Original Research Article

The ameliorative effect of Ashwagandha- *Withania somnifera (L.)* Dunal on *park*^{25} induced neurodegeneration in *Drosophila melanogaster* Parkinson's disease model

ABSTRACT

Ashwagandha - *Withania somnifera (L.)* Dunal is a perennial shrub belongs to family Solanaceae. Ashwagandha has been used from over 3000 years in traditional Indian Ayurveda for treatment of various neurological, and stress disorders. The root of Ashwagandha (ASH) is regarded as tonic, aphrodisiac, narcotic, diuretic, anthelmintic, astringent, thermogenic and stimulant. The plant (aerial, root and both) is recommended for health and healing when consumed orally and on topical application. The number of single and compound formulation is prescribed rationally to treat emaciation in children, debility from old age, constipation, flatulent colic, rheumatism, vata vitiation, leucoderma, goiter, insomnia, nervous breakdown, etc. The topical application cures carbuncles, ulcers and painful swellings, boils, pimples, etc. The ASH root in combination with other drugs is recommended to treat snake venom and also scorpion-sting. Ashwagandha with other herbal decoction was recognized to treat Kampavatha (Parkinson's Disease) since 18th century. With this wide array of ethnopharmological relevance, Ashwagandha has been recognized as one of the prominent complementary and alternative medicine to treat many neurodegenerative diseases like Alzheimer's and Parkinson's disease. Parkinson's (PD) has been recognized as the second most occurring neurodegenerative disease worldwide after Alzheimer's (AD) with complex multifactoral phenomena and limited approaches for pharmacotherapy. Many genetic factors are responsible for onset and progression of PD. Loss-of-function mutations in the *parkin* gene are a major cause of early onset of autosomal recessive juvenile parkinsonism. In *Drosophila park*^{25} flies exhibits loss of function mutations and show significantly increased number of mitochondria-endoplasmic

reticulum contacts and significantly decreased number of dopaminergic neurons in the adult brain which is the main cause of PD condition. There is a prominent increase the cases of AD and PD all over the world and it demands the requirement for complementary and alternative herbal remedies with no/minimal side effects. Several studies have demonstrated the ability of Ashwagandha in imparting neuroprotection, improve locomotory ability, memory and learning abilities. The challenge lies in scrutinizing the mechanism and the pathways involved in the neuroprotective properties of this well known herbs. Here in our study we test the possible neuroprotective effect of Ashwagandha on *park*^{25} mutants of *Drosophila* using climbing ability as a disease marker.

Key words: Ashwagandha/ *Withania somnifera*, Neurodegeneration, Parkinson's disease, *Drosophila* disease model, *parkin*, *park*^{25}, Motor dysfunction, Lifespan

1. INTRODUCTION

Withania somnifera (L.) Dunal commonly known as Ashwagandha or Indian Ginseng has been widely used in Ayurveda as a nervine tonic to treat many neurological disorders like anxiety, memory loss, sleep disorders, Parkinson's etc. It is rich in active phytochemicals isolated from its root and leaves which attribute to the medicinal property of this shrub. The plant (aerial, root and both) is recommended for health and healing, and the number of single and compound formulation is prescribed rationally. The biologically active chemical constituents of *Withania somnifera* (WS) include alkaloids (isopelletierine, anaferine, cuseohygrine, anahygrine, etc.), steroidal lactones (withanolides, withaferins) and saponins [1]. Sitoindosides VII–X and Withaferin-A, have been shown to have significant anti-stress activity against acute models of experimental stress [2]. Many of its constituents support immunomodulatory actions [3]. The aerial parts of WS yielded 5-dehydroxy withanolide-R and withasomniferin-A [4]. *Withania somnifera* is rich in active phytochemicals isolated from its root and leaves which attribute to the medicinal property of this shrub. There are about 12 alkaloids, 35 withanolides and several sitoindosides have been isolated and structurally elucidated till date [1,5]. HPTLC studies quantification of withanolodes from roots measured the presence of withaferin A, 1,2 deoxywithastramonide, withanolide A and withanolide B [6]. With this wide array of ethnopharmacological relevance and medicinal property Ashwagandha has been recognized as one of the prominent complementary and alternative medicines to treat many neurodegenerative diseases like Alzheimer's and Parkinson's disease.

