Exploring the effects of *Mucuna pruriens* seed powder on Parkinson's disease management: Insights from *Drosophila* mutant models

A THESIS SUBMITTED TO

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

FOR THE PARTIAL FULFILLMENT OF THE AWARD OF THE DEGREE OF
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BY

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UNDER THE GUIDANCE OF

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THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY
Private University Established in Karnataka by ACT 35 of 2013
BENGALURU - 560064

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DECLARATION BY THE CANDIDATE

I declare that this thesis "**Exploring the effects of *Mucuna pruriens* seed powder on Parkinson's disease management: Insights from *Drosophila* mutant models**" submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of Dr. Megha (and co-supervision of Dr. Aman Aggarwal and Dr. Prasan Shankar). I confirm that no part of the work reported herein has been submitted for a degree or examination at any other university. References, funding and material obtained from other sources have been duly acknowledged, and no part of this dissertation has been plagiarised.

Place: Bengaluru **Signature of the Candidate**

Date: 15-06-2023

Name of candidate: Vikrant Chandel

Reg. No.: 2021MSCAB15

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CERTIFICATE FROM THESIS SUPERVISOR/S

This is to certify that the work incorporated in this thesis **"Exploring the effects of *Mucuna pruriens* seed powder on Parkinson's disease management: Insights from *Drosophila* mutant models"** submitted by Vikrant Chandel was carried out under my/our supervision. No part of this thesis has been submitted for a degree or examination at any other university. References, help, and material obtained from other sources have been duly acknowledged. I confirm the originality of the work and that there is no plagiarism in any part of the thesis.

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I also express my sincere thanks to TDU for providing me with an opportunity to perform this work.

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Lastly, I would like to thank *Drosophila* for your existence, and R.I.P. to the flies whom I have killed on this journey.

SUMMARY

Parkinson's disease (PD) is a neurodegenerative disorder that affects millions of people worldwide. Plant-based therapeutics form one arm of treatment strategies for PD. The current understanding of their mechanism of action rests on the presence of therapeutically significant levels of Dopamine precursors. However, plant formulations are a cocktail of several chemicals, which can potentially address not just proximal neurological symptoms, but also distal tissues/ organs that regulate brain function. Hence, the effectiveness of *Mucuna pruriens* (MP) seed powder, also a clinically and commercially available therapeutic agent, was tested. PD mutants of *Drosophila melanogaster* (vinegar/ fruit fly) were used as a model animal to observe motor and non-motor symptoms of Parkinson's disease (PD). The L-Dopa levels in a commercially available MP powder were measured using high-performance thin-layer chromatography (HPTLC) and the dosage of feeding flies *ad libitum* was standardized. Motor symptoms were assessed using high-throughput, high-resolution FlyVRL (Fly vertically rotating arena for locomotion). Upon intervention, there was no improvement in locomotion behavior. Interestingly, there was a significant increase in the lifespan of the *park25/+* and moderately in *park13/+* mutants.

Non-motor symptoms were assessed by measuring TAG (triacylglyceride) and protein concentrations, as well as the gene expression of a transcription factor *relish* involved in the inflammatory response in flies. There is a significant increase in the TAG levels in mutants when fed on MP seed powder, in terms of gene expression of *relish* after feeding MP seed powder it is decreasing in *park25/+* males.

The objective of the study was to provide preclinical evidence regarding the efficiency of MP in managing PD symptoms. Overall, this study contributes to the growing body of research focused on developing effective therapeutical strategies for PD and sheds light on the benefits of traditional medicine, such as MP, in managing the symptoms of this neurodegenerative disease.

PERSONAL REFLECTION

This dissertation has had a profound impact on shaping my personal growth and development as an individual. It has instilled in me the importance of planning and patience throughout any endeavor I undertake. Science, as I have come to understand, requires meticulous planning, keen observation, and thorough analysis, all of which are not only essential in the pursuit of knowledge but also in leading a meaningful and fulfilling life.

Through this dissertation, I have gained a deeper understanding of the importance of time management. I have learned how to effectively utilize the stipulated time I have, enabling me to be highly productive in various aspects of my life. The experience has taught me to prioritize tasks, set goals, and allocate time wisely, resulting in increased efficiency and achievement of desired outcomes.

Moreover, this dissertation has fostered in me a strong passion for science. Witnessing the planning, execution, and analysis involved in research has ignited a curiosity within me to delve deeper into scientific exploration. Engaging in brainstorming sessions during lab meetings and acquiring new skills have been beneficial in expanding my knowledge. This not only enhanced my critical thinking but also provided a platform for intellectual exchange and collective problem-solving, fostering a sense of camaraderie and teamwork.

The lessons I have learned, along with my newfound skills and passion for science, have paved the way for my future endeavors. I am now more driven and motivated to pursue a career that allows me to contribute to the field of research and make a positive impact on society.

Contents

1. Introduction

1.1 Overview of Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, primarily affecting locomotion. Early clinical symptoms include tremors, stiffness, and difficulty initiating and controlling movements (Roselli & Caroni, 2015). Non-motor symptoms of PD include sensory, cognitive, mood and sleep changes. PD is known to be caused by selective degeneration of dopaminergic (DA) neurons in the substantia nigra compacta region of the human brain. PD is usually sporadic and is known to be hereditary in about 10-12% of cases. Late-onset PD develops after a median age of to 45 years, while early-onset PD has a median age of onset of 16 years. The main genetic factors involved in late-onset PD are LRRK2 and α-synuclein, whereas PINK1, Parkin and DJ-1 are involved in juvenile PD.

1.1.1 Significance of Understanding Motor and Non-Motor Symptoms in Parkinson's Disease

PD is a complex neurodegenerative disorder that affects multiple aspects of an individual's health and well-being. An understanding of both the motor and non-motor symptoms, provide more effective management strategies and improve the overall quality of life for the PD patients.

Accurate Diagnosis and Early Intervention: Motor symptoms such as resting tremor, bradykinesia, rigidity, and postural instability are the hallmark signs of PD. Recognizing these symptoms and distinguishing them from other conditions is crucial for an accurate diagnosis. Additionally, identifying non-motor symptoms, which can manifest before motor symptoms, enables early intervention and treatment initiation, potentially improving long-term outcomes (Schapira et al., 2017).

Comprehensive Treatment Approaches: Motor symptoms are typically the primary focus of PD treatment, with medications targeting dopamine deficiency to alleviate motor dysfunction. However, non-motor symptoms significantly contribute to the overall burden of the disease and can greatly impact patients' quality of life. By considering and addressing non-motor symptoms, healthcare providers can develop comprehensive treatment strategies tailored to the individual needs of patients, providing holistic care (Schapira et al., 2017; Martinez-Martin et al., 2011).

Improved Symptom Management: Tailoring treatments to address specific symptoms, such as cognitive impairment, depression, sleep disturbances, or autonomic dysfunction, can lead to better symptom control and improved overall functioning (Martinez-Martin et al., 2011).

Prediction of Disease Progression: Motor and non-motor symptoms in PD can vary among individuals and change over time. Monitoring and understanding the progression of these symptoms can help predict disease trajectory and identify potential complications. This information aids in adjusting treatment plans, optimizing care, and preparing patients and caregivers for future challenges (Kalia & Lang, 2015).

Impact on Quality of Life: PD is a chronic condition that significantly impacts patients' quality of life. Motor symptoms can affect mobility and independence, while non-motor symptoms can contribute to emotional, cognitive, and social challenges. By addressing and managing both types of symptoms, healthcare professionals can improve the overall well-being and quality of life for individuals living with PD (Martinez-Martin et al., 2011).

In conclusion, understanding the motor and non-motor symptoms in Parkinson's disease is crucial for accurate diagnosis, comprehensive treatment approaches, improved symptom management, and prediction of disease progression. Hence, by addressing the wide spectrum of symptoms, healthcare providers and researchers can provide more personalized and effective care, ultimately improving the quality of living in individuals with this complex neurodegenerative disorder.

1.2 The Parkin Gene

1.2.1 Introduction to the Parkin Gene (PARK2)

The Parkin gene, also known as PARK2, is one of the key genes associated with Parkinson's disease (PD). It was first identified in 1998 as a causative gene for autosomal recessive juvenile parkinsonism (AR-JP) (Kitada et al., 1998). PARK2 is located on chromosome 6 and encodes the Parkin protein, which plays a crucial role in cellular processes such as protein degradation and mitochondrial homeostasis.

1.2.2 Role of Parkin in Parkinson's Disease Pathogenesis

Parkin is primarily involved in the ubiquitin-proteasome system (UPS), which is responsible for the targeted degradation of damaged or misfolded proteins. Mutations in the Parkin gene lead to dysfunction of the Parkin protein, impairing its ubiquitin ligase activity and resulting in defective protein degradation. This disrupts cellular homeostasis and can contribute to neuronal cell death in PD (Dawson & Dawson, 2003). Several mechanisms have been proposed to explain Parkin dysfunction, including impaired substrate recognition, abnormal protein folding, and altered interaction with other components of the ubiquitin–proteasome system.

1.3 *Drosophila* as a Model Organism in Parkinson's Research

Figure 1.1 *Drosophila melanogaster* (Fruit fly)

1.3.1 Introduction to *Drosophila melanogaster* as a Model Organism

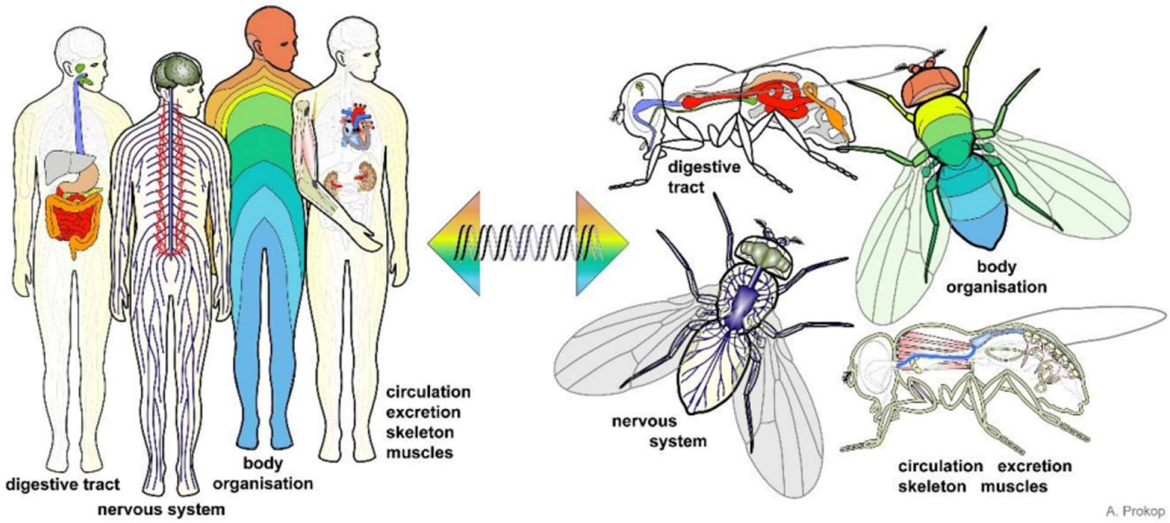


Figure 1. 2 Similarity in system organizations and genetic architecture between *Drosophila* and humans made *Drosophila* an excellent model system to study human diseases and disorders (Prokop et al 2020).

The majority of neurodegenerative disorders are idiopathic; however, multiple genes have been associated with them. This has allowed us to investigate the genetics and molecular mechanisms of these disorders. Animal models have been contributing to the study of the genetics of molecular mechanisms for many decades. The ability to modify or knockout specific genes or chromosomal loci, and to generate point mutations and copy number variants has increasingly evolved with the rapid advancements in genetic engineering technologies. Although the production of the animal models is the first step, the establishment and validation of the model systems are crucial for understanding the manifestation of the disease. For over a century, *Drosophila* has proven to be an excellent model organism for studying genetics (Figure 1.2). Owing to its strong repertoire of genetic tools and short life cycle, various models of different neurodegenerative diseases have been studied in *Drosophila* over many decades. *Dropdead*, one of the genes involved in brain deterioration was studied in one of the early genetic screens to identify *Drosophila* mutants with abnormal behaviour (Benzer, 1971). Further, *Drosophila* shares more than 77% homology for genes implicated in human diseases (Bier, 2005; Rubin et al., 2000), and its brain structure, similar to the human brain, is composed of a series of functionally specialized substructures (Figure 1.3). This makes *Drosophila* the perfect model organism to study neurodegenerative diseases such as Parkinson's disease (PD).

