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The Ameliorative Effect of Ashwagandha- *Withania somnifera (L.)* Dunal on *park*^{25} Induced Neurodegeneration in *Drosophila melanogaster* Parkinson's Disease Model

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MNM, VC and HDN performed investigation, experimentation, data analysis, compilation of results, draft manuscript preparation and funding acquisition. Author BVS did the conceptualization and design of the study, supervision, critical analysis, and interpretations, critical review and editing of original draft and funding acquisition. All authors read and approved the final manuscript.

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ABSTRACT

Ashwagandha - *Withania somnifera (L.)* Dunal is a perennial shrub belonging to the family Solanaceae. Ashwagandha has been used for over 3000 years in traditional Indian Ayurveda for treatment of various neurological, and stress disorders. The root of Ashwagandha (ASH) is regarded as a tonic, aphrodisiac, narcotic, diuretic, anthelmintic, astringent, thermogenic and stimulant. Ashwagandha with other herbal decoctions was recognized to treat Kampavatha (Parkinson's Disease) since 18th century. With this wide array of ethnopharmacological relevance, Ashwagandha has been recognized as one of the prominent complementary and alternative medicine to treat many neurodegenerative diseases like Alzheimer's (AD) and Parkinson's disease (PD). There is a prominent increase in the cases of AD and PD all over the world and it demands the requirement of complementary and alternative herbal remedies with no/minimal side effects. Many genetic factors are responsible for the 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 (AR-JP). *Drosophila park^{25}* loss of function mutants exhibit significantly increased number of mitochondria-endoplasmic reticulum contacts and a significantly decreased number of dopaminergic neurons in the adult brain which is the main cause of PD condition. Several studies have demonstrated the ability of Ashwagandha in imparting neuroprotection, improved 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 herb. Here in our study, we test the possible neuroprotective effect of Ashwagandha on *park^{25}* mutants of *Drosophila* using lifespan analysis and climbing disability as a disease marker. Parkinson's mimicking flies were administered with aqueous extraction of Ashwagandha-root mixed with the fly food and subjected to negative geotaxis assay. We observed that there is a prominent increase in the climbing ability in *park^{25}* treated flies compared to its age-matched untreated flies. This is the first report showing that, aqueous extraction of Ashwagandha-root extract was able to ameliorate the disease phenotype in the *park^{25} Drosophila* Parkinson's disease model.

Keywords: Ashwagandha / Withania somnifera; neurodegeneration; Parkinson's disease; Drosophila disease model; park^{25}; motor dysfunction; lifespan.

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 treatment;
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.

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 and Parkinson's [1]. 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 number of single and compound formulations are prescribed rationally. The biologically active chemical constituents of *Withania somnifera* (WS) include alkaloids (isopelletierine, anaferine, cuseohygrine, anahygrine, etc.), steroidal lactones (withanolides, withaferins) and saponins [2]. Sitoindosides VII–X and Withaferin-A, have been shown to have significant anti-stress activity against acute models of experimental stress [3]. Many of its constituents support immunomodulatory actions [4]. The aerial parts of WS yielded 5-dehydroxy withanolide-R and withasomniferin-A [5]. There are about 12 alkaloids, 35 withanolides and several sitoindosides which have been isolated and structurally elucidated till date [2,6]. HPTLC quantification of withanolides from Ashwagandha roots measured the presence of withaferin A, 1,2 deoxywithastramonide, withanolide A and withanolide B [7]. With this wide array of pharmacological 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 [8,9]. PD is age related progressive movement disorder, characterized by the loss of dopaminergic (DA) neurons in substantia nigra [10]. 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 a familial form, and of this, mutation in one of the Parkinson's genes, the *parkin* contributes to approximately 50% of all early onset of PD called autosomal recessive juvenile parkinsonism (AR-JP) [11]. Parkin is an E3 ubiquitin protein ligase encoded by *parkin*. Parkin and PINK1 (PTEN induced putative kinase 1) have essential role in maintaining mitochondrial integrity and function [12,13,14,15]. 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 the discovery of new effective drugs for treatment. In recent years complementary and alternative medicines have gained worldwide attention due to their health benefits and lesser or no side effects [16].