Parkinson's disease (PD) is a neurodegenerative disorder described by multiple motor and non-motor symptoms, affecting the socio-physical wellness of the patients [7,8]. PD is the age related progressive movement disorder, characterized by the loss of dopaminergic (DA) neurons in substantia nigra [9]. Most cases of PD are idiopathic and mitochondrial dysfunction is one of the prominent features of idiopathic PD. About 3-5% of PD cases are known to have familial form, of this genetic origin, mutation in one of the Parkinson's gene, the *parkin* contributes to approximately 50% of all early onset of PD called autosomal recessive juvenile parkinsonism (AR-JP) [10]. Parkin is an E3 ubiquitin protein ligase encoded by *parkin*. Parkin along with PINK1 have essential role in maintaining mitochondrial integrity and function [11,12,13,14]. Since, there aren't any curative treatments for PD, and having the most available treatments meant for providing only symptomatic relief, there is an increasing demand for discovery of new effective drugs for the treatment. In recent years complementary and alternative medicines have gained world-wide attention due to its health benefits and lesser or no side effects [15].

Several studies carried out in vitro and rodent models have implied the ameliorative effect of ASH on chemically induced or stress induced neurodegenerative models [16]. However, there are very few studies made on the neuroprotective effect of ASH in connection of specific disease causing genetic pathways. In this direction, *Drosophila* melanogaster, with its remarkable potential, as an *in vivo* drug screening model will be a better targeted approach with higher success rate. Mutation in the *Drosophila* ortholog of the *PRKN* gene, *parkin (park)*, has many similarities to PD patients: decreased motor function, reduced lifespan, selective loss of dopaminergic neurons, loss of olfaction, mitochondrial dysfunction and others [17,18,19]. Several studies on *Drosophila* Parkin model demonstrated the enhancement in lifespan and amelioration in climbing deficits in *parkin* mutants when supplemented with antioxidants and other molecules. The dietary supplementation of metabolite stearic acid significantly increased life span and rescued fragmented mitochondria [20], nicotine ameliorated the defect in flying ability [21], zinc chloride, ascorbic acid and N-acetylcysterine significantly increased the median half life of *parkin* mutants when supplemented with normal fly food in a dose dependant manner [22,23]. In the present study we have used $park^{25}$, a *Drosophila* model of AR-JP to study the neuroprotective effect of Ashwagandha using locomotor deficit and lifespan as parameters.

2. MATERIALS AND METHODS

2.1. Drosophila stocks and maintenance:

Wild type Oregon-K, obtained from Drosophila stock centre (DSC), University of Mysore. This served as control for all experiments. w,*park*^{25}/TM6B.GFP flies were generous gift from Dr Alex Whitworth which is previously described [24,25]. The non tubby *park*$^{25/25}$ homozygous flies were separated from *park*^{25}/TM6B tubby flies at pupal stage (here after referred to as *park*$^{25/+}$ heterozygotes). Stocks were reared at 12 hr light/dark cycle at 22°±2° C with 60% relative humidity on standard wheat cream agar medium with yeast granules.

2.2. Preparation of Ashwagandha aqueous extraction:

Ashwagandha root powder (choorna) is commercially available and purchased from The Nikhila Karnataka Central Ayurvedic Pharmacy Ltd (NKCA), Mysuru. We employed decoction and filtration method [26] to extract water soluble constituents of Ashwagandha. 10g of powder was weighed and mixed with 10 ml of water to make a thick paste. This paste was placed in a clean wet muslin cloth of 10X10 sq.cm and suspended into boiling water. The cloth was tied around the mouth of the beaker so that the middle part containing mixture is immersed in the boiling water. When the water boils, contents from the ASH were released into water. 300ml more water was added to the mixture at the intervals of 15 to 20min and stirred slowly and continuously using a glass rod. The fine powder passes through the pores of muslin cloth leaving behind large debris. This procedure was continued for one hour. The muslin cloth was removed carefully and squeezed gently to obtain maximum amount of crude extract from the powder. The extract thus obtained was boiled for another 60 to 90 minutes until a thick paste is formed. The obtained paste was weighed and stored in refrigerator for further use. Dry weight of the extract per 1g paste was calculated after every extraction.

