients and also in other experimental models of PD (Johnson *et al*, 2012; Zhu *et al*, 2007).

Reduced GSH level was decreased under PD conditions further supporting the evidence of OS generation (fig.11). Earlier investigation have also suggested the ROS to be the major factor contributing to DAergic neuronal loss in the PD brain, as a consequence of DA metabolism, low GSH, and high levels of iron and calcium in the SN*pc* (Jenner and Olanow, 2006). GSH confers the neuronal cells with multiple defenses not only to sequester but also against different types of toxic products and by products of cellular mechanism. Reduction in GSH levels as compared to control subjects in SN in PD patients have been reported (Sian *et al*, 1994). Depletion in GSH level may impair mitochondrial function via inactivation of complex I and increase in ROS production and the relation between decreased GSH function and severity of PD has been reported (Smeyne and Smeyne, 2013). In the present study K therapeutic intervention markedly rescued the GSH level in both the age groups (fig.11a,b). Efficacy of K to elevate the reduced GSH activity

against Acrylamide induced toxicity has also been reported (Prasad and Muralidhara, 2014).

Measurement of total Thiols showed reduced activity under PD condition and K could marginally increase the total thiol levels (fig.12). Thiol groups are highly reactive and susceptible to oxidation that may cause significant loss of biological activity. A critical function of cell-based thiol redox buffering systems is to protect thiol groups from oxidation and to repair those that may have become oxidized as a result of normal or aberrant cellular metabolism. Studies have shown that thiols levels are reduced under disease conditions (Isik *et al*, 2017).

12.4. Acteylcholine esterase as Neurotoxicity Marker

Neurotransmitter enzyme AChE activity was also estimated in the present study. It was found that the enzyme activity was inhibited under PD condition in both the age groups (fig.13a,b). Such action will impair the neurotransmission process since AChE is involved in maintaining the free Acetylcholine in the synaptic region. Earlier studies have shown reduced AChE activity in several brain disorder including neurodegenerative diseases (Mendez *et al*, 2011; Shinotoh *et al*, 2003). The alteration in activities of these enzymes is indirectly responsible for neuronal loss and probably plays a role in pathogenesis of PD. AChE inhibition has been used as a biomarker for the influence of organophosphate and carbamate pesticides in invertebrate and vertebrate model. PQ exposure lead to marginal inhibition of AChE activity and inhibited levels were rescued by K co-treatment in both the age groups (fig.13) reaffirming the efficacy of K to attenuate the PQ induced toxicity in both the age groups. It has been proposed that exposure to low levels of pesticides leads to several neurological and neurobehavioral changes that cannot be accounted for AChE

inhibition only (Salvi *et al*, 2003; Stephens *et al*, 1995). The particular enzyme associated with the different sub-cellular fractions can exert different effects.

The present results show that K can sequester the enhanced levels of OS not only during early phase (health stage) but importantly, also during late phase (transition stage) of adult life stage in fly PD model as seen from the above results (fig.4-13). Further it has been observed that at higher concentration treatment, K *per se* can act as potential pro-oxidant, suggesting the necessity to properly screen the compounds before prescribing them as a therapeutic agent.

13. Oxidative Stress markers-based studies alone may not be sufficient to decipher the Neuroprotective efficacy of Nutraceuticals

Most of the laboratories analyze the neuroprotective action of nutraceuticals using OS markers as a gold standard in their studies considering the OS as a factor responsible for neurodegenerative diseases such as PD (Niveditha *et al*, 2017; Ravi *et al*, 2017; Jhonsa *et al*, 2016). Employing the same approach, I found that K can mitigate the OS induced by PQ exposure in health stage flies as evident from the above results. This finding confirms with several other studies of K neuroprotection employing health stage in adult *Drosophila* PD model (Nguyen *et al*, 2018). Also K could reduce the OS even in the transition stage flies as seen from the above study on stress markers, antioxidant and neurotransmitter enzyme activities which is an important and interesting phenomenon. It is shown that dietary feeding of K and other phytochemicals modulate PQ induced alteration in the expression levels of several genes that are associated with anti-oxidant and anti-aging effect in 2-3 days old flies (Park *et al*, 2012). K also is reported to improve PQ induce motor deficit and replenish the decreased DA level in adult health stage (Abbaiou *et al*, 2017; Phom *et al*, 2014). However, K is unable to rescue either the motor deficits or