Several studies carried out *in vitro* and in rodent models have implied the ameliorative effect of ASH on chemically induced or stress-induced neurodegenerative models [17]. However, there are very few studies made on the neuroprotective effect of ASH in connection with specific disease causing genetic mutations. In this direction, *Drosophila melanogaster*, with its remarkable potential, as an *in vivo* drug screening model can be a better targeted approach with a higher success rate. Mutation in the *Drosophila* ortholog of *PRKN* gene, *parkin (park)*, shows many similarities to PD patients: decreased motor function, reduced lifespan, selective loss of dopaminergic neurons, loss of olfaction, mitochondrial dysfunction and defects in indirect flight muscle [18,19,20]. Several studies on *Drosophila* Parkin model have demonstrated the enhancement in lifespan and amelioration in climbing deficits in *parkin* mutants when supplemented with antioxidants: the dietary supplement of metabolite stearic acid significantly increased life span and rescued fragmented mitochondria [21], nicotine ameliorated the defect in flying ability [22], 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 [23,24]. In the present study, we have used $park^{25}$ *, a Drosophila* model of AR-JP to study the ameliorative effect of Ashwagandha-root extract 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. These flies served as control for all experiments. w;$park^{25}$/TM6B.GFP [25,26] flies were generous gift from Dr Alex Whitworth. 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

Pure root powder of Ashwagandha is commercially available and purchased from The Nikhila Karnataka Central Ayurvedic Pharmacy Ltd (Batch number EM-4-16), Mysuru. We employed decoction and filtration method [27] 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. 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 was 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 per 1gm wet extract, an appropriate amount of crude extract was dissolved in freshly prepared standard wheat cream agar media and mixed well. This is referred to as treatment medium. Based on our earlier pilot study, we used 0.6% w/v of ASH-root extract treatment for all the experiments. Both homozygous and heterozygous *park*^{25} 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 at 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 [28]. Flies were collected at pupal stage. After eclosion (0-24 hrs old), a group of 10 age and sex matched 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 within 10 seconds were counted and tabulated. The mean value was considered for each control, mutant-untreated and treatment groups (n=50).

2.6 Statistics

Statistical analyses were done using Statistical Package for Social Sciences (SPSS), Version. 21.0 software. Two groups were analysed using Independent t test. Data are presented as mean ± SEM. Significance level was marked based on probability values (*P*). $^{**}P \leq .01$ and $^{***}P \leq .001$.

3. RESULTS AND DISCUSSION

3.1 The Lifespan of Homozygous *park*$^{25/25}$ *Drosophila* Flies was Increased by Ashwagandha-Root Extract Supplement

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 *park*^{25} flies as well as the w^{1118} control flies [29]. In order to determine the life enhancing property of ASH on homozygous *park*^{25}/^{25} 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 results showed increase in the total lifespan as well as the survivorship in treated group as compared to the untreated group. The survival curve in Fig. 1 depicts the percentage of survivorship of wild-type 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 (day 8 and later) there was a significant increase in the number of flies surviving on ASH supplement each day when compared to its untreated fellow group. The 0.6% ASH-root treated flies showed extended life span by about 14% (17 days v/s 15 days) when compared to untreated group. In considering with the survivorship, about 12% of untreated flies were still alive on day 15th whereas there were no survivors in the untreated group. This implies that the age related decline in lifespan and survivorship of *park*$^{25/25}$ homozygous *Drosophila* flies are corrected by 0.6% ASH-root extract treatment.

3.2 Ashwagandha ameliorates climbing deficit in both *park*^{25} Homozygous and Heterozygous flies

A relatively low locomotory function is the major motor symptom of both familial and sporadic forms of PD [8,9,11]. Due to the loss of dopamine producing neurons and muscular degeneration in *parkin* mutants of *Drosophila,* along with other genetic and environmental factors exhibit a great decline in their climbing ability from day 1 of post eclosion [26,30,31]. 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 loss of *park*^{25} gene function. The flies were subjected to negative geotaxis assay to assess the loss of motor activity in the flies. 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 was 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