2.3. Feeding of Ashwagandha:

In all experiments, normal wheat cream agar medium served as control medium. The Oregon-K, *park*$^{25/25}$ homozygous and *park*$^{25/+}$ heterozygous flies which fed on normal medium were referred to as negative and positive control groups respectively. Based on the obtained dry weight of ASH, an appropriate amount of crude extract was dissolved in freshly prepared standard wheat cream agar media and combined well. This referred to as treatment medium. We used 0.6% w/v of ASH-root extract treatment for all the experiments. Both homozygous and heterozygous *park25* flies were reared on 0.6% ASH treatment medium and referred to as treatment group. ASH-root extract was administered from larval to adult stage (L^+/A^+) without yeast. Flies were transferred to freshly prepared respective medium once in every 2 days.

2.4. Survival assay

Oregon-K, *park*$^{25/25}$ homozygous and *park*$^{25/+}$ heterozygous flies were collected at pupal stage to avoid the effect of anesthetic ether. After emergence (0-24h old), a total of 50 flies from each genotype were housed in a density of 10 flies per vial without yeast at 22^o C. Flies were shifted to respective fresh media vials every other day, and the number of dead flies was tabulated each day until the last fly was deceased (n=50).

2.5. Negative geotaxis Assay

We followed the procedure described by Feany and Bender with minor modifications [27]. Flies were collected at pupal stage. After eclosion (0-24 hrs old), a group of 10 age and sex matches flies were placed in a glass vial marked at 8 cm height. After 15 minutes of acclimatization, flies were gently tapped down to the bottom. The flies which escaped 8cm at 10 seconds were tabulated. The mean value was considered for each control, mutant-untreated and treatment groups (n=50).

2.6. Statistics

Statistical analyses were done using SPSS 21.0 software. Two groups were analysed using Independent t test. Data are presented as mean ± SEM. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$

3. RESULTS AND DISCUSSION

3.1. The lifespan of homozygous *park$^{25/25}$* *Drosophila* flies was marginally increased by Ashwagandha root extract supplementation:

Ageing is the greatest risk factor for the progression of AR-JP condition. The *Drosophila* null mutant *park$^{25/25}$* homozygous flies have shown shortened life span when compared to heterozygous park25 flies as well as the w^{1118} control flies [28]. In order to determine the life enhancing property of ASH on homozygous park25/park25 flies, we have supplemented flies with 0.6% w/v of ASH root extract from larval stage till the last day of their survival (L+/A+). The survival curve in **figure 1** depicts the percentage of survivorship of wildtype Oregon-K (control), homozygous *park$^{25/25}$* untreated and ASH-root extract supplemented group of flies. During the initial days, the effect of ASH was not prominent in the treated group but after the median lifespan (day8 and later) there was a significant increase in the number of flies surviving on ASH supplement, when compared to its untreated fellow group. The lifespan of ASH-root treated flies increased to 17 days from 15 days when compared to untreated group. Though there is not much difference between the untreated and treated group in considering with age, 12% of untreated flies were still alive on day 15th where as there were no survivors in the untreated group. This implies that the effect of 0.6% ASH-root extract on survival of *park$^{25/25}$* homozygotes is limited when supplemented with normal fly food.

3.2. Ashwagandha ameliorates climbing deficit in both *park25* homozygous and heterozygous flies:

Relatively low locomotory function is the major motor symptom of both familial and sporadic forms of PD. Due to the loss of dopamine producing neurons in *parkin* mutants of *Drosophila* along with other genetic and environmental factors exhibit a great decline in their climbing ability from day1 of post eclosion [25, 29,30]. In our present study, first we have used *park$^{25/25}$* homozygous *Drosophila* null mutants as a PD model to study the effect of Ashwagandha root extract treatment on the locomotor defect caused due to dopaminergic neuron degeneration. The flies were subjected to negative geotaxis assay to assess the