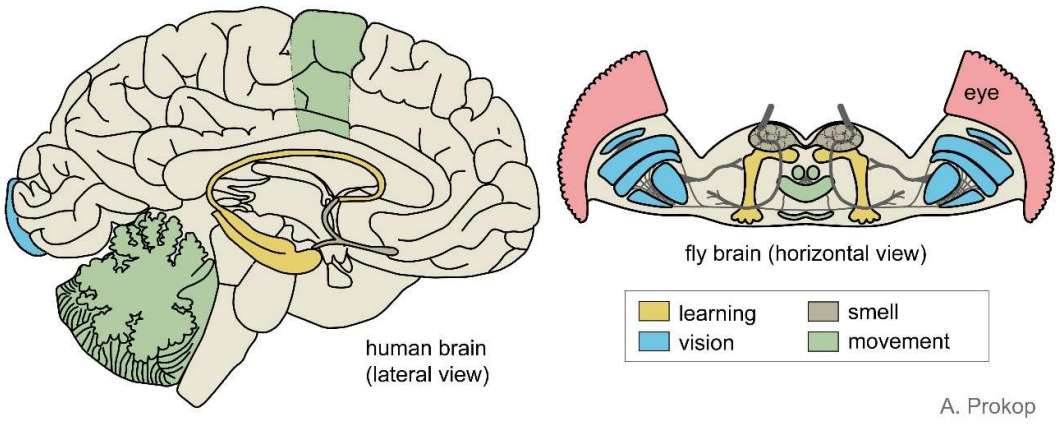


Figure 1. 3 A comparison of human and *Drosophila* brains shows the presence of similar functionally specialized substructures, which makes *Drosophila* a perfect model for studying neurodegenerative diseases. (Adapted from Prokop et al. 2020)

Many *Drosophila* models of PD have been established over the last couple of decades. These models have proven to be of immense use in elucidating the cellular and molecular mechanisms responsible

for the loss of dopaminergic neurons (Feany & Bender, 2000). Studies, including ectopic expression of α-synuclein (Auluck et al., 2002), mutations in *Drosophila* homologue of *LRRK2* (Z. Liu et al., 2008), and endogenous *PINK1* and *parkin* genes (I. E. Clark et al., 2006; Park et al., 2006) have been crucial in understanding the molecular mechanisms of familial PD. Similar to human pathological marker, loss of DA neurons in specific brain regions, flies also show degeneration of DA neurons in specific pockets of the brain (Riemensperger et al., 2013).

1.3.2 Utilization of *Drosophila* Models in Parkinson's Research

Drosophila models have been crucial in unravelling the impact of PD-associated genes on protein aggregation, mitochondrial dysfunction, oxidative stress, neuroinflammation, and neurodegeneration. In addition, behavioral assays in flies allow for the assessment of motor impairments and other disease-related phenotypes (Bove et al., 2011).

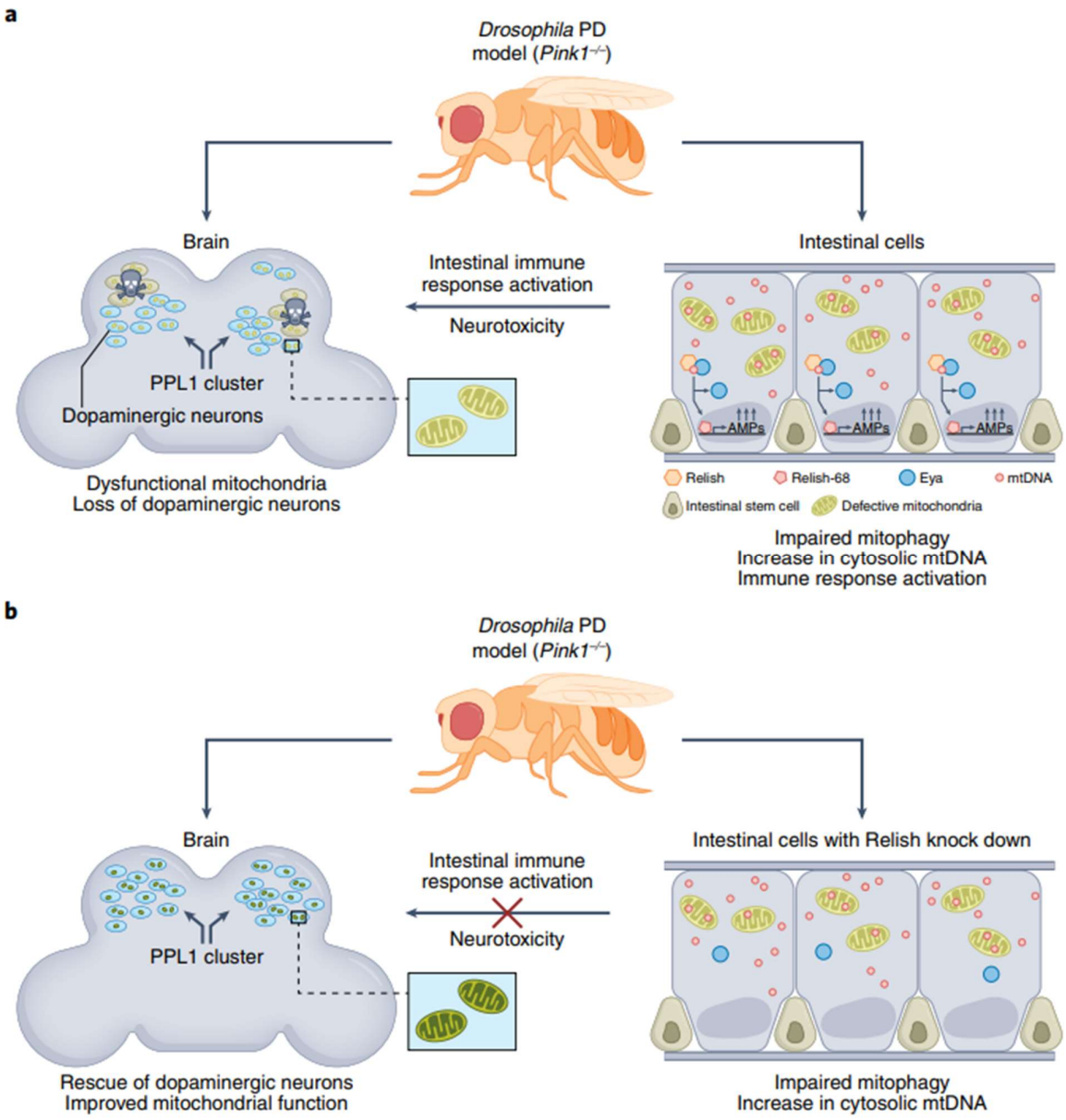


Figure 1.4 In a *Drosophila* model of Parkinson's disease, activation of the immune response in intestinal cells is essential for neurotoxicity (Aparicio et al., 2022).

pink1b9-mutant flies (a Parkinson's disease model) had large quantities of cytosolic mtDNA in intestinal cells due to faulty mitochondria. Relish-68, the activated N-terminal cleaved domain of RELISH, translocate from the cytoplasm to the nucleus as the transcription factor RELISH. Mitochondrial DNA triggers the immunological response in intestinal cells via Relish and alterations in RELISH-Eya interactions. The expression of immune genes encoding antimicrobial peptides

(AMPs) in gut cells causes neurotoxicity in the brain and cell death in the PPL1 cluster of dopaminergic neurons (Figure 1.4 a). *relish* knockdown in intestinal cells in this Parkinson's disease model suppresses immune response activation in the gut, even in the context of gut mitochondrial dysfunction. As a result, the number of dopaminergic neurons in the PPL1 cluster and mitochondrial activity in the brain are restored. *pink1b9* mutant suppresses immune response activation in the gut, even when there is gut mitochondrial dysfunction (Figure 1.4 b).

1.3 *Drosophila* Model used in this study

1.3.1 Overview of *park25* (PARKIN) and *park13* (HTRA2)

For this study, we used *park25* and *park13 Drosophila* mutants. *park25* is a gene homologous to *Parkin* gene in humans which is associated with autosomal recessive juvenile Parkinson's disease. Mutations in the *parkin* gene result in dysfunctional PARKIN protein, which plays a crucial role in the clearance of damaged mitochondria through a process called mitophagy. (Kitada et al., 1998; Valente et al., 2004).

park13, homologous gene of HTRA2, is another gene linked to Parkinson's disease. Mutations in the HTRA2 gene have been associated with both familial and sporadic forms of Parkinson's disease. The HTRA2 protein is a serine protease involved in protein quality control and regulation of cell death pathways. (Strauss et al., 2005; Winklhofer, 2010).

1.4 *Mucuna pruriens* Seeds: Literature Survey on Parkinson's Management in *Drosophila*

1.4.1 Introduction to *Mucuna pruriens*

Mucuna pruriens, also known as velvet bean, is a legume belonging to the Fabaceae family. The plant has typical fabaceae characteristics like long branches and alternate leaves. The legumes are violine-shaped and velvet-like texture on the outer covering. The plant has wide atmospheric tolerance and only cannot grow in cold weather mostly grown in the Indian sub-continent.

Figure 1.5 *Mucuna pruriens* fruit (left) and seed (right) collected from IAIM hospital garden. Image captured on Feb 2023

The taxonomic characteristics of *Mucuna pruriens* is as follows:

Kingdom-Plantae

Subkingdom-Tracheobionta

Superdivision-Spermatophyta

Division--Magnoliophyta

Class-Magnoliopsida

Subclass-Rosidae

Order-Fabales

Family-Fabaceae Lindl.

Genus-Mucuna Adans.

Species-*Mucuna pruriens* (L.) DC.

Chemical Constituents:

Chemical	**Plant Part**
5-hydroxytryptamine	Seed
5-methoxy-N, N-dimethyltryptamine-N-oxide	Seed
5-oxyindole-3-alkylamine	Seed
Alanine	Seed

Arachidic-acid	Seed
Arginine	Seed
Ash	Seed
Aspartic-acid	Seed
Behenic-acid	Seed
Beta-carboline	Seed
Beta-sitosterol	Seed
Bufotenine	Seed
Calcium	Seed
Carbohydrates	Seed
Choline	Seed
Cis-12,13-epoxyoctadec-trans-9-cis-acid	Seed
Cis-12,13-epoxy octadec-trans-9-enoic-acid	Seed
Cystine	Seed
Dopa	Seed
Fat	Seed
Fiber	Seed
Gallic-acid	Seed
Glutamic-acid	Seed
Glutathione	Seed
Glycine	Seed
Histidine	Seed
Indole-3-alkylamine	Seed
Iron	Seed
Isoleucine	Seed
Kilocalories	Seed
Lecithin	Seed
Leucine	Seed
Linoleic-acid	Seed
Linolenic-acid	Seed
Lysine	Seed

Methionine	Seed
Mucunadine	Seed
Mucunain	Seed
Mucunine	Seed
Myristic-acid	Seed
N, N-dimethyltryptamine	Seed
N, N-dimethyltryptamine-n-oxide	Seed
Niacin	Seed
Nicotine	Seed
Oleic-acid	Seed
Palmitic-acid	Seed
Palmitoleic-acid	Seed
Phenylalanine	Seed
Phosphorus	Seed
Proline	Seed
Protein	Seed
Prurienidine	Seed
Prurienine	Seed
Riboflavin	Seed
Saponins	Seed
Serine	Seed
Serotonin	Seed
Stearic-acid	Seed
Thiamin	Seed
Threonine	Seed
Tryptamine	Seed
Tyrosine	Seed
Valine	Seed
Vernolic-acid	Seed
Water	Seed

Table 1.1 Molecules found in *Mucuna pruriens* seed (Dr. Duke's Phytochemical and Ethnobotanical Databases).

1.4.2 *Mucuna pruriens* in medicinal use

Mucuna pruriens beans are rich in protein and have been traditionally consumed by south Indian hill tribes the kanikkars. Moreover, various parts of *Mucuna pruriens* have been widely used in traditional medicine for its therapeutic properties like in the management of male infertility, and nervous disorders, anti-venom activities, anti-helminthic activity, analgesic and anti-inflammatory, and also as an aphrodisiac. Other than these recent reports have shown *Mucuna pruriens* have anti-diabetic, anti-neoplastic, anti-epileptic, and anti-microbial activities. (Lampariello et al., 2012).