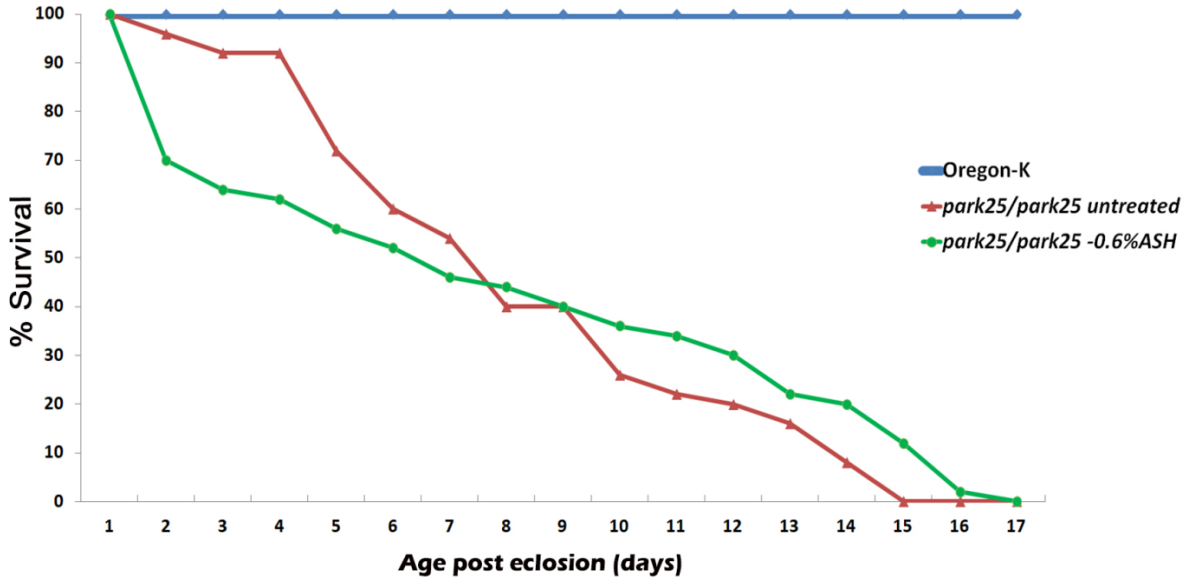

Fig. 1. Ashwagandha-root extract supplementation enhances life span in *park*$^{25/25}$ homozygous null mutants of *Drosophila melanogaster*. Graph depicts the percentage of survivorship of wild-type Oregon-K (control), homozygous *park*$^{25/25}$ untreated and *park*$^{25/25}$ flies supplemented with 0.6% w/v ASH-root extract. From day 1 to 7, the effect of ASH was not prominent in the treated group but on 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 the untreated group. 12% of untreated flies were still alive on day 15th unlike there were no survivors in the untreated group