loss of motor activity in flies due to loss of dopaminergic neurons. Oregon-K and untreated *park*$^{25/25}$ flies served as negative and positive controls respectively. *park*$^{25/25}$ flies were subjected to 0.6% ASH-root extract treatment and referred to as treatment group. A cohort of 10 flies, in 5 trials (total 50 flies) were assayed (n=5) in each group. Age matched flies from each group were analysed for climbing performance each day from day 1 of post eclosion until day 8. The flies were acclimatized in the assay tube for about 15 minutes and later gently tapped to the bottom and allowed to climb the assay tube. The number of flies which reached/crossed 8 cm height within 10 sec were tabulated each day for control, untreated-mutant and 0.6% ASH-root extract treated groups. The results are represented in figure 2. The wild type Oregon-K (control) did not show any decline in their climbing function till the 8th day post eclosion *park*$^{25/25}$ untreated mutant flies showed adult onset of climbing disability from day 1 post eclosion with only about 61% of flies reaching 8 cm height and the progressive decline was observed each day when compared to control group. At 8th day, none of the flies from the mutant untreated group showed locomotory function and were unable to move against gravity. Interestingly, in 0.6% ASH-root extract treated group, the flies showed significantly higher performance in their climbing function when compared to its fellow untreated-mutant group. On day 1, nearly 74% were able to cross the marked target height, by the 8th day, where untreated flies completely stopped climbing, about 38% of ASH treated flies have showed significantly higher climbing ability when compared to its positive control (*park*$^{25/25}$ untreated mutant).

In case of *park*$^{25/+}$ heterozygotes, we performed locomotory assay each day from day 1 to day 20 for each control, untreated- mutant and 0.6% ASH-root extract treated group of flies. The results are represented in figure 3**.** *park*$^{25/+}$ heterozygotes itself showed better climbing ability compared to homozygous but significantly reduced performance in comparison with Oregon-K control flies. On day 1 itself, 0.6% ASH-root extract treated flies showed statistically highly significant improvement (100% climbing ability) in comparison to untreated group (77%). The progression of decline in climbing ability in untreated *park*$^{25/+}$ heterozygotes continued with age and complete decline in its climbing function was observed at 19th day. Surprisingly, the flies with 0.6% ASH-extract treatment retained their climbing ability from day1 and were significantly improved when compared to age matched untreated flies. On 19th day, where untreated heterozygotes stopped climbing, about 38% flies in the treated group still showed climbing function. Thus, our results clearly demonstrate that 0.6% ASH-extract treatment is able to provide strong protection against *park*^{25} null mutation induced neurodegeneration in *Drosophila melanogaster* model of PD.

3.3. DISCUSSION

Since there is a prominent increase in neurodegenerative diseases like Alzheimer's and PD cases all over the world, it demands the requirement for complementary and alternative herbal remedies with no/minimal side effects. Many research studies have demonstrated the ability of Ashwagandha in imparting neuroprotection, improve locomotory ability, memory and learning abilities. The challenge lies in scrutinizing the mechanism and the pathways involved in the neuroprotective properties of this well known herbs [1,2,31]. HPTLC studies quantification of ASH roots measured the presence of phytochemical active constituents like withaferin A, 1,2 deoxywithastramonide, withanolide A and withanolide B which are known to be neuroprotective [6]. Due to the ability of withanamides of ASH to cross blood brain barrier [32] and no notable toxic effect made Ashwagandha a widely used neuroprotective agent in recent decades using *in vitro* and *in vivo* models.