1.4.3 Levodopa Content and Dopaminergic Effects of *Mucuna pruriens*

Mucuna pruriens contains a significant amount of levodopa (L-Dopa), which is the precursor of dopamine (Manyam et al., 2004). Levodopa is the primary medication for Parkinson's disease as it helps replenish dopamine levels in the brain and alleviate motor symptoms (Cilia et al., 2020). Studies have shown that the levodopa present in *Mucuna pruriens* is bioavailable and can effectively cross the blood-brain barrier (Katzenschlager et al., 2004).

1.4.4 Studies Utilizing *Mucuna pruriens* in *Drosophila* Model of Parkinson's Disease

Several studies have explored the effects of *Mucuna pruriens* in the *Drosophila* model of Parkinson's disease. These studies have demonstrated that *Mucuna pruriens* can improve motor function, protect against dopaminergic neuron degeneration, and reduce oxidative stress in $PINK1^{B9}$ mutant (Nassel et al., 2021; Sanyal et al., 2020).

(Poddighe et al., 2014) had shown that feeding of MP media, $PINK1^{B9}$ mutants significantly improved climbing ability, olfaction, rescued damaged mitochondria. They suggest MP as an effective medication with intrinsic ability of delaying the onset of chronic L-Dopa-induced long-term motor complications.

1.4.5 Neuroprotective and Symptomatic Relief Properties of *Mucuna pruriens* in *Drosophila*

Previous studies in *Drosophila* model have provided evidence of the neuroprotective and symptomatic relief properties of *Mucuna pruriens* in Parkinson's disease. It has been shown to mitigate neuronal damage, increase dopamine levels, and improve locomotor activity in fruit flies (Khurana et al., 2017;

Khare et al., 2015). Moreover, *Mucuna pruriens* has exhibited antioxidant and anti-inflammatory effects, contributing to its neuroprotective properties (Katzenschlager et al., 2004).

2. Materials and methods

2.1 Media

Media composition (1000mL)	**Normal diet**	**Experimental diet**
CORN FLOUR	80g	80g
DEXTROSE(GLUCOSE)	40g	40g
SUCROSE	20g	20g
YEAST EXTRACT	30g	30g
AGAR	8mg	8mg
PROPIONIC ACID	4mL	4mL
O-PHOSPHORIC ACID	6mL	6mL
Vaidya Ratnam Kapikachu powder (Batch no-22A0362)		0.68g
Distilled water	1000mL	1000mL

Table 2.1 Composition of media.

2.2 Fly husbandry

For the experiment purpose, *park25, park13,* and *CS* (Canton Special) flies were obtained from the fly facility of the National Centre for Biological Sciences (NCBS). The genotype for the *park25* mutant fly is *w−;;park25/TM3-Ser* and the *park13* mutant fly was *w*;;park13/TM6B,Tb*, where *TM3* and *TM6* are the balancer chromosomes expressing the dominant phenotype of Serrate (*Ser*) and Tubby (*Tb*). The mutant fly was generated through the deletion of the *parkin* gene as shown in (Figure 2.1).

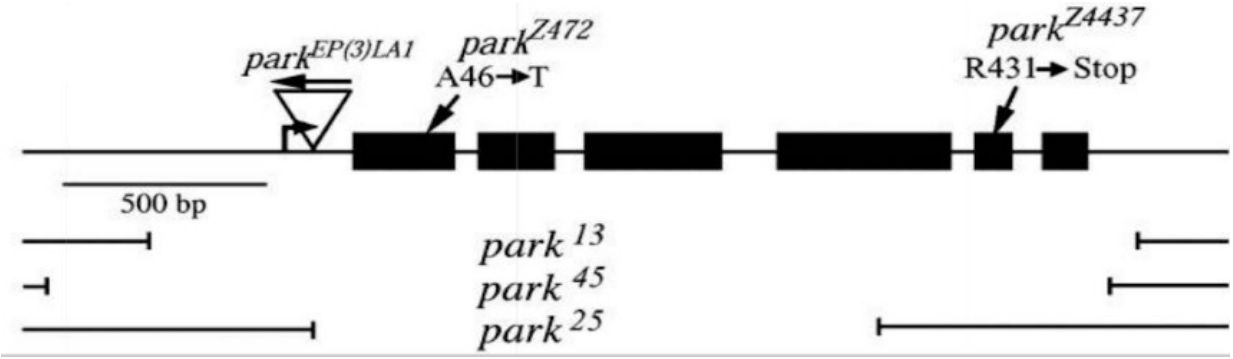


Figure 2.1 depicts the deletion of *park25*, and *park13* (Green et al., 2003).

The fruit flies were maintained in plastic bottles and flipped over alternate days to avoid overcrowding and all the experiments were performed in vials. The flies were reared at 25°C and relative humidity ranged between 60%-80%.

2.3 Experimental setup

The experimental setup was followed by the collection of *CS* (Canton Special) virgin female flies, males of *park25, park13,* and *CS*. While the *CS* flies were utilized as wild-type flies for the study, *park25/+,* and *park13/+* mutants were heterozygous as this study was based on understanding the disease in humans, where humans mainly consist of the heterozygous gene encoding for this protein. The following crosses were set up:

♀*CS* X ♂*CS* (control)

♀*CS* X ♂*park25*

♀*CS* X ♂*park13*

♂ \ ♀	+	+
park25	+; *park25*	+; *park25*
TM3-Ser	+; *TM3-Ser*	+; *TM3-Ser*

Table 2.2 shows the Punnett square for the probability of desired progenies from the cross of ♀*CS* X ♂*park25*.

♂ \ ♀	+	+
park13	+; *park13*	+; *park13*
TM6-Tb	+; *TM6-Tb*	+; *TM6-Tb*

Table 2.3 shows the Punnett square for the probability of desired progenies from the cross of ♀*CS* X ♂*park13*.

From the above tables, it can be calculated that the probability of obtaining *park25*/+, and *park13*/+ was 50%. The F1 generation flies were collected after 12-14 days, followed by sorting of the desired progenies. The progenies were collected by eliminating those flies who expressed balancer markers, like, Serrate (*Ser*) wings for *park25* and Tubby (*Tb*) abdomen for *park13*. The flies were sorted for three consecutive days, each day having 8 replicates n=160, for 3 Days n=480 for each genotype.

To get the activity window of *Drosophila melanogaster* across the day, the flies were incubated for three-time points 1 am - 1 pm (*park13*/+), 4 am - 4 pm (+/+), and 7 am – 7 pm (*park25*/+).

2.4 HPTLC

L-DOPA standard was purchased from Sigma-Aldrich (D9628-5G), other solvents and reagents were of analytical grade, and HPTLC plates silica gel 60F254 (20 cm X 20 cm) were procured from E. Merck (Darmstadt, Germany). Different concentrations of L-DOPA ranging from 1mg/mL to 5mg/mL were used for the standard curve and quantification of the desired solution was done.

Steps followed-

- Seeds of *Mucuna pruriens* were dried and powdered after removing seed coats.
- Different concentrations (10mg/mL, 20mg/mL, and 50mg/mL) of the VaidyaRatnam Kapikachu powder (Batch no-22A0362) were refluxed with 50 mL distilled water in a boiling water bath for 30 min.
- The extract obtained was filtered using Whatman filter paper and the residue was again, refluxed with fresh 50 mL of distilled water for another 30 min.
- The extract was filtered and both the filtrates were pooled to make the total volume of the extract 100 mL with distilled water for estimation of L-DOPA.
- An aliquot of sample 2 µl was applied to the HPTLC plate.

- A Camag HPTLC system equipped with Linomat V automatic TLC sample spotter, glass twin trough chamber (20×10 cm), Scanner 3, and integrated Wincats 4.03 Software was used for the analysis.
- Experimental conditions were optimized. The samples were spotted in the form of 8 mm bands on HPTLC plates (20×10 cm) using Linomat V under a flow of N2 gas, 10 mm from the bottom.
- The linear ascending development was carried out in a twin trough chamber which was pre-saturated with 12 mL mobile phase of n-butanol: acetic acid glacial: water (4:1:1, v/v) for 30 min.
- The plates were developed up to 8 cm and then air-dried.
- Quantitative evaluation of the plate was performed in absorption-reflection mode at 280 nm, using a Camag TLC scanner 3 integrated with WINCATS software at a slit width of 6×0.45 mm and data resolution 100 µm step and scanning speed 20 mm s^{-1}.

2.5 Locomotion Behaviour

The locomotory behavior of fruit flies was studied using a vertical rotating arena for locomotion assay (Fly-VRL), as described in (Aggarwal et al., 2019). The setup consisted of a cassette with three sections, and an infrared light source was used for illumination. Images were captured using 250 frames per second (FPS) Point Grey camera with a Canon 18-55ES lens, providing a resolution of 55 micrometres per pixel.

Data was captured using the FlyContra program. The behavior of the flies was examined in accordance with the LD (light-dark) period. Therefore, three phases of the LD cycle were followed to assess the flies' behavior. The behavior of +/+ and *park25/+* flies was observed during the 3rd week (pre-treatment) and 7th week (post-treatment). For *park13/+* flies, the behavior was observed during the 1st week (pre-treatment), 3rd week (post-treatment), and 3rd week (post-treatment).

Each day, 10 flies per genotype were used for behavior analysis. Since there were three technical replicates, the behavior experiments were conducted for three days each week. In total, 30 flies were used for behavior analysis over the course of three days.

Three main parameters, namely the number of tracks covered, distance covered, and speed covered, were used to determine the fitness, tenacity (persistence), and agility of the fruit flies. A track was defined as the path fly travelled continuously for more than one body length unit (BLU) without stopping for more than 1.5 seconds at a time.

The parameters were defined as follows:

1. Total distance travelled: The distance fly travelled is estimated by adding the lengths of all tracks in BLUs within 5 minutes. The total distance travelled in 5 minutes for a certain genotype is estimated as the mean of the total distance travelled by each fly of that genotype.
2. Average speed: The speed of a fly for each track is calculated as the average instantaneous speed during that track. The mean speed of the fly is then determined as the average of the speeds for all tracks. The average speed for a genotype is computed as the mean of the mean speeds of each fly.
3. Straightness: we determined the straightness of the fly's path, which is a measure of gross motor control and body orientation. Track straightness is determined by creating a regression model of the track and computing the co-efficient of determination, r2, for that model. The value of r2 reflects the straightness of the track; a greater r2 value corresponds to a straighter track. The mean r2 for all pathways covered by a fly is used to assess its average track straightness.
4. Track duration: The duration of a track is the average of the durations, in seconds, of each track once the fly begins to move. The duration of tracks in a particular time for a genotype is computed by taking the mean of the median track.
5. Geotactic index: The geotactic index (GTI) is a measure of a fly's capacity to sense and respond in relation to gravity. Each track in which the fly moves against gravity (from the bottom to the top of the arena) is worth one point. Similarly, each track where a fly advances along gravity (from top to bottom of the arena) is scored +1. Tracks with no vertical displacement above a certain threshold are scored zero (0). GTI is calculated as
   Geotactic index = $(\Sigma Tup + \Sigma Tdown)/(\Sigma Tup - \Sigma Tdown + \Sigma TNone)$,
   where ΣTup is the total score of all tracks against gravity, $\Sigma Tdown$ is the total score of all tracks along gravity, and $\Sigma TNone$ is the total score of tracks with no vertical displacement.

To ensure accurate distance measurements and account for allometric relations that could bias linear locomotion parameters, the data were scaled based on individual body length. Therefore, all distances are reported in frames and speed is reported in per second.

2.6 Survival

The mortality rate of the flies was assessed by counting the number of deceased flies each day. This information was used to analyse the trend in the survival rates over time for each genotype and diet condition by plotting the survivorship graph. By comparing the survival rates of flies fed the normal

diet versus those fed the experimental diet with Vaidya Ratnam Kapikachu powder, the potential impact of the powder on longevity could be determined.

2.7 Metabolic assay

Sample preparation for Bicinchoninic acid assay (BCA) and Triacylglycerol assay (TAG) has been done and performed on whole fly (3 flies/sample).