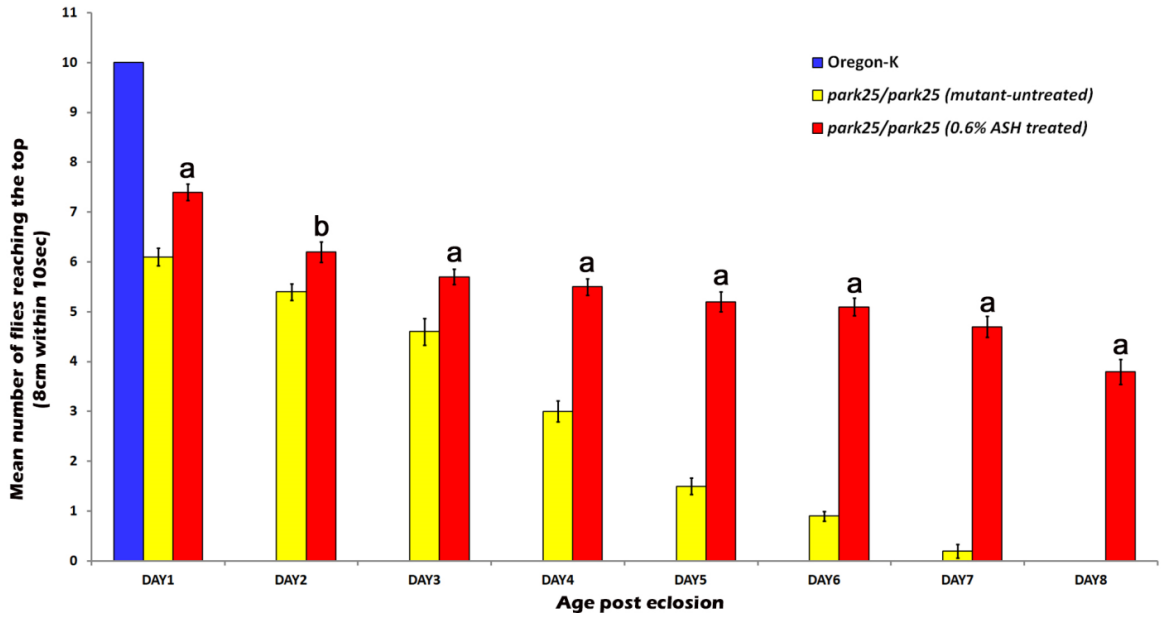

Fig. 2. Negative Geotaxis assay demonstrates the ameliorative effect of Ashwagandha on motor dysfunction in homozygous *park*$^{25/25}$ *Drosophila.* Graphical representation of the mean number of flies reaching the top (8 cm) of the assay tube within 10 sec, in negative control (Oregon-K), positive 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 day 1 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 the 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 indicate significant difference with a=*$P \leq .001$ and b=**$P \leq .01$**

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 was tabulated each day for the control, untreated-mutant and 0.6% ASH-root extract treated groups. The results are represented in Fig. 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 that of the control group. On the 8th day, none of the flies from the mutant untreated group could climb to the top, as they were unable to move against gravity. Interestingly, in the 0.6% ASH-root extract treated group, the flies showed significantly higher performance in their climbing ability when compared to their 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.

In case of *park*$^{25/+}$ heterozygous flies, 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 Fig. 3. *park*$^{25/+}$ heterozygous flies showed better climbing ability compared to that of homozygous flies, but had 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

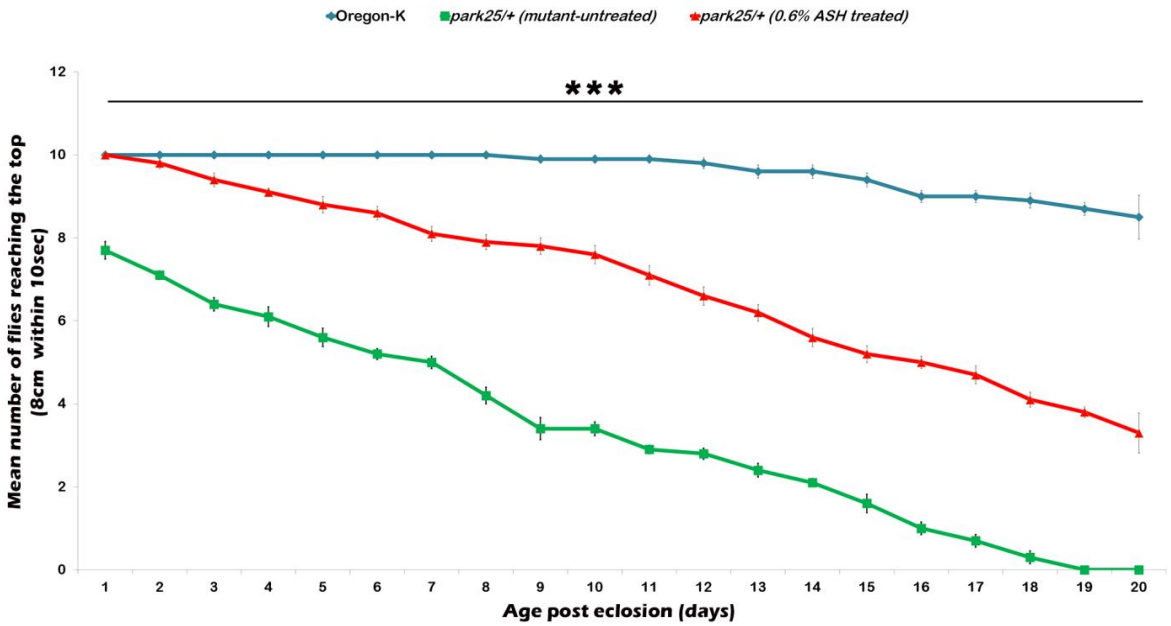

Fig. 3. Ashwagandha-root extract ameliorates motor dysfunction in heterozygous *park*$^{25/+}$ *Drosophila* flies. Graphical representation of the mean number of flies reaching the top (8cm) of the assay tube within 10 sec, in the negative control (Oregon-K), positive control (*park*$^{25/+}$ untreated) 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 on the 19th day. The flies with 0.6% ASH-extract treatment retained their climbing function from day 1 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. The line graph represents mean values, error bars represent standard error of the mean and asterisks indicates significant difference with ***$P \leq .001$