diminished DA level in adult transition stage of *Drosophila* exposed to PQ (Phom *et al*, 2014). But contrary to this finding, K could reduce the OS levels in both the fly age groups as seen from the above results, clearly suggesting that sequestration of boosted OS alone is not enough to prevent the DAergic neurodegeneration in PD. Therefore apart from OS, there is the synergic effect of other pathways that could be responsible for the DAergic neurodegeneration in PD. Several laboratories have shown the effective role of various therapeutic compounds that modulates the altered enzymatic functions arising out of OS in model organisms. They suggested for therapeutic approach using such active compounds in NDD such as PD. However, according to the present findings it is clear that accessing OS level alone is not sufficient enough to understand the neuroprotective efficacy of therapeutic molecule in late onset NDD such as PD. Studies in addition have also shown that K increases longevity when administered in the developmental of health stage but exerts negative result when administered in other life stage, suggesting that target molecules of K are present in the health stage (Soh *et al*, 2013) but not in transition and senescence stage.

14. Conclusion

Present study reveals that OS may be a necessary factor, but alone it may not be sufficient enough for DA degeneration which is characteristic pathological feature of PD. Therefore, studies in animal models through which neuroprotective efficacy of multiple natural products being assessed based on OS markers alone may not be good enough to recommend them as neuroprotective agents. Hence, further it is necessary to look into the molecular targets along with the oxidative stress markers.

SUMMARY

Parkinson's disease is the second most common neurodegenerative disease and it is characterized by degeneration of DAergic neurons in the SN of mid brain that lead to impairments of motor functions (Cacabelos *et al*, 2017). Progressive loss of DAergic neurons and intraneuronal protein inclusions, called LB in the mid brain are characteristic of PD (Olanow and Tatton, 1999). The neuronal loss in this region gives rise to motor symptoms like bradykinesia, rest tremor, postural instability, and gait impairment whereas non-motor symptoms like impaired olfaction, constipation, depression, increased daytime sleep, rapid eye movement sleep disorder, and behavioral deficits are commonly observed (Saleem *et al*, 2013). During the time of diagnosis of the symptoms, about 50 to 60% of the DAergic neurons have already been degenerated. Over the time, individuals suffering from PD are unable to perform even the basic function and they become completely dependent on care and support.

Although the disease was first described in the 18th century, there is no therapy available that will cure the disease and medication is limited only to alleviate the progressing symptoms and with more of side effects. Presently, the treatment strategies for PD include Deep Brain Stimulation (DBS) and Levo-dopa (L-dopa) therapy. These therapies are considered as the most effective, but placement of lead in DBS often result in infection and intracranial hemorrhage (Zrinzo *et al*, 2012) and long-term treatment of L-dopa leads to abnormal involuntary movement known as L-dopa induced dyskinesia (Fahn *et al*, 2000). Developing a therapeutic strategy for NDD such as PD remains a challenge till date.

Here lies the importance of model organism based development of therapeutic strategies and their further validation in humans.

Over the year's researchers have developed several animal models including *Drosophila* while attempting to understand the PD progression and thereby find means to its therapeutic approach. A suitable model for sporadic PD should show histopathologically characterizable progressive loss of DA neurons together with other neurons and significant reduction in DA level. Moreover, the onset of the disease should be in a stage of adulthood. The model animal should also manifest disease in such a way that it would mimic the PD affected human motor symptom such as bradykinesia, rigidity, postural instability and resting tremor, with motor features being responsive to L-DOPA (L-dopa, the precursor of DA) or any anti PD drug therapy. Low cost of maintenance, shorter life cycle and defined neuropathological profile is making *Drosophila* amongst the emerging and more interesting model of PD, though nonhuman primate and mouse has been the traditional model of PD (Pienaar, 2010).

Environmental toxins are implicated to be the causative agents of idiopathic PD. Several case studies have reported that the subjects having exposure to pesticides, herbicides showed symptoms similar to Parkinsonism. Laboratory exposure of model organisms to environmental toxins like PQ is therefore productively employed to study the disease progressions that would help in understanding the mechanisms involved. Animal models such as *Drosophila*, allow researchers to replicate human diseases symptoms or abnormal behavior, enabling to understand the many biological functions without ethical concerns inherent of human studies. Complex human behavior such as aggression, mating, circadian rhythms, sleep, learning and memory are also observed in animals. Therefore, by studying these processes and behaviors in animal models, one can understand the basic biology underlying them and thus applying this information to figure out how diseases progression take place and find the remedial measures.