- 3 flies have been taken in 200µl of 0.2% tween-20.
- then crush the flies with the help of a manual homogenizer.
- add 300µl of 0.2% tween-20, and heat inactivate for 10 minutes at 70℃.
- Centrifuge at 4000 rpm for 4 minutes at RT.
- Take 200µl of supernatant and put it in a separate 1.5mL centrifuge tubes.

BCA assay – for the protein estimation Bovine Serum Albumin (BSA) standard protein of 1mg/mL was used as known concentration to obtain standard curve, and desired sample concentration was calculated accordingly.

Component	Blank	1	2	3	4	5	10µl (Sample)
0.2% tween-20 (µl)	10	9	8	7	6	5	0
Enzyme buffer mix (µl)	80	80	80	80	80	80	80
Total (µl)	90	90	90	90	90	90	90

TAG assay – for the estimation of Triglyceride we used benespera avantor kit using following methodology.

Reaction mixture – to obtain standard curve standard triglycerides solution was diluted 1:1 concentration. The reaction mixture was as follows:

Component	Blank	1	2	4	8	50µl (Sample)
0.2% tween-20 (µl)	50	49	48	46	42	0

Enzyme buffer mix (µl)	150	150	150	150	150	150
Total (µl)	200	200	200	200	200	200

2.8 Gene expression study

RNA isolation from whole flies

- Add 200µl trizol and put 3 flies and homogenized and then add 300µl trizol.
- Then add 100µl (one-fifth volume) of Chloroform HPLC grade.
- Mix thoroughly by inversion (20 times).
- Incubate at RT for 10 minutes.
- Centrifuge the tube at 14000 rpm for 15 minutes set at 4°C. At this stage, phase separation occurs.
- Carefully remove the upper aqueous phase (200 -250µl) using a micropipette into a fresh 1.5mL microfuge tube. Leave behind 20 – 30µl of the aqueous phase (to prevent DNA contamination).
- Add equal volume of Isopropanol (~200-250µl), HPLC grade. Mix by inversion.
- Incubate at -20 for 10 minutes.
- Centrifuge the tube at 14000 rpm for 30 minutes set at 4 °C
- A white smear should be visible at the bottom (or on the wall toward the bottom) of the tube.
- Discard the supernatant carefully without disturbing the pellet. Pellets can be loose and can be easily sucked up by pipetting action.
- Wash the pellet with 500µl of 70% DEPC treated ethanol. Centrifuge at 14000 rpm, 10 minutes at 4°C.
- Repeat wash.
- After the last wash, carefully remove all the solvent. Air dry the pellet.
- Dissolve the pellet in ~15-20µl Nuclease free water (DEPC treated water/ Sigma nuclease free water), depending on the sample.
- To quantify, nanodrop 1µl and run 1µl on a 1% Agarose/TAE gel.

cDNA synthesized by using Takar PrimeScript™ RT Reagent Kit (Perfect Real Time) Catalog number RR037A.

1. Prepare the reaction mixture on ice according to the following table:

Reagent	**Volume**
5X PrimeScript Buffer	2 µl
PrimeScript RT Enzyme Mix I	0.5 µl
Oligo dT Primer (50 µM)	0.5 µl
Random 6 mers (100 µM)	0.5 µl
Total RNA	500 ng
RNase Free dH2O	to 10 µl

2. Mix the reaction mixture gently by pipetting up and down.
3. Dispense 10 µl aliquots of the reaction mixture into PCR microtubes.
4. Incubate the reaction mixture under the following conditions:
    - Reverse Transcription: 37°C for 15 minutes
    - Inactivation of Reverse Transcriptase: 85°C for 5 seconds

Quantitative real time PCR

The reaction mixture included:

Reagent	Volume (1X µl)
cDNA	2
ddH_2O	2.8
Forward primer	0.1
Reverse Primer	0.1
Kapa SYBR	5

Total reaction mixture= 10 µl

Primer pairs used are:

rp49 Forward- CGGATCGATATGCTAAGCTGT,

Reverse- GCGCTTGTTCGATCCGTA

relish – Forward- ACAGGACCGCATATCG;

Reverse- GTGGGGTATTTCCGGC

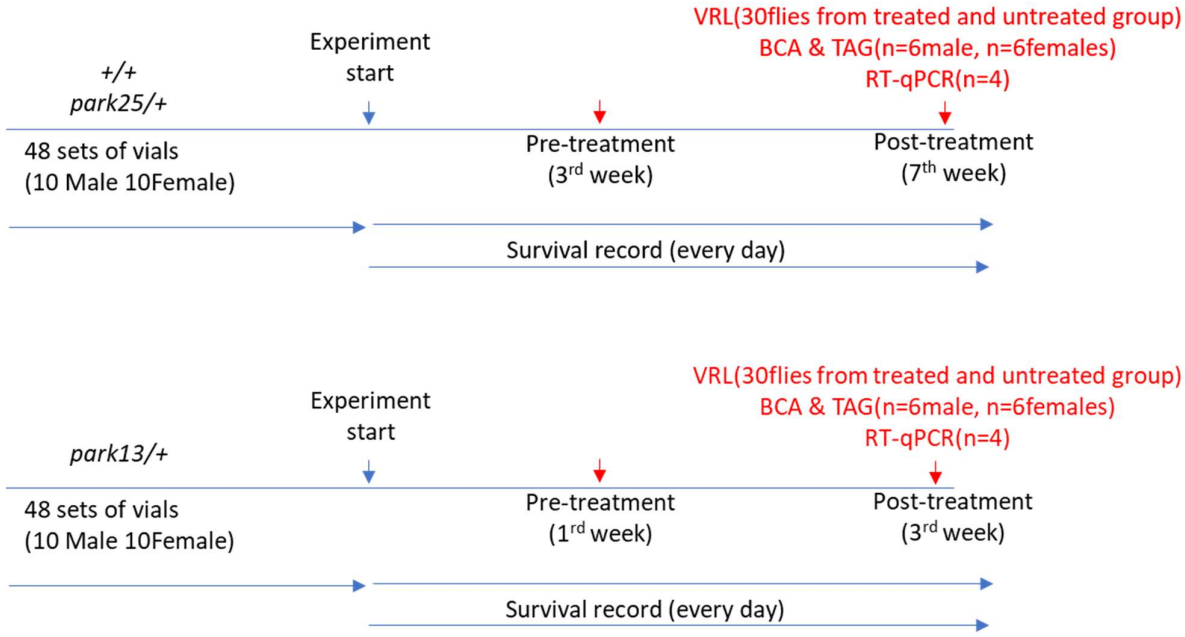
The reaction condition used was

95ºC for 20 seconds, 95ºC for 10 seconds, 60ºC for 20 seconds, 95ºC for 1 second, 60ºC for 20 seconds, and 95ºC for 1 second. For 45 cycles.

2.9 Statistical analysis

The statistical analysis for Triacylglyceride assay and qRT- PCR was done using the unpaired Student's t-test. The significance of the survival curve was analysed using Kaplan-Meier estimate (log-rank test). Error bars in all the graphs represent standard error of the mean (SEM). For locomotion behavior assay, One-way ANOVA- Non-parametric (Kruskal-Wallis test and Dunn's multiple comparison test) was performed.

2.10 Experimental Plan



3. Results and Discussion

3.1 Diet standardization

Vaidyaratnam-branded kapikachu powder diet intervention was standardized in the *CS* flies. For an adult of 70 kg who is having PD have given 24g of kapikachu powder a day (Dr. Prasan Shankar). We have calculated it for weight of fly and were given three different seed powder concentrations (0.34mg/mL, 1.7mg/mL, and 6.8mg/mL) during a five-day period to check the survivability of all the flies, and the flies remained alive after 5 days of kapikachu powder ingestion. The metabolic profile was investigated using a TAG and protein quantification assay. It was discovered that by elevating the *Mucuna pruriens* diet concentrations, protein concentration is the same (Figure 3.2) but TAG concentration was observed to increase significantly (Figure 3.3). When normalized with protein TAG

levels are increasing significantly in females but not males, with increasing concentration of *Mucuna pruriens* in diet (Figure 3.4). Hence, we chose an intermediate concentration of 0.68mg/mL *Mucuna pruriens* in diet for further experiments.

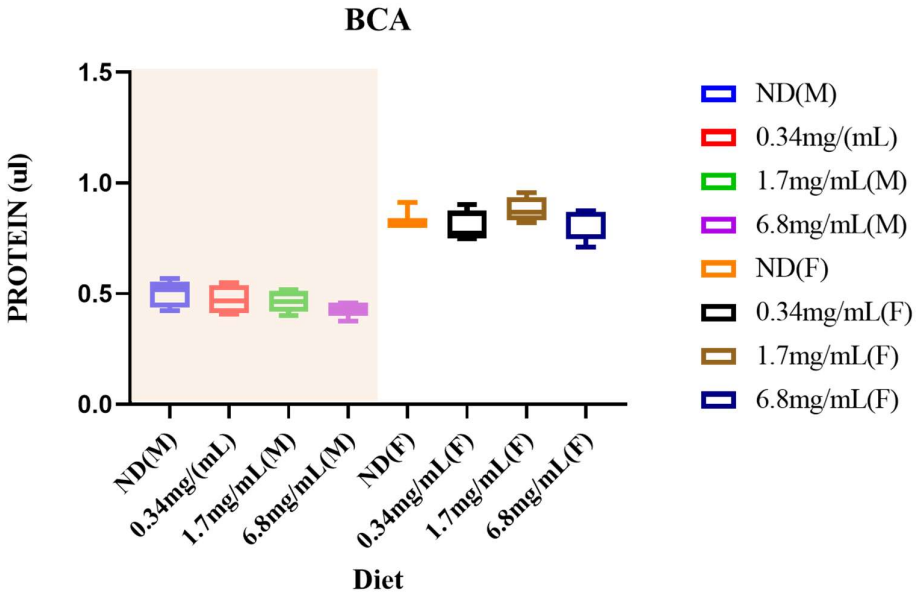


Figure 3.1 - Quantification of protein in flies fed on M.P. diet of different concentrations. The flies were fed on kapikachu powder media for 5 days and the flies of 10 day old was taken for the assay. M= Males, F = Females (n=6 sets of 3 flies each).

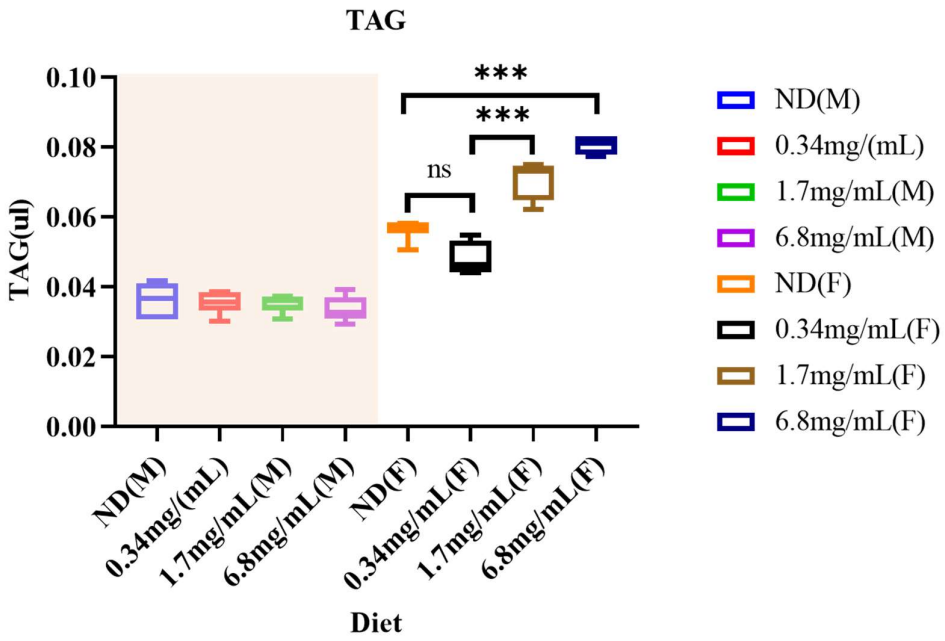


Figure 3.2 - Quantification of TAG in flies fed on kapikachu powder diet of different concentrations. The flies were fed on kapikachu powder media for 5 days and the flies of 10 day old was taken for the assay. M= Males, F = Females (n=6 sets of 3 flies each), Unpaired t-test P<0.05.