comparison to the untreated group (77%). The progression of decline in climbing ability in untreated *park*$^{25/+}$ heterozygotes continued with age and a complete decline in its climbing function was observed on the 19th day. Surprisingly, the flies with 0.6% ASH-extract treatment continued to retain their climbing ability which significantly improved, when compared to age matched untreated flies. On the 19th day, whereas untreated heterozygotes stopped climbing, about 38% of flies in the treated group were able to climb the top. Thus, our results clearly demonstrate that 0.6% ASH-extract treatment is able to provide strong protection against *park*^{25} null mutation induced motor disability in *Drosophila melanogaster* model of PD.

3.3 Discussion

Since there is a prominent increase in neurodegenerative diseases like Alzheimer's and Parkinson's all over the world, it demands the requirement for complementary and alternative herbal remedies with no/minimal side effects. Many studies have demonstrated the ability of Ashwagandha to impart neuroprotection, and improve locomotory ability, memory and learning abilities. The challenge lies in scrutinizing the mechanisms and the pathways involved in the neuroprotective properties of this well known herb [2,3,32]. 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 [7]. Due to the ability of withanamides of ASH to cross the blood-brain barrier [33] and no notable toxic effect made Ashwagandha a widely used neuroprotective agent in recent decades using *in vitro* and *in vivo* models of neurodegenerative diseases [3].

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 [34,35,36]. Parkin along with PINK1 has an 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 normal cellular function. Uncontrolled or abnormal fission-fusion could lead to its damage subsequently altering its morphology and function. This results in impairment in numerous cellular functions like ATP/energy synthesis, ROS control and finally leads to cellular death [37]. Function of Parkin in association with the PINK1 protein is necessary in the cellular mitochondria, to regulate proteasomal degeneration of abnormal mitochondria. Thereby the fusion of unhealthy mitochondria with healthy, functional mitochondria is prevented. This rescues the cells from mitochondrial dysfunction induced apoptosis. Any mutation in the *parkin* gene disrupts this repair mechanism leading to cellular death [38,39]. *park*$^{25/25}$ homozygous mutants of *Drosophila* have exhibited "onion"-like and a"dumbbell" shaped defective mitochondria in the muscle tissue of third instar larval body wall and in indirect flight muscles of the adults [40]. Studies also have demonstrated that *park*$^{25/25}$ homozygous flies have significant defects in climbing and flight ability, muscle degeneration and mitochondrial disruption, compared to heterozygotes [40].

In *Drosophila*, several studies have demonstrated the role of Parkin in maintaining the integrity of mitochondria in larval and adult stages. The *Drosophila park*^{25} flies show a significantly increased number of mitochondria-endoplasmic reticulum contacts and a significantly decreased number of dopaminergic PPL1 neurons in the adult brain [39]. They also exhibit a severe disruption in the mitochondrial network structure and indirect flight muscles, accompanied by a significant reduction in ATP levels, as compared to controls [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*. Parkin-mediated mitochondrial ubiquitination was observed in mitochondrial damaging agents (MPTP, Rotenone) treated cells and overexpression of dominant negative ubiquitin mutants prevented Parkin-induced mitophagy, which demonstrates the strong relationship between mitophagy and Parkin [41,42,30]. In consideration with these information, we tried to decipher the ameliorative effect of Ashwagandha on lifespan and climbing dysfunction as parameters.