Parkinson's disease has been recognized as the second most occurring neurodegenerative disease worldwide with complex multifactoral phenomena and limited approaches for pharmacotherapy. Among many other genes responsible for PD, loss-of-function mutations in the *parkin* gene are a major cause of early onset of AR-JP and Parkin dysfunction may also lead to late-onset sporadic PD. Parkin is a E3 ubiquitin protein ligase, consisting of N-terminal ubiquitin like domain and C-terminal RING finger domain [33,34,35]. Parkin along with PINK1 have essential role in maintaining mitochondrial integrity and function by controlling its fusion and fission. Mitochondria being the energy producing organelle, dynamically undergo fusion and fission to maintain its structure and stability which is essential for the normal cellular function. Genetic factors MFNs and GTPase OPA1 are involved in the fusion and fission respectively. Uncontrolled or abnormal fission-fusion could lead to its damage subsequently altering its morphology and function [36]. This results in impairment in numerous cellular functions like ATP/energy synthesis, ROS control and finally leads to cellular death. To degrade these abnormal or unwanted mitochondria, a special mechanism mitophagy is involved in the turnover of deformed mitochondria. Under the abnormal cellular condition in mitochondria, PINK1 selectively recruits Parkin to damaged mitochondria and induces turnover of impaired mitochondria through an autophagy related 5 (ATG5) gene-ubiquitin like conjugation system mechanism [37]. The mitophagy machinery recognizes abnormal mitochondrial by its lost

membrane potential. The loss of membrane integrity leads to accumulation of mitochondrial dynamic control protein PINK1 on outer mitochondrial membrane (OMM). Phosphorylation of Ser65 of ubiquitin by PINK1 on OMM further recruits Parkin [38]. Parkin also contains Ser65 like domain, which is further phosphorylated by PINK1, which activates its E3 ubiquitin activity. This positive loop mechanism expands to form polyubiquitin chain which enhances parkin to ubiquinate the componts/ targets like MFN1 and MFN2 (Marf in *Drosophila*) for protosomal degradation of unwanted mitochondria. This repair machinery protects cells by avoiding the fusion of abnormal mitochondria into healthy mitochondria in the cellular environment thus protecting the cell undergo apoptosis. Any mutation in *parkin* gene disrupts this repair mechanism leading to cellular death.

In *Drosophila*, several studies demonstrated the role of Parkin in maintaining integrity of mitochondria in larval and adult stage. The *Drosophila park*^{25} flies exhibits loss of function mutations and show significantly increased number of mitochondria-endoplasmic reticulum contacts and significantly decreased number of dopaminergic PPL1 neurons in the adult brain. They also exhibit a severe disruption in the mitochondrial network structure, indirect flight muscles, accompanied by a significant reduction in ATP levels, as compared to controls [39,40]. The early onset of climbing disability in both homo and hetero park25 flies may be due to the accumulation of several of these pathogenic conditions caused due to the loss of function of *parkin*. Several *in vitro* and *in vivo* studies have demonstrated the role of *parkin* in quality control of mitochondria. Mutation in this gene greatly affects the mitochondrial stability and leads to cellular death. Parkin-mediated mitochondrial ubiquitination was observed in mitochondrial damaging agents (MPTP, Rotenone etc) treated cells and overexpression of dominant negative ubiquitin mutants prevented Parkin-induced mitophagy, which demonstrates the strong relation between mitophagy and Parkin [41,42,30]. *park*$^{25/25}$ homozygous mutants of *Drosophila* have exhibited "onion"-like and a"dumbbell" shaped mitochondrial defects in the muscle tissue of third instar larval body wall and in indirect flight muscles [43]. Studies also demonstrated that, *park*$^{25/25}$ homozygous flies have significant defects in climbing and flight ability, muscle degeneration and mitochondrial disruption, compared to heterozygotes.

Here in our study we test the possible neuroprotective effect of Ashwagandha on *park*^{25} homozygous and heterozygous null allele mutants of *Drosophila* using climbing ability as a disease marker. Parkin could be

a good therapeutic target to test any compound due to its low basal activity. A small increase in wild type Parkin activity could be sufficient to slow down the progression of sporadic forms of PD [44]. Our study demonstrated that, administration of 0.6% ASH extract could enhance the lifespan of *park*$^{25/25}$ homozygous only by 3 days, however the number of flies survived upon ASH treatment was significantly higher (13%) on 15th day when compared to untreated group. In consideration with climbing ability, *park*$^{25/25}$ homozygous flies with 0.6% ASH-root extract treatment showed statistically highly significant improvement from day 1 (61% v/s 74% between untreated and 0.6% ASH root extract treatment group) and the climbing function was significantly increased in treatment group till 8th day (0% v/s 38% between untreated and 0.6% ASH root extract treatment group) compared to its untreated fellow group flies. Similarly, in *park*$^{25/+}$ heterozygotes the climbing ability in treated flies showed statistically highly significant increase in their locomotory function when compared to *park*$^{25/+}$ heterozygotes.