Several researchers have suggested the neuroprotective efficacy of natural products showing modulation of biochemical markers, antioxidant enzymes and phenotype associated rescue upon exposure to neurotoxic agents in different disease models. Natural product K is largely used in food as spices, coloring agent, and traditional medicines in India, China, Southeast Asia (Aggarwal *et al*, 2007) and properties of K performing neuroprotective effect, anti-oxidant, anti-inflammatory and anti-cancer are well known. The most central biological role of K associated to neuroprotection has been shown to be its anti-oxidant effect which protects the SN neurons and increases striatal DA count and chelates Fe^{2+} in the 6-OHDA (6-hydroxyDA) rat models of PD (Mythri *et al*, 2012).

Using *Drosophila*, it is reported that K have gender and genotype specific life span extension and sequester OS mediated free radicals, enhance locomotor ability and show chemo preventive property, suggesting its potential use in treatment applicability in higher organisms (Lee *et al*, 2010). K decreases death of SH-SY5Y human neuroblastoma cells induced by rotenone; improve characteristic symptoms associated with PD in *Drosophila* via reduction in intracellular and mitochondrial ROS levels and acting against the caspase-3/caspase-9 activity (Liu *et al*, 2013). Transgenic fly expressing human α-synuclein was exposed to different concentrations of K and found considerable delay in the loss of activity pattern, decrease in the level of OS and apoptosis, and extended life span (Siddique *et al*, 2014) suggesting potential role of K in neuroprotection.

For understanding the pathology of late onset neurodegenerative diseases like PD, it is important to employ organisms at the adult phases where the disease sets in, such as the transition stage of *Drosophila* PD model. Because different stages of the life stage have different patterns of gene expression and the cellular, physiological and molecular phenomenon at old stage may be completely different from young stage. There exists significant change of about 23% in genome-wide transcript profiles with age in *Drosophila*

(Pletcher *et al*, 2002) suggesting that targets of genotropic compounds under study may well not be present in all life stages. Genotropic drugs would be effective only during those life cycle stages when their target molecules are available (Soh *et al*, 2013). Therefore, it is possible that targets of genotropic compounds such as K may not be present in all life stages, which is an interesting paradigm. However, no reports are available regarding the efficacy of K in PD models during later phases of adult life. Therefore, it is necessary to understand the neuroprotective efficacy of compounds at transition phase because this is the period during which late-onset neurodegenerative diseases such as idiopathic PD sets in.

Oxidative modifications of enzymes and structural proteins play a significant role in the etiology and progression of several human neurodegenerative diseases. Accumulation of free radicals and subsequent neurodegeneration in specific brain regions have been proposed as the underlying factors in neurodegenerative diseases such as Alzheimer's and PD (Halliwell, 2006). Studies on post-mortem brains from PD patients have implicated the role of oxidative damage in the pathogenesis of PD (Yuan *et al*, 2016; Zeevalk *et al*, 2008; Bosco *et al*, 2006).

K rescues the mobility defects induced by PQ during health stages of *Drosophila* but fails to rescue the mobility defects induced by PQ during transition phase of *Drosophila*. This finding is well substantiated by results that K could replenish decreased DA levels caused by PQ exposure in *Drosophila* during health stage but not in transition phase (Phom *et al*, 2014). But K effectively sequester the elevated level of OS and attenuated levels of antioxidant enzyme activities and neurotransmitter enzyme in PD condition during both health span and transition phases of adult life span *Drosophila* suggesting that for a therapeutic compound such as K, the OS pathway may be one of the factors through which

it act to attenuate the disease aggravated abnormal cellular and neural pathways, including one responsible for DAergic neuronal degeneration.

The study reveals that OS may be a necessary factor, but alone it may not be sufficient enough for DA degeneration which is characteristic pathological feature of PD. Therefore, studies in animal models through which neuroprotective efficacy of multiple natural products are being assessed based on OS markers alone may not be good enough to recommend them as neuroprotective agents. Hence, further it is necessary to look into the molecular targets along with the oxidative stress markers.

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CONFERENCE/SEMINAR/WORKSHOP ATTENDED

- Presented a paper on 'Understanding Neurodegeneration and Rescuing Pathology Associated with Parkinson's Disease in *Drosophila* Model' during Workshop on Introduction to Basic and Advanced Biomedical Approaches for Enhancing Quality Of Life in Aging Societies. Advanced Industrial Science and Technology Biomedical Research Institute (AIST), Tsukuba, Japan. 14-21st October, 2018.

