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Effect of *Withania somnifera* leaf extract on the dietary supplementation in transgenic *Drosophila* model of Parkinson's disease

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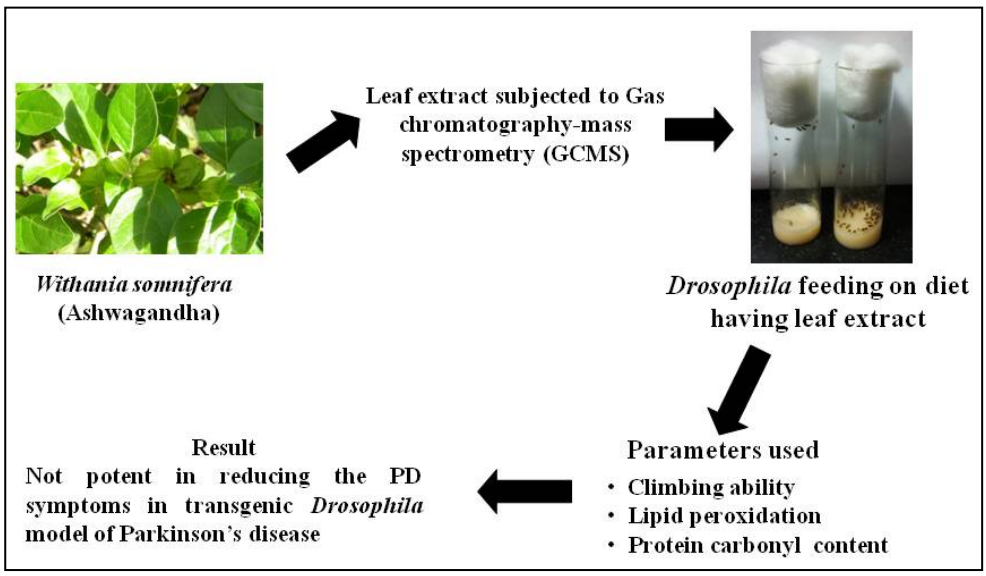
Graphical Abstract



Abstract: The role of *Withania somnifera* L. leaf extract was studied on the transgenic *Drosophila* model flies expressing normal human alpha synuclein (h-αS) in the neurons. The leaf extract was prepared in acetone and was subjected to GC-MS analysis. *W. somnifera* extract at final concentration of 0.25, 0.50 and 1.0 µL/mL was mixed with the diet and the flies were allowed to feed for 24 days. The effect of extract was studied on the climbing ability, lipid peroxidation and protein carbonyl content in the brains of transgenic *Drosophila*. The exposure of extract to PD model flies did not show any significant delay in the loss of climbing ability nor reduced the oxidative stress in the brains of transgenic *Drosophila* as compared to untreated PD model flies. The results suggest that *W. somnifera* leaf extract is not potent in reducing the PD symptoms in transgenic *Drosophila* model of Parkinson's disease.

Keywords: *Withania somnifera*; Lipid peroxidation; Protein carbonyl content; *Drosophila*; Climbing ability.

Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the progressive loss of the dopaminergic neurons of substantia nigra pars compacta in the ventral midbrain.[1] One of the pathological features of PD is the presence of Lewy bodies.[2] The function of alpha synuclein (αS) is not fully understood but it has been reported to bind with lipid membranes, forming an amphipathic helix.[3] It has been suggested that there may be synaptic role for αS.[4] Lewy bodies contain other proteins including neurofilaments and other cytoskeletal proteins, suggesting the presence of co-precipitants that might be important in aggregation.[4] The aggregation of αS leads to the toxicity and oxidative stress,[5] but it is still unclear whether misfolded proteins directly cause toxicity or damage cells via the formation of protein aggregates.[6] The neurons being lost as the progression of PD have been reported to generate endogenous toxins (hydrogen peroxides) and free radicals that may further lead to the loss of neurons.[7] In recent years, use of natural antioxidants from food and other biomaterials have been increased due to their presumed safety, nutritional and therapeutic values.[8] Ayurveda has been in practice in India for more than 3500 years, and traditional healers have used this system since time immemorial for the benefit of mankind.[8] The dietary habits are specific to population and vary widely hence it is necessary to study the disease- prevention potential of functional micronutrients in the regional diet.[9] *Withania somnifera* or Ashwagandha is widely used in Ayurvedic medicine and the traditional medical system of India.[10] *W. somnifera* (Indian Ginseng) is a subtropical under shrub that belongs to the family Solanaceae.[11] Its root powder has been reported to posses free radical scavenging,[12] improvement of motor neurons function,[13] formation of dendrities,[14] stimulating thyroid function,[15] inhibition of tumor cell lines[16] catecholamines and physiological abnormalities in PD model mouse,[13,17] inhibits amyloid-β fibril,[18] anti-diabetic,[19] antigenotoxic,[20] and other pharmacological properties. Most of the studies have been performed on the root extract. However, little is known about possible effects of leaf extract. Hence**,** an attempt has been made to study the effect of the leaf extract of *W. somnifera* on the PD model transgenic flies.