Ashwagandha root extract with more than 50 phytoconstituents mainly consists Withanolides as the active steroids of ASH with structural diversity based on the nature and member of oxygenated substitutes and the saturation level of the rings. This great structural diversity of withanolides probably enables them to target different cellular degenerative molecules under diverse situations and participate in multiple biologically ameliorative functions. In our present study we demonstrated that, ASH-root extract dietary supplement is able to target mutant park25 induced neurodegeneration in *in vivo* loss of function model of *Drosophila melanogaster*.

4. CONCLUSION

In short, our study elucidates that Ashwagandha serves as a comprehensive, multipotent phytotherapeutic herb to combat neurodegeneration, targeting causative genetic conditions. Due to its low basal activity, Parkin could be a good therapeutic target to test any natural or synthetic therapeutic agent. A small increase in wild type parkin activity could be sufficient to slow down the progression of sporadic forms of PD. Our experiments demonstrated that, Ashwagandha aqueous extract treatment greatly reduces motor dysfunction caused due to loss of function of *parkin* gene. Thus, Ashwagandha with all its bioactive constituents serves as neuroprotective therapeutic agent.

ETHICAL APPROVAL

Not applicable

List of Abbreviations

AD	Alzheimer's Disease
AR-JP	Autosomal recessive juvenile parkinsonism
ASH	Ashwagandha
ATG5	Autophagy protein 5
DA	Dopaminergic neurons
GTPase	Hydrolase enzymes that bind to the nucleotide guanosine triphosphate (GTP)
HPTLC	High-performance thin-layer chromatography
L^+/A^+	Larval and adult stage
MFN	Mitofusin
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy-1
PD	Parkinson's Disease
PINK1	PTEN induced putative kinase 1
PPL1	Posterior inferior lateral protocerebrum
PRKN	Human *parkin* gene
ROS	Reactive oxygen species

w/v Weight per Volume

WS *Withania somnifera*

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FIGURES AND LEGENDS

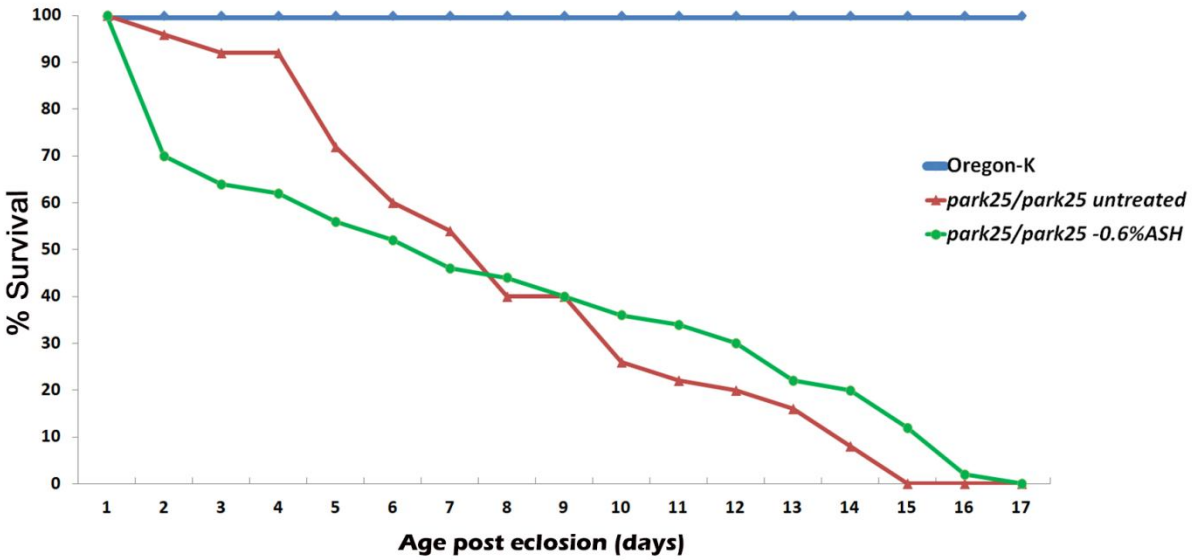

Figure 1: Ashwagandha-root extract supplementation enhances life span in *park*$^{25/25}$ homozygous null mutants of *Drosophila melanogaster*.