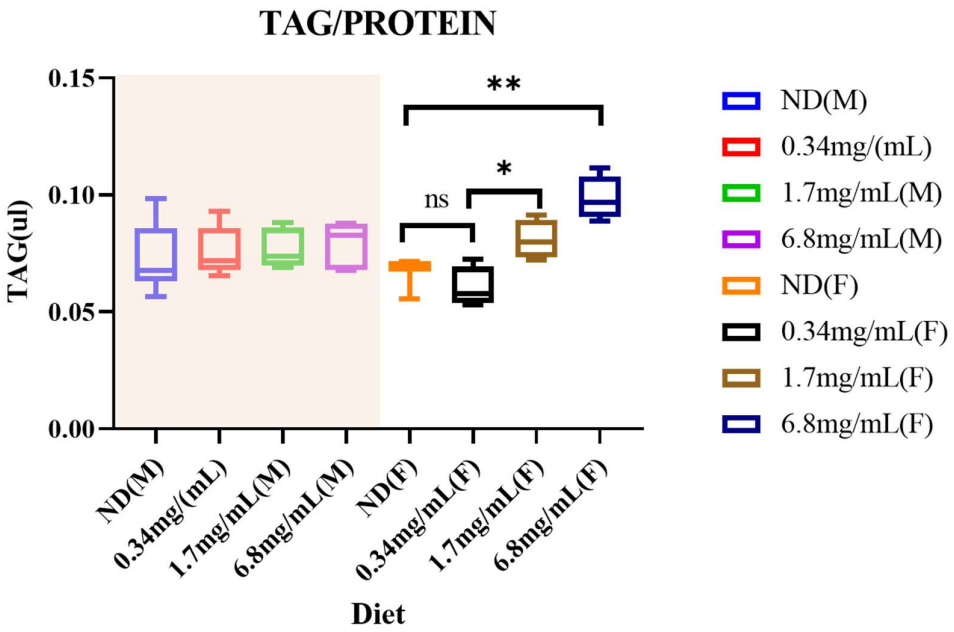


Figure 3.3 - TAG/protein ratio of flies fed on kapikachu powder diet of different concentrations. M= Males, F = Females (n=6 sets of 3 flies each), Unpaired t-test P <0.05.

3.2 Detection and quantification of L-DOPA in VaidyaRatnam Kapikachu powder

To detect L-DOPA in VaidyaRatnam Kapikachu powder (M.P) HPTLC was performed. The sample was prepared as shared in materials and methods. We observed that the sample had L-DOPA with the retention factor of 0.39, same as L-DOPA standard (Figure 3.1). similar to Raina AP et al., (2011). For quantification we used ImageJ software, we measured the area density of L-DOPA standard and sample (5mg/mL). Based on the standard curve obtained, it is estimated that there is 0.06mg/mL of L-DOPA in 0.68mg/mL of VaidyaRatnam Kapikachu powder.

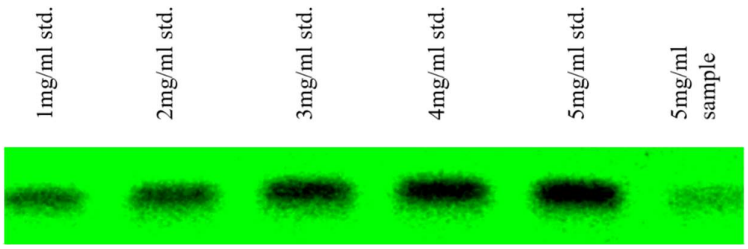


Figure 3.4 - Detection of L-DOPA in kapikachu powder using HPTLC. The sample was prepared in H_2O and the absorbance was taken at 254nm.

3.3 Metabolic assays

Emerging evidence has suggested that lipid metabolism is correlated with Parkinson's disease onset and progression (Zhang et al, 2022). To understand the metabolic status of PD mutant flies TAG and BCA were performed. In this study we compared TAG level in both male and female mutant and wild type flies in pre-treated and post-treated condition. In pre-treated condition, there is no significance difference of TAG level in both male and female flies of 3-week-old *park25*/+ mutant flies compared to +/+ flies (figure 3.5.)

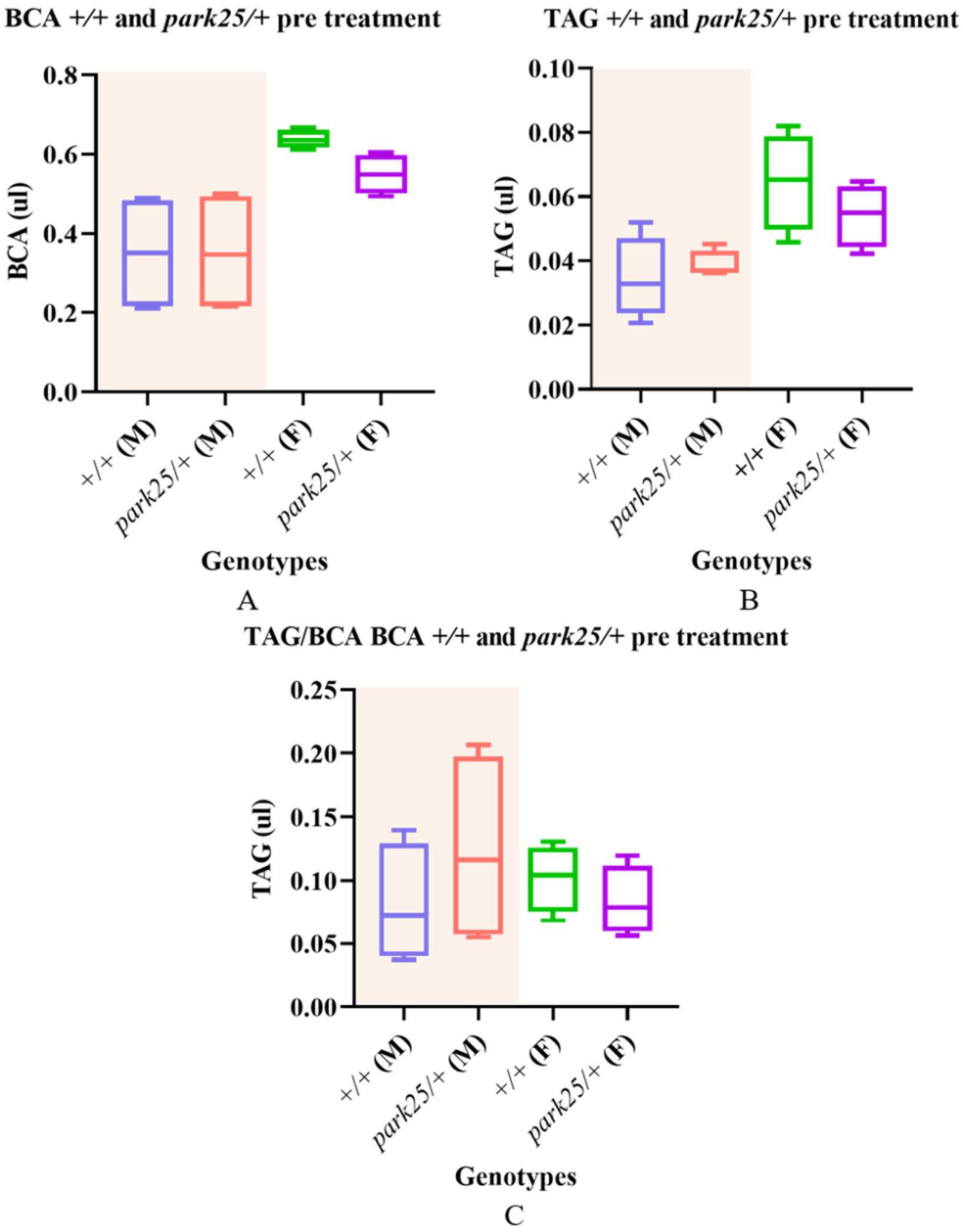


Figure 3.5 –Metabolic activity of +/+ (control) and *park25/+* (heterozygous mutant PD model) flies, pre-treatment condition. Flies were 3-week-old flies, both males (M) and females (F) were taken n=6. **A**. Protein estimation using BCA assay. **B**. TAG quantification in 3-week-old flies. **C**. TAG/protein ratio of +/+ and *park25/+* mutant flies.

Then the post treatment metabolic analysis of +/+ and *park25/+* was done on 7thweek, there was no significant changes in TAG and protein levels was observed in +/+ flies

(Figure 3.6), however in *park25*/+ treated males 26.67% increase in TAG levels when compared with non-treated group (Figure 3.7).

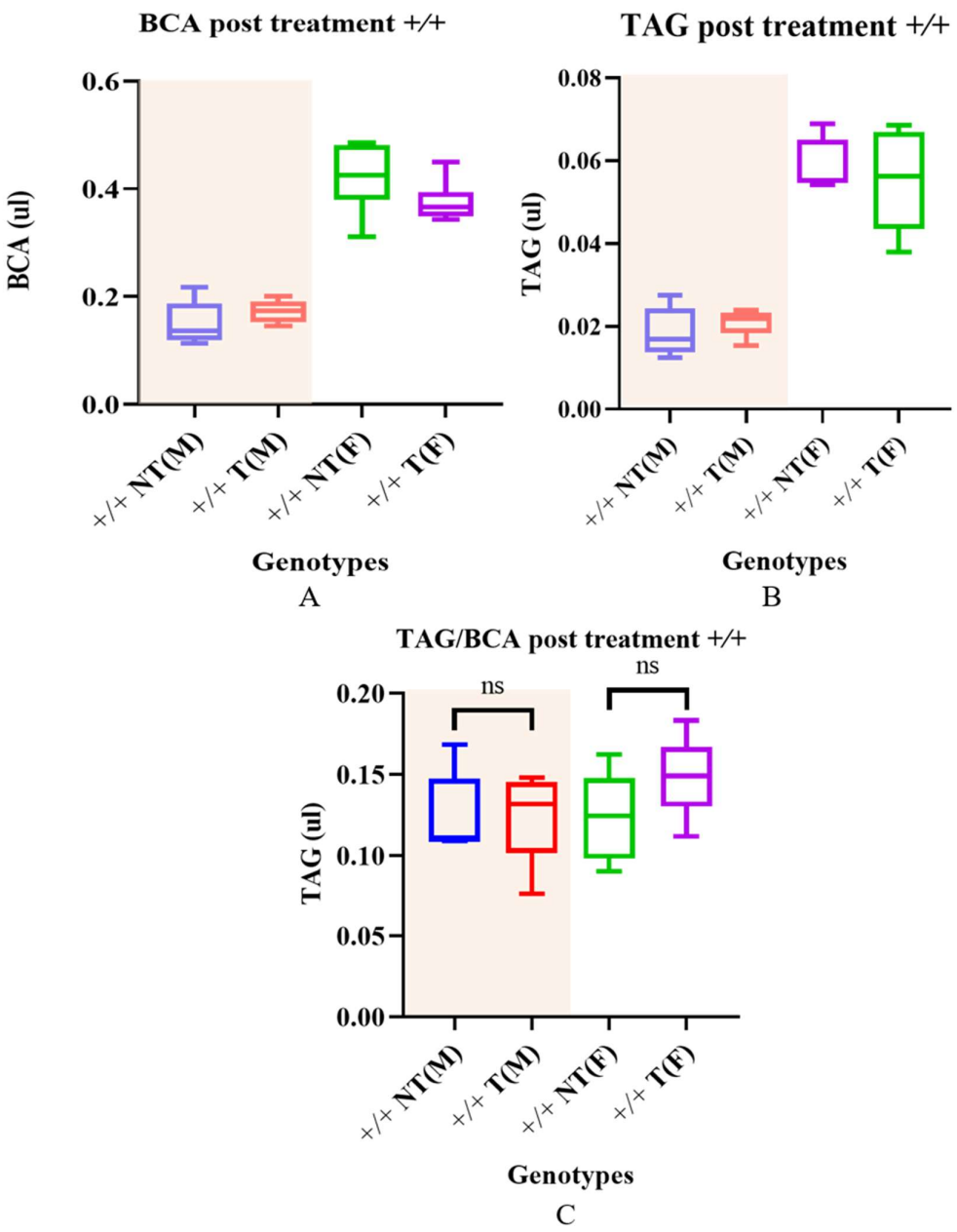


Figure 3.6 – Metabolic activity of +/+ (control) flies, post-treatment condition. Flies were 7-week-old flies, both males and females were taken n=6. **A**. Protein estimation using BCA assay. **B**. TAG quantification in 3-week-old flies. **C**. TAG/protein ratio of +/+ flies.