The primary risk factor for the onset of the AR-JP condition is ageing. Oxidative stress is the major cause in accelerating age by causing damage in DNA, proteins, and cells. The two main evolutionary theories to explain the fundamental mechanisms of the changes that take place with ageing are mutation accumulation and antagonistic pleiotropy [41,42]. It has been discovered that *Drosophila* laboratory stocks that have been selected for longer lifespans have increased tolerance to various types of stress, such as heat, starvation, desiccation, oxidative damage, etc [43,44,45]. *The 'ageing gene' methuselah, Indy, InR, chico, and superoxide dismutase* are thought to increase *Drosophila* lifespan by up to 85% [46]. *Drosophila melanogaster* has a number of advantages for ageing research, including a short lifespan (mean lifespan, 2-3 months), low maintenance requirements, an array of genetic resources, and ease of genetic manipulation [47].

In our experiment, *park*^{25} homozygous flies exhibited a reduced lifespan about 15 days under normal laboratory conditions. These flies when supplemented with 0.6% of Ashwagandha with fly food, showed prominently increased survivorship. The effect of ASH was seen after the median lifespan (day 8 and later). There was a significant increase in the number of flies surviving on ASH-root extract 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 the untreated group. While the untreated and treated groups did not differ significantly in terms of age (in days), about 12% of the untreated flies were still alive on day 15, while there were no survivors in the untreated group. This suggests that when added to regular fly food, the effect of 0.6% ASH-root extract on the survival of *park*$^{25/25}$ homozygotes is minimal.

In our study, we tested the possible neuroprotective effect of Ashwagandha on *park*^{25} homozygous and heterozygous null allele mutants of *Drosophila* using climbing ability as disease parameter. Parkin could be a good target to test the neuroprotective efficacy of 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 [48]. In consideration of climbing ability, *park*$^{25/25}$ homozygous flies with 0.6% ASH-root extract treatment showed statistically highly significant improvement in climbing ability and survivorship from day 1 and the climbing function was significantly increased in the treatment group till 8th day compared to its untreated fellow group flies. Similarly, in *park*$^{25/+}$ heterozygotes, the climbing ability in treated flies showed a statistically highly significant increase in their locomotory function when compared to untreated *park*$^{25/+}$ heterozygotes.

The *Drosophila park*^{25} null mutant flies exhibit a severe disruption in the mitochondrial structure. Due to the failure in mitophagy, a mitochondrial degradation mechanism in the cell leads to the accumulation of abnormal mitochondria and its fusion with normal healthy mitochondria. This leads to the building up of ROS, failing to rescue the cells from free radicals [18,19]. Secondly, indirect flight muscles, which have a significant number of mitochondria for continuous energy production are largely affected by *parkin* mutation with significant reduction in ATP levels, as compared to controls [49,50,51,52]. Last, but not least, there is a significantly decreased number of dopaminergic PPL1 neurons in the adult brain of *parkin Drosophila* mutants [19,20]. We assume that the early onset of climbing deficit in *park*^{25} flies might be due to the accumulation of these several pathogenic conditions in mitochondria finally leading to cellular death. Our experimental results in both lifespan measurement and locomotory function, suggest that ASH dietary supplement is able to increase the lifespan and survivorship in *park*^{25} homozygous flies and enhancement in climbing ability in both homozygous and heterozygous *park*^{25} flies. Ashwagandh may address one or many of these multiple pathogenic conditions like increased oxidative stress, mitochondrial fission-fusion dysfunction, dysregulated mitophagy and degeneration of dopaminergic PPL1 neurons in *park*^{25} null mutant of *Drosophila melanogaster*.

4. CONCLUSION

The applications of herbal remedies are growing along with the modern medicine. The herb Ashwagandha has much of potential as a safe and effective neuroprotective agent [7]. Ashwagandha serves as a comprehensive, multipotent phytotherapeutic formulation to combat neurodegeneration, targeting the causative genetic conditions. Parkin might make a good therapeutic target to test any natural or synthetic therapeutic agent because of its low basal activity [48]. Our experiments demonstrated that Ashwagandha aqueous extract treatment greatly reduces motor dysfunction caused due to loss of function of the *parkin* gene. Thus, the different bioactive compounds present in the whole Ashwagandha extract function in synergy to promulgate lifespan promoting effects and neuroprotection. Our results provide a basis for further investigation into the mechanism of action of Ashwagandha in targeting biomolecules in disease causing pathway, and HPLC analysis of active component which binds and bring about neuroprotection.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMNTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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