Graph depicts the percentage of survivorship of wild-type Oregon-K (control), homozygous *park*$^{25/25}$ untreated and *park*$^{25/25}$ flies supplemented with 0.6% w/v ASH-root extract. From day 1 to 7, the effect of ASH was not prominent in the treated group but day 8 and later there was a significant increase in the number of flies surviving on ASH supplement, when compared to its untreated fellow group. The lifespan of ASH-root treated flies increased to 17 days from 15 days when compared to untreated group. 12% of untreated flies were still alive on day 15th unlike there were no survivors in the untreated group.

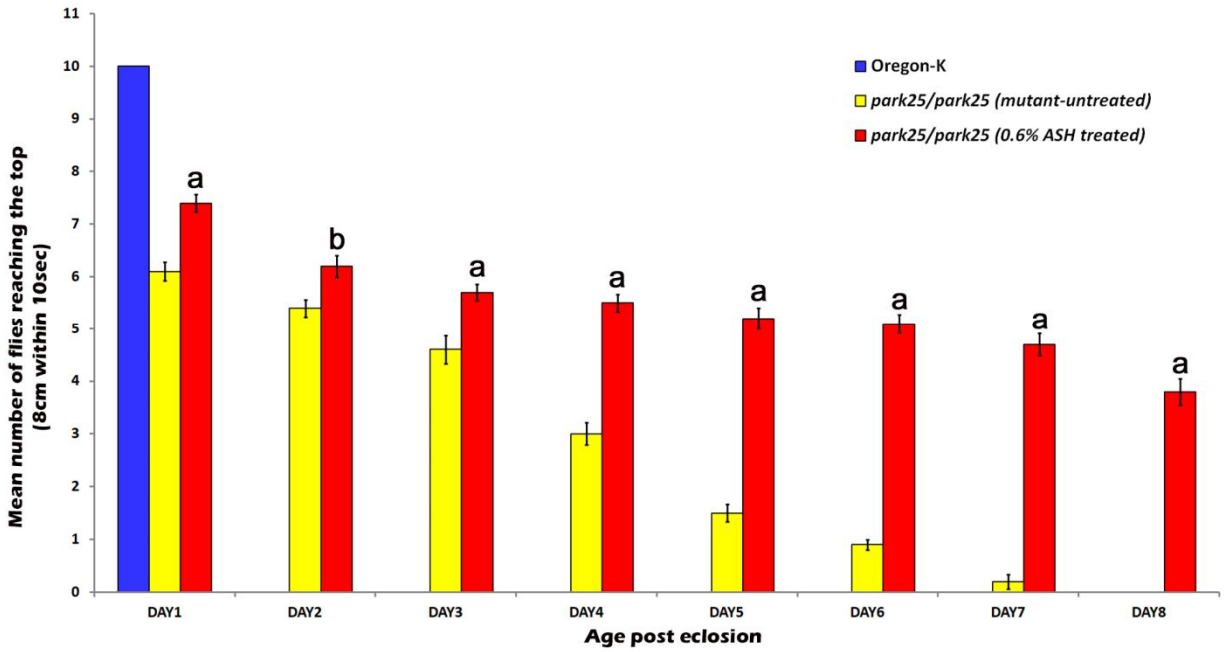

Figure 2: Negative Geotaxis assay demonstrate ameliorative effect of Ashwagandha on motor disfunction in homozygous *park*$^{25/25}$ *Drosophila*.