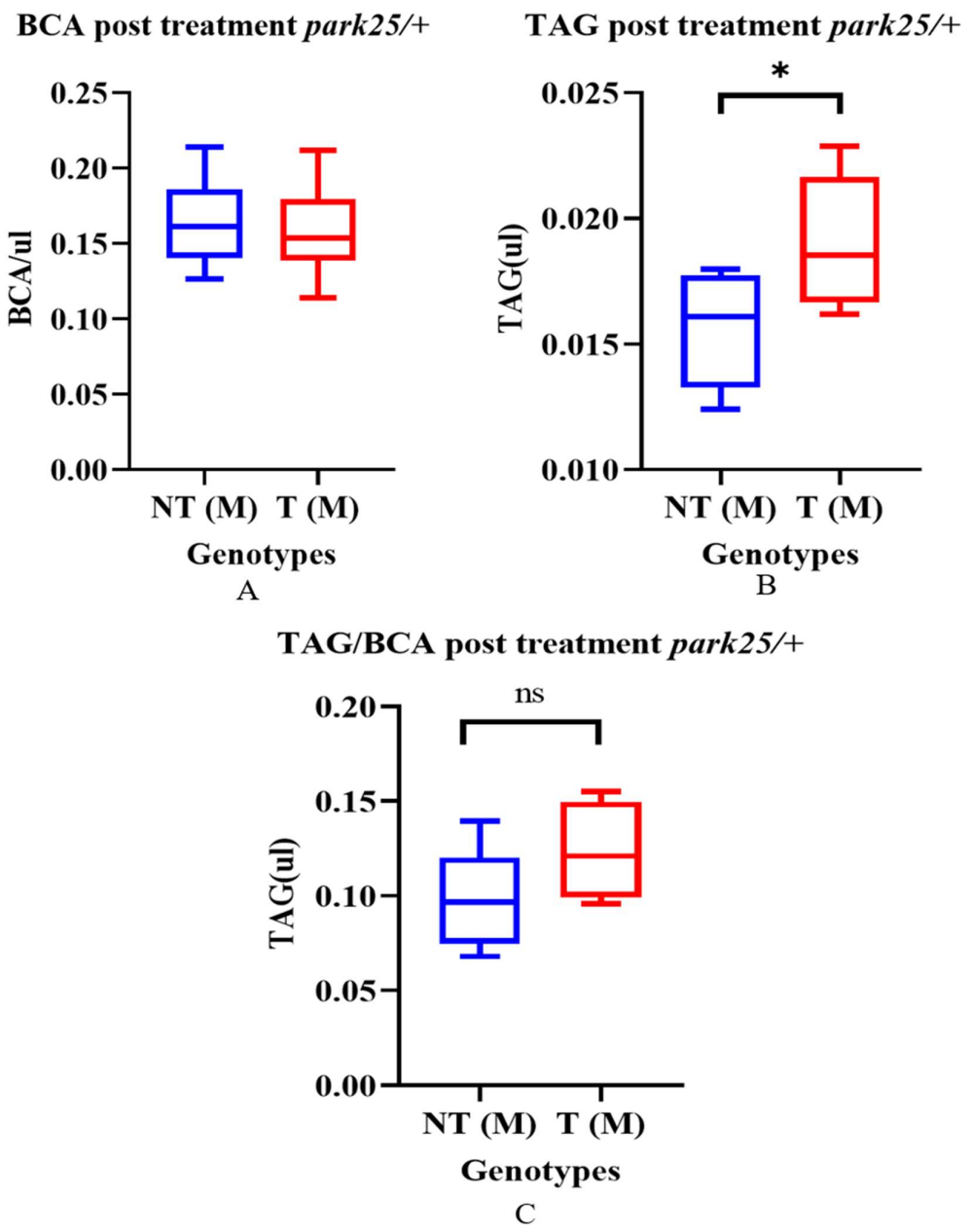


Figure 3.7 –Metabolic activity of *park25/+* flies, post-treatment condition. Flies were 7-week-old flies, both males and females were taken n=6. **A**. Protein estimation using BCA assay. **B**. TAG quantification in 3-week-old flies. **C**. TAG/protein ratio of *park25/+* mutant flies, Unpaired t-test $P<0.05$.

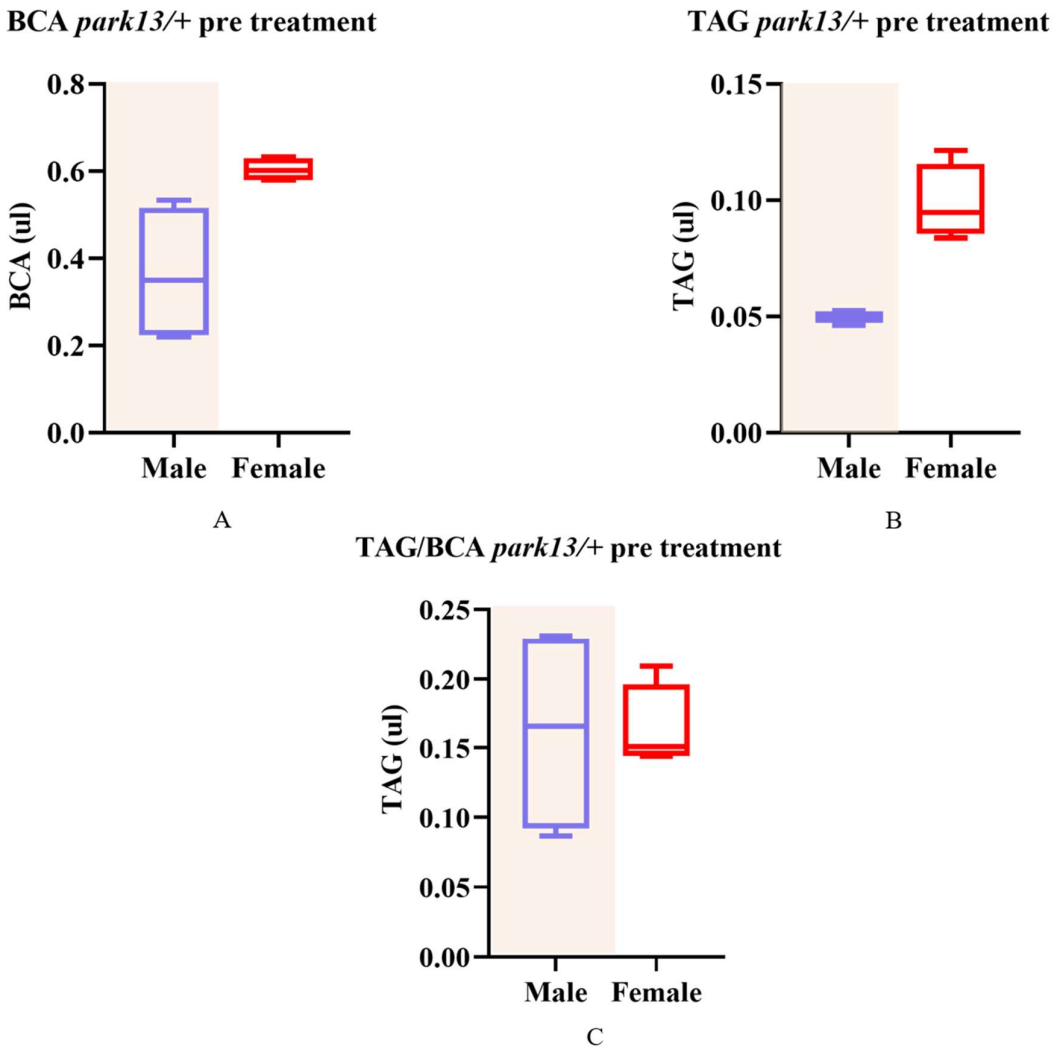


Figure 3.8 –Metabolic activity of *park13/+* flies, pre-treatment condition. Flies were 1-week-old flies, both males and females were taken n=6. **A**. Protein estimation using BCA assay. **B**. TAG quantification in 3-week-old flies. **C**. TAG/protein ratio of *park13/+* mutant flies.

The post treatment metabolic analysis of *park13/+* on the 3rd week shown an increase of TAG 67.1 % in females compared with non-treated females and there is no significant difference in males (Figure 3.9).

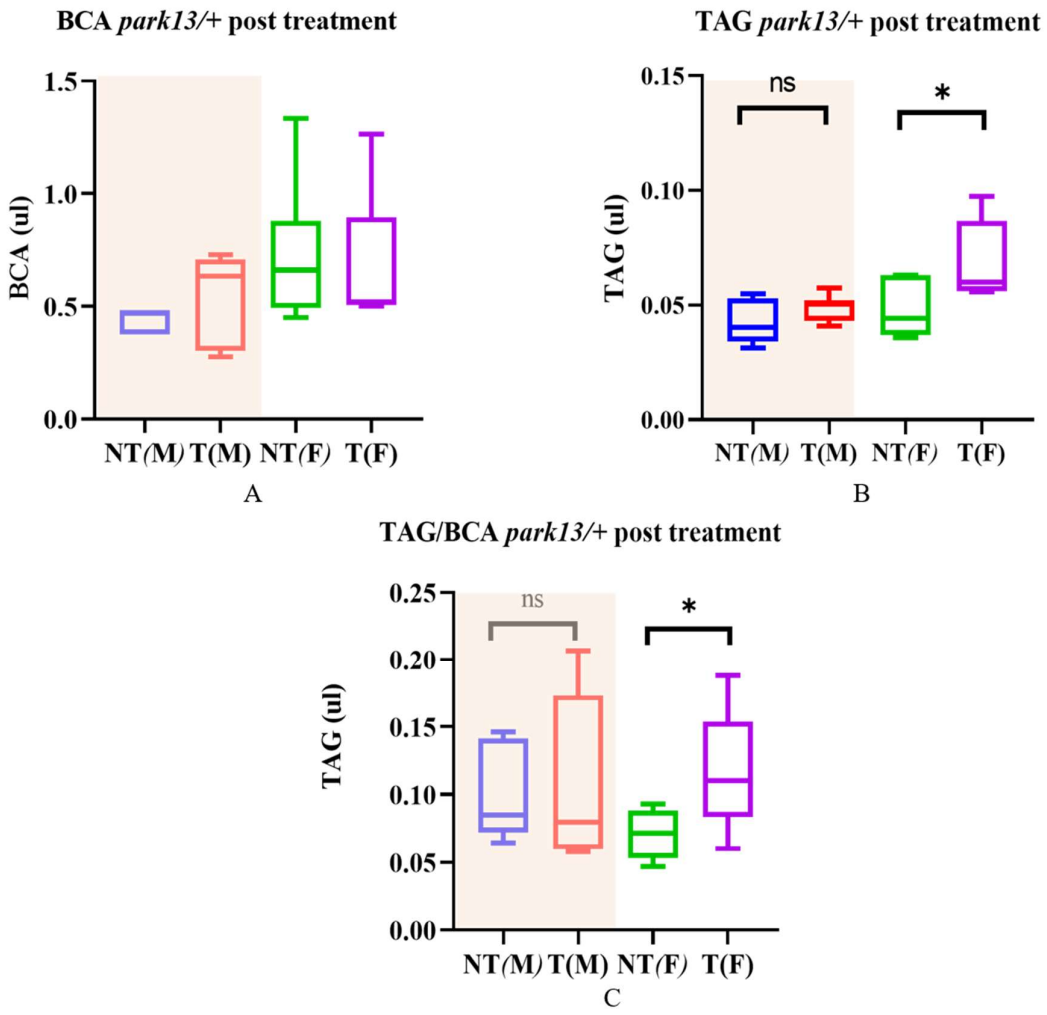


Figure 3.9 –Metabolic activity of *park13/+* flies, post-treatment condition. Flies were 3-week-old flies, both males and females were taken n=6. **A**. Protein estimation using BCA assay. **B**. TAG quantification in 3-week-old flies. **C**. TAG/protein ratio of *park13/+* mutant flies, Unpaired t-test $P<0.05$.