- Presented a paper titled, 'Curcumin Improves Paraquat Induced Mobility Defects During Health Span and Modulates Brain Nitrite Levels in *Drosophila* Model of Parkinson's Disease' at National Seminar On Climate Change and Sustainable Development with Special Focus on North East India. Nagaland University, Nagaland, India. 17-18th May, 2017.

- Presented a poster titled, 'Curcumin's Neuroprotective Efficacy in *Drosophila* Model of Idiopahic Parkinson's Disease is Phase Specific: Implications of its Therapeutic Effectiveness' at International Conference on Parkinson's Disease and Movement Disorder. Frankfurt, Germany. 11 -13th August, 2015.

- Presented a poster titled, 'Curcumin Improves Paraquat Induced Mobility Defects During Health Span and Transition Phase in *Drosophila* Model of Parkinson's Disease' at National Seminar on Metabolomics: A New Frontier in Natural Products Research. NEHU, Meghalaya, India. 23-24th May, 2014.

- Abstract accepted titled, 'Understanding the Neuroprotective Properties of Curcumin During Health and Transition Stage: Insights from *Drosophila* Model of Parkinson's Disease' for National Conference on Contemporary Excitement in New Biology. Department of Zoology, Nagaland University, India. 30-31st October, 2018.

HONORS AND AWARDS

- Awarded with Department of Biotechnology (DBT), New Delhi - Junior Research Fellowship and Senior Research Fellowship, 2012-2015.

- Awarded with Indian Council of Medical Research (ICMR), New Delhi - Young Scientist International Travel Grant, 2015.

- Awarded with Indian Council of Medical Research (ICMR), New Delhi - Senior Research Fellowship (not availed), 2018.

- Selected for Japan-Asia Youth Exchange Program in Science (Sakura Exchange Program in Science), 2018, through the Department of Biotechnology (DBT), New Delhi.

LIST OF PUBLICATIONS

- Phom L, Achumi B, Alone D, Muralidhara, and Yenisetti SC (2014). Curcumin's Neuroprotective Efficacy in *Drosophila* Model of Idiopathic Parkinson's Disease is Phase Specific: Implication of its Therapeutic Effectiveness. *Rejuvenation Research*. 17(6); 481-9.

- Modi P, Ayajuddin M, Phom L, Koza Z, Das A, Chaurasia R, Samadder S, Achumi B, Muralidhara, Pukhrambam RS, Yenisetti SC (2016). Understanding Pathophysiology of Sporadic Parkinson's Disease in *Drosophila* Model: Potential Opportunities and Notable Limitations. In Challenges in Parinson's Disease. Ed. Jolanta Dorszewska. *Intech Open*. 11; 217-44.

- Pukhrambam PR, Thepa A, Jamir N, Phom L, Yenisetti SC (2017). Parkinson's Disease and Therapeutic Strategies. *Int J of Neurology and Neurosurgery*. 9(2); 172-86.

- Pukhrambam PR, Koza Z, Phom L, Lal P, Yenisetti SC (2017). Parkinson's Disease - An Overview. *Fazl Ali College Journal.* 7;1-17.

- Ayajuddin M, Das A, Phom L, Modi P, Chaurasia R, Koza Z, Thepa A, Jamir N, Singh PR, Longkumer S, Lal P, Yenisetti SC (2018). Parkinson's Disease: Insights from *Drosophila* Model. In *Drosophila melanogaster*: Model for Recent Advances in Genetics and Therapeutics. Ed. Farzana Khan Perveen. *Intech Open*. 8;157-92.

- Sub-Editor. Dopamine - Health and Disease (In Press). *Intech Open*. ISBN 978-953-51-6729-7.

PUBLICATIONS THROUGH COLLABORATION WITH OTHER LAB MEMBERS

- Achumi B, Phom L, Zevelou, Ayajuddin M, Lal P, Yenisetti SC (2014). Eco-geopraphic Pattern of Genus *Drosophila* (Insecta, Diptera: Drosophilidae) in Nagaland State, India. *Nagaland University Research Journal*. 7; 320-37.

- Achumi B, Ayajuddin M, Phom L, Hegde SN, Lal P, Singh OP, Yenisetti SC (2016). Drosophilid (Insecta, Diptera: Drosophilidae) Biodiversity of North-East India. *Bioprospecting of Indigenous Bioresources of North East India*. 14; 231-51.