Graphical representation of mean number of flies reaching the top (8cm) of the assay tube within 10 sec, in negative control (Oregon-K), positive control (*park*$^{25/25}$ untreated) and the 0.6% ASH-root extract treated groups. The wild type Oregon-K (control) did not show any decline in their climbing function throughout the experiment (the day1 data (blue bar) remains the same till 8th day). Mutant *park*$^{25/25}$ untreated flies showed adult onset of climbing disability from day 1 post eclosion. (61% at top) and the progressive decline was observed each day when compared to control group. At 8th day, none of the flies from the mutant untreated group could climb the assay tube (0%). Interestingly, in 0.6% ASH-root extract treated group, the flies showed significantly higher performance in their climbing function when compared to untreated- mutant group (74% on day 1 and 38% on day 8). Bar graphs represent mean values, error bars represent standard error of the mean and asterisks indicates significant di□erence with a=***$P \leq .001$ and b=**$P \leq .01$.

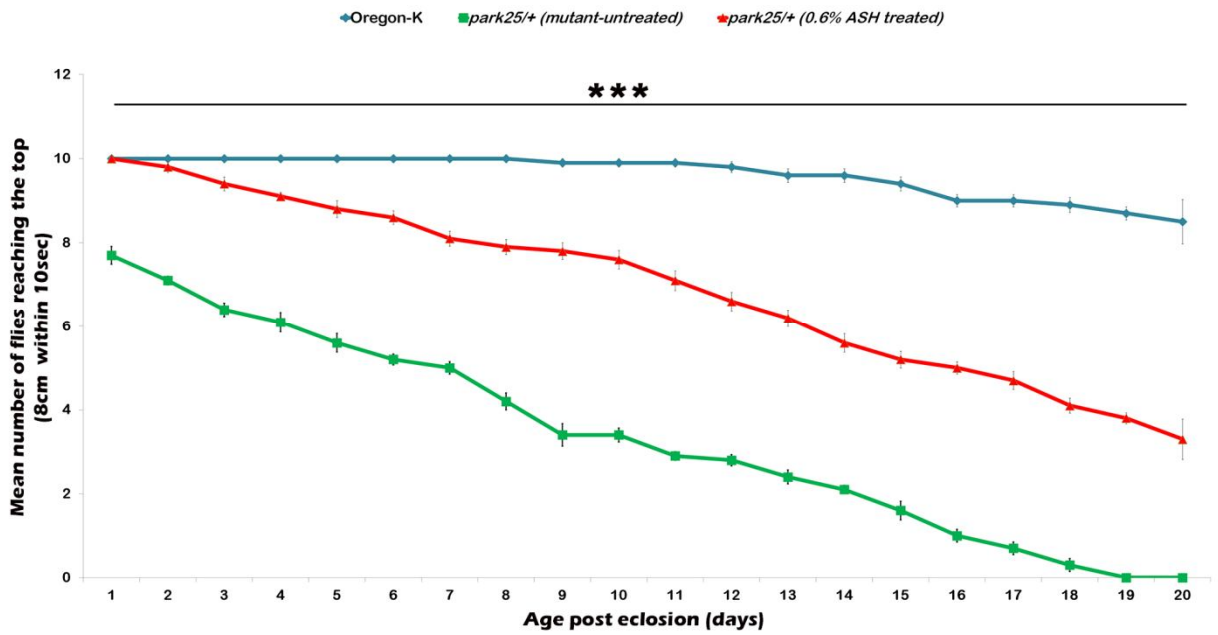

Figure 3: Ashwagandha-root extract ameliorates motor disfunction in heterozygous *park*$^{25/+}$ *Drosophila* flies.

Graphical representation of mean number of flies reaching the top (8cm) of the assay tube within 10 sec, in negative control (Oregon-K), positive control (*park*$^{25/+}$ untreated) and *park*$^{25/+}$ flies treated with 0.6% ASH-root extract. On day 1, 0.6% ASH-root extract treated flies showed statistically highly significant improvement (100% climbing ability) in comparison to untreated group (77%). The progression of decline in climbing ability in untreated heterozygotes continued with age and complete decline in its climbing function was observed at 19th day. The flies with 0.6% ASH-extract treatment retained their climbing function from day1 and were significantly improved when compared to *park*$^{25/+}$ untreated flies. On 19th day, untreated *park*$^{25/+}$ heterozygotes stopped climbing; about 38% flies were able to reach the top of the assay tube. Line graph represents mean values, error bars represent standard error of the mean and asterisks indicates significant di□erence with ***$P \leq .001$.