3.4 Survival

To understand if the drug treatment improves longevity of flies, survival assay was performed. Poddighe et al., (2014) studied *PINK1*^{B9} flies by feeding 0.1% *M. pruriens* seed powder and did not observe change in survivability. We observed that, between treated and untreated groups of +/+ flies, there was no change in the longevity. Interestingly, an improved survival was observed in the mutants, *park25/+* and *park13/+*, upon treatment with *Mucuna pruriens*.

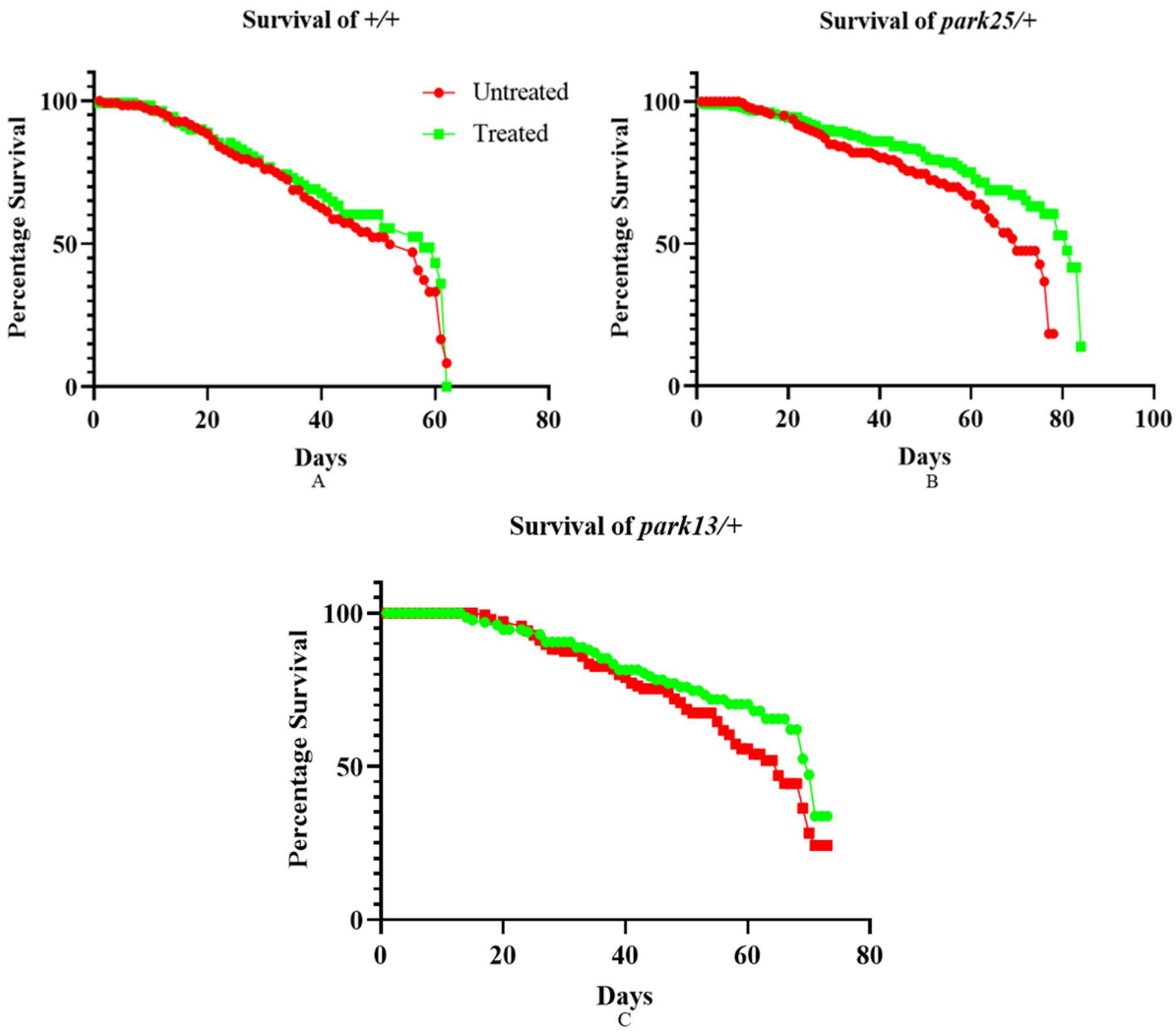


Figure 3.10 – Survivorship curve of A. +/+ flies feeding on M.P media (Treated) and Normal Diet media (Untreated) n=180. B. *park25/+* flies feeding on M.P media (Treated) and Normal Diet media (Untreated). C. *park13/+* flies feeding on M.P media (Treated) and Normal Diet media (Untreated). Both males and females were reared together.

3.5 Gene expression of inflammatory marker *relish*

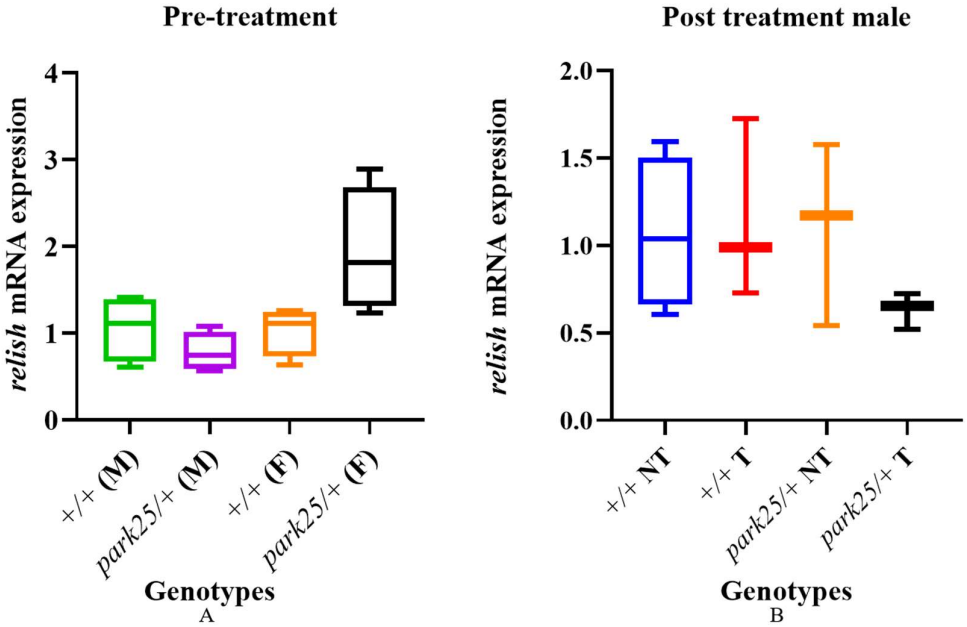


Figure 3.11- Expression of *relish* in +/+ and *park25*/+ flies A. Pre-treatment- 3-week-old flies were taken B. Post treatment of males- 7-week-old flies were taken. M= male, F= Female, T= Treated, NT= Non treated, n= 4, set of 3 flies.

Gene expression of *relish* has been checked for inflammatory status of mutants. Aparicio et al., (2022) reported *relish* gene is associated with activation of immune responsive genes in $PINK1^{B9}$ flies. Fedele et al., (2022) discovered 45 immune-response genes upregulated in $PINK1^{B9}$ mutants, with Relish (which encodes a *Drosophila* NF-B transcription factor) essential for the activation of the majority of these immune genes.

In pre-treatment condition, there was no change in the expression levels of *relish* in *park25*/+ males when compared to +/+ male flies. But, in females the *relish* was upregulated as compared to +/+ flies, however, this was not statistically significant (Figure 3.11.A). After the intervention of 4 weeks, it has been seen that there is no change in the +/+ males, but there is downregulation of the *relish* in the treated males of *park25*/+ (Figure 3.11.B).

The expression levels of *relish* in *park13*/+ did not change upon treatment with kapikachu powder (Figure 3.12).

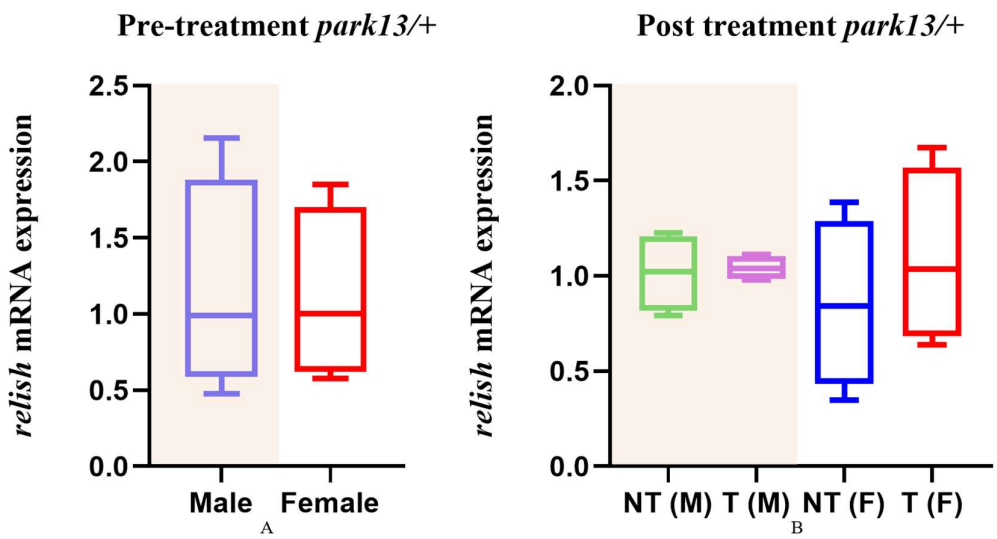
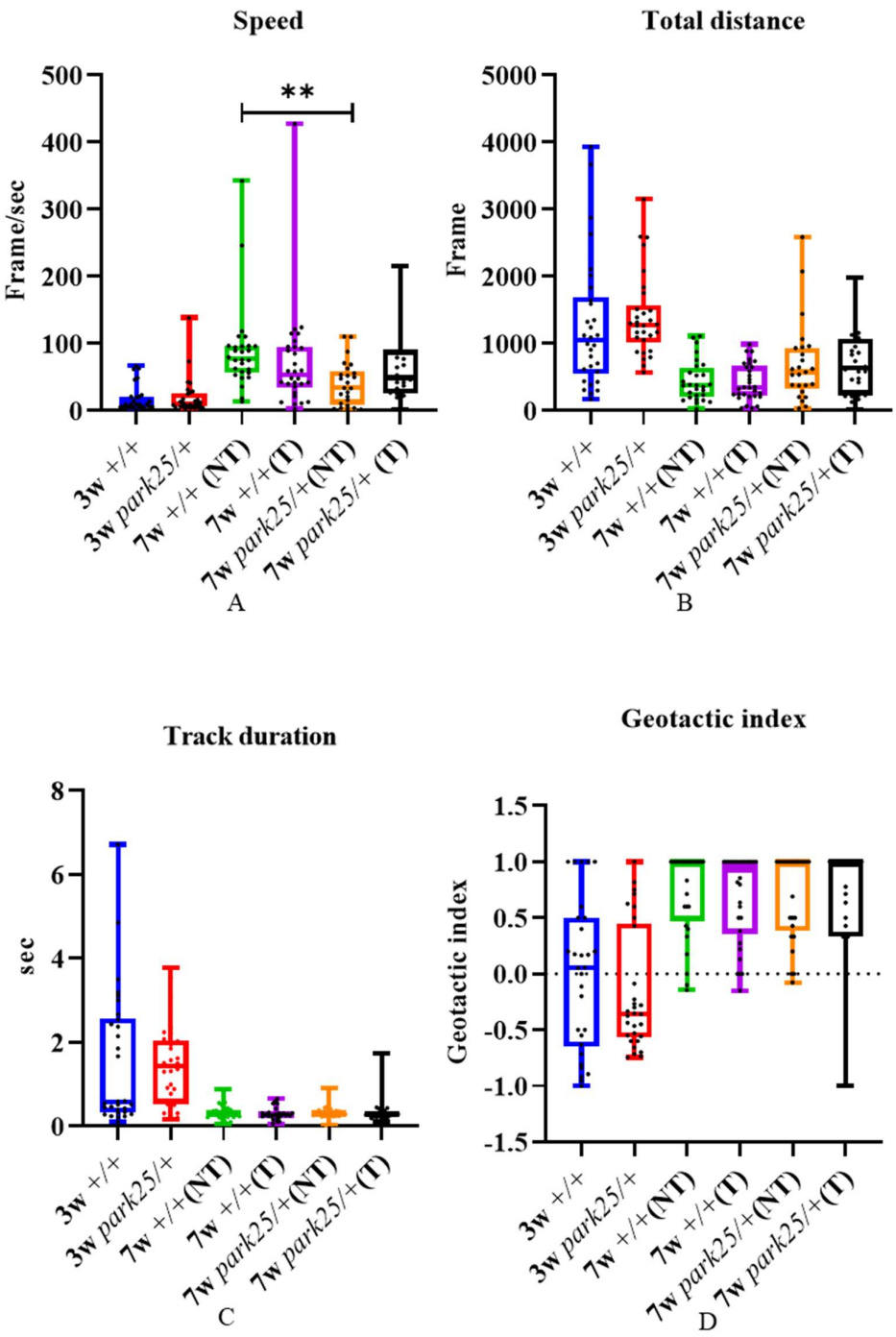


Figure 3.12- Expression of *relish* in *park13/+* flies A. Pre-treatment- 1-week-old flies were taken B. Post treatment of males- 3-week-old flies were taken. M= male, F= Female, T= Treated, NT= Non treated, n= 4, set of 3 flies.

3.6 Locomotion assay

To determine the locomotion behavior the following parameters were measured using Fly-VRL: Speed, Track duration, Total distance, Geotactic index and Straightness. We have taken pre- and post-treatment conditions for the assay (Figure 3.13). The *park25/+* had a locomotion deficit, where the 7-week-old *park25/+* flies had a significant reduction of speed and straightness when compared to wildtype.

Upon treatment, there has been a moderate increase in the speed and straightness of 7-week-old *park25/+* flies, however, it was not statistically significant.


Speed
Frame/sec
500
400
300
200
100
0
**
3w +/+
3w park25/+
7w +/+ (NT)
7w +/+(T)
7w park25/+(NT)
7w park25/+ (T)
A
Total distance
Frame
5000
4000
3000
2000
1000
0
3w +/+
3w park25/+
7w +/+(NT)
7w +/+(T)
7w park25/+(NT)
7w park25/+(T)
B
Track duration
sec
8
6
4
2
0
3w +/+
3w park25/+
7w +/+(NT)
7w +/+(T)
7w park25/+(NT)
7w park25/+(T)
C
Geotactic index
Geotactic index
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
3w +/+
3w park25/+
7w +/+(NT)
7w +/+(T)
7w park25/+(NT)
7w park25/+(T)
D

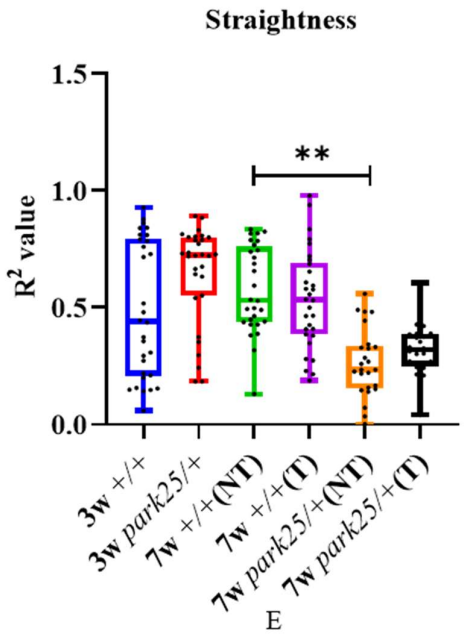
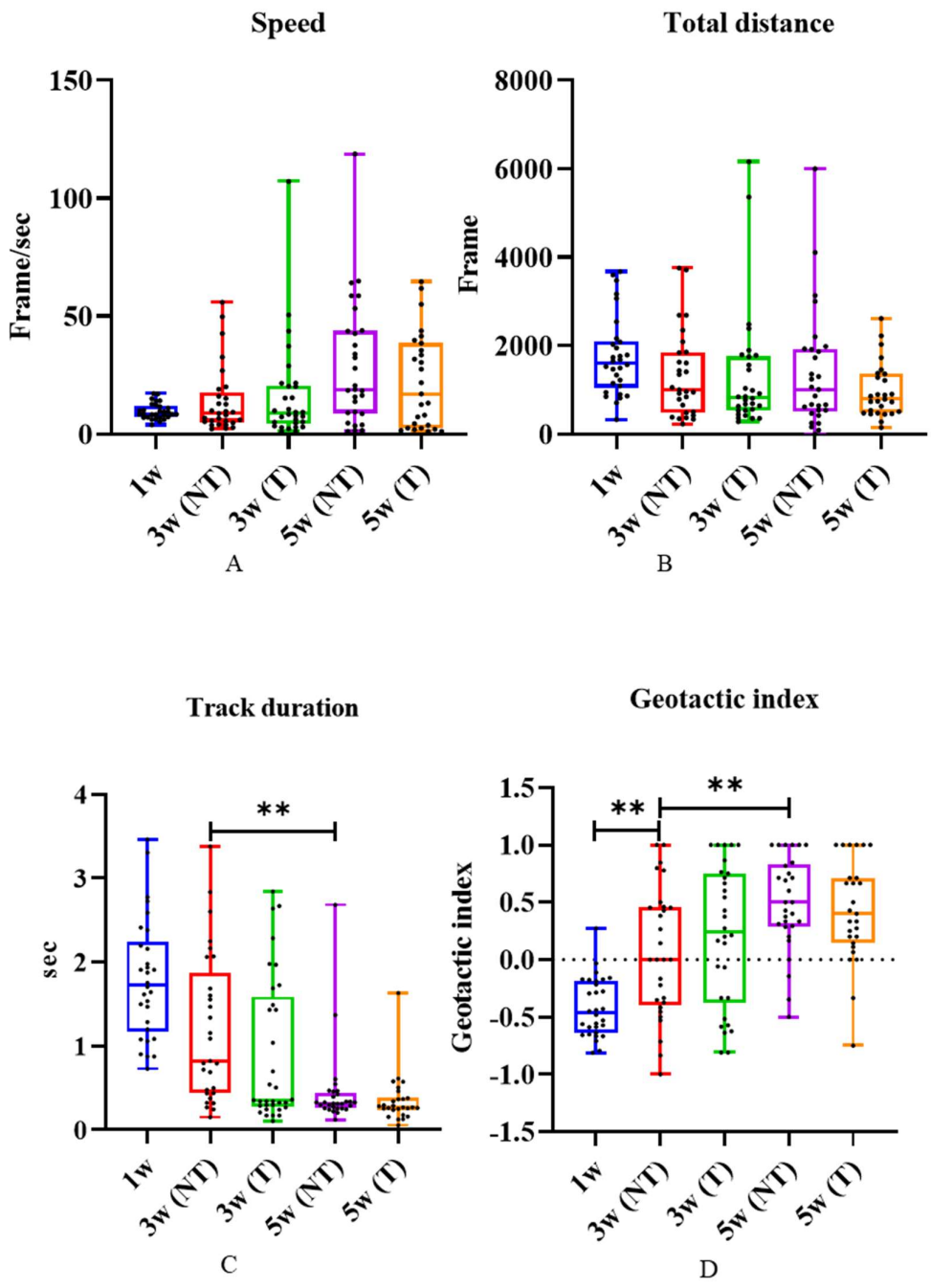


Figure 3.13- Locomotion behaviour of *park25*/+ and +/+ flies. Using fly-VRL the following parameters were measured: (A) Speed, (B) Track duration, (C) Total distance, (D) Geotactic index and (E) Straightness. 3-week-old flies were taken for Pre-treatment and 7-week-old flies were taken for post treatment. NT= Non treated, T= Treated, 3w= 3-week-old flies, 7w= 7-week-old flies, n=30 One-way ANOVA- Non-parametric (Kruskal-Walli's test and Dunn's multiple comparison test) was performed, P<0.01= **.

For *park13*/+, locomotion deficit was studied in pre- and post-treatment conditions as examined on week 1 (pre-treatment), 3, and 5 for the assay (Figure 3.14). We observed a decline in locomotion behaviour with time in non-treated flies, which is evident in the track duration, geotactic index and straightness. However, treatment did not have a significant impact in rescuing the locomotion deficit in these flies.


Speed
Frame/sec
150
100
50
0
1w
3w (NT)
3w (T)
5w (NT)
5w (T)
A
Total distance
Frame
8000
6000
4000
2000
0
1w
3w (NT)
3w (T)
5w (NT)
5w (T)
B
Track duration
sec
4
3
2
1
0
**
1w
3w (NT)
3w (T)
5w (NT)
5w (T)
C
Geotactic index
Geotactic index
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
**
**
1w
3w (NT)
3w (T)
5w (NT)
5w (T)
D

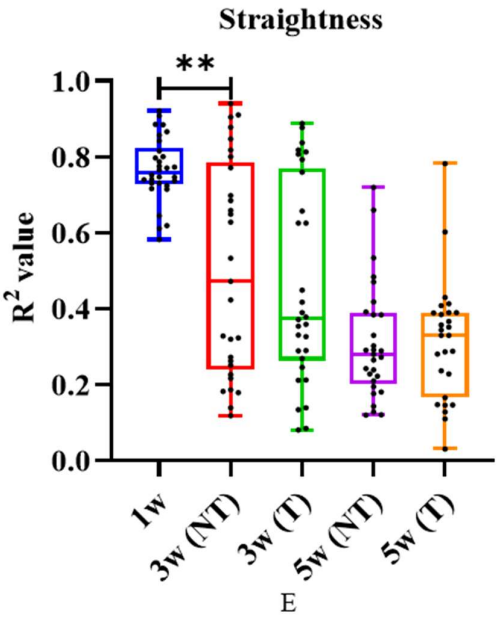


Figure 3.14- Locomotion behaviour of *park13/+* flies. Using fly-VRL the following parameters were measured: (A) Speed, (B) Track duration, (C) Total distance, (D) Geotactic index and (E) Straightness. 1-week-old flies were taken for Pre-treatment, 3-week-old and 5-week-old flies were taken for post treatment. NT= Non treated, T= Treated, 1w= 1-week-old flies, 3w= 3-week-old flies, 5w= 5-week-old flies, n=30 One-way ANOVA- Non-parametric (Kruskal-Walli's test and Dunn's multiple comparison test) was performed, P<0.01= **.

4. Conclusion

The present study provides preliminary evidence supporting the potential use of kapikachu powder in traditional medicine as a medication for Parkinson's disease (PD). The study focused on both motor and non-motor symptoms of the disease.

By analysing the powder using HPTLC, we established the baseline L-DOPA content of the kapikachu powder used in the study. This step ensures consistency in the composition and dosage of the kapikachu powder across different experimental conditions.

The observations revealed that when fed on a kapikachu powder-supplemented diet, mutant flies exhibited increased levels of TAG compared to flies fed on normal diet. This increase in TAG levels is significant because low levels of tag have been associated with disease progression in PD patients. Therefore, the ability of kapikachu powder to increase TAG levels suggests that it may have a role in

managing the development of PD. However, further studies are needed to determine the effectiveness of this management and the rate at which it occurs.

The gene expression data indicated that treatment with kapikachu powder for a period of 4 weeks resulted in lower levels of *relish* in *park25/+* mutant flies. However, this effect was not observed in *park13/+* flies, which were treated with kapikachu powder for only 2 weeks. This suggests that a longer treatment duration may be necessary to observe a significant impact on inflammation in *park13/+* flies.

The locomotion behaviour study using Fly-VRL suggested that the disease progression happened with time. Despite observing a moderate improvement in the speed and straightness of flies, no significant changes were observed in other parameters following the treatment. Based on previous literature, we hypothesis that the quantity of L-DOPA present in the *Mucuna pruriens* seed powder used in this study was inadequate to effectively rescue the locomotor function.

In summary, the findings of this study suggest that kapikachu powder may have potential therapeutic benefits for PD by increasing TAG levels and potentially managing disease progression. However, further research is required to fully understand the effectiveness of this treatment and its impact on inflammation in different PD genetic models.

5. References:

Aggarwal, A., Reichert, H., & Vijay Raghavan, K. (2019). A locomotor assay reveals deficits in heterozygous Parkinson's disease model and proprioceptive mutants in adult *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America, 116(49), 24830–24839. https://doi.org/10.1073/pnas.1807456116.

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