Experimental

Preparation of leaf extract: The leaves of *W. somnifera* were collected from the nursery of Forest Research Institute (FRI), Dehradun (Accession No: 143201).The extract was prepared according to the protocol described in **an** earlier published work.[21]

Analysis of *W. somnifera* extract through GC-MS: GC-MS analysis was performed using Trace GC ultra gas chromatograph connected to a Quantum XLS mass spectrometer (Thermo Scientific, FL, USA). GC was equipped with TG-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) consisting of a stationary phase 5% phenyl and 95% methyl polysiloxane. The injection was carried out in CT splitless mode at an injector temperature of 260°C Helium gas used as a carrier gas with a flow rate of 1.1 mL/min. The oven temperature programming was as follows: the initial oven temperature was held at 70°C for 2.0 min, and then increased to 210°C at a rate of 20°C/min and then increased to 290°C at a rate of 10°C/min held for 13.0 min. The ion source and transfer line temperature were 220°C and 290°C respectively. Identification of the compounds was performed by comparing their mass spectra with the NIST library available in the instrument.

***Drosophila* stocks:** Transgenic fly lines that expresses wild-type h-αS under UAS control in neurons ''(w[*]; P{w[+mC]=UAS–Hsap/SNCA.F}''5B and GAL4 ''w[*]; P{w[+mC]=GAL4- elavL}''3 were obtained from Bloomington *Drosophila* stock center (Indiana University, Bloomington, IN). When the males of UAS (upstream Activation Sequence)-Hsap/SNCA.F strains are crossed with the females of GAL4-elav. L (vice-versa) the progeny will express the human αS in the neurons.[22]

***Drosophila* culture and crosses:** The flies were cultured on standard *Drosophila* food containing agar, corn meal, sugar and yeast at 25°C (24 ± 1).[23] Crosses were set up as described in an earlier published work.[23] First, the climbing assay was

performed for the PD flies and UAS-Hsap/SNCA.F (control). The other group of PD flies was exposed separately to different doses of *W. somnifera* extract mixed in the culture medium. *W. somnifera* extract was added in the medium at final concentration of 0.25, 0.50 and 1.0 µL/mL. The PD flies were also exposed to 10^{-3} M of L-dopamine. The UASHsap/ SNC.F acts as a control. The control flies were separately exposed to the selected doses of *W. somnifera* extract.

Drosophila **climbing assay:** The climbing assay was performed as described by Pendleton et al.[24] Ten flies were placed in an empty glass vial (10.5 cm × 2.5 cm). A horizontal line was drawn 8 cm above the bottom of the vial. After the flies had been acclimated for 10 min at room temperature, both controls and treated groups were assayed at random, to a total of 10 trails for each. The mean values were calculated and then averaged, and a group mean and standard error were obtained. All behavioral studies were performed at 25°C under standard lightning conditions.

Lipid peroxidation assay: Lipid peroxidation assay in the brain homogenate was performed according to the procedure described by Siddique et al.[25] Reagent 1 (R1) was prepared by dissolving 0.064 g of 1-methyl-2-phenylindole into 30 mL of acetonitrile to which 10 mL of methanol was added to bring the volume to 40 mL. The preparation of 37% HCl served as the reagent R2. The brain of flies was isolated under stereozoom microscope in ice cold Tris HCl (20 mM) (10 brain/group; five replicates/group). Homogenate was prepared in Tris HCl and centrifuged at 3000g for 20 min and subsequently the supernatant was collected. In a microcentrifuge tube 1300 µL of R1 was taken. A volume of 1µL of supernatant was added along with 300 µL of R2 vortexed and incubated at 45°C for 40 min. After incubation, the tubes were cooled in ice and centrifuged at 15,000g for 10 min at 4°C. All samples were read at 586 nm.

Estimation of Protein Carbonyl content: The protein carbonyl content was estimated according to the protocol described by Hawkins et al.[26] The brain homogenate was diluted to a protein concentration of approx. 1mg/mL. About 250µL of each diluted homogenate were taken in eppendorf centrifuge tubes separately. To it 250µL of 10mM 2, 4-dinitrophenyl hydrazine (dissolved in 2.5M HCl) was added, vortexed and kept in dark for 20 min. About 125µL of 50% (w/v) trichloroacetic acid (TCA) was added, thoroughly mixed and incubated at -20°C for 15min. The tubes were then centrifuged at 4°C for 10 min at 9000 rpm. The supernatant was discarded and the pellet obtained was washed twice by ice cold ethanol: ethylacetate (1:1). Finally**,** the pellets were re-dissolved in 1mL of 6M guanidine hydrochloride and the absorbance was read at 370nm.

Statistical analyses: The statistical analyses were done using Statistica Soft Inc. The mean values of various fly groups were statistically compared using an unpaired group of the student ‘‘t’’-test.

Results and Discussion

The compounds present in acetone extract of leaves of *W. somnifera* were identified by GC-MS analysis (Figure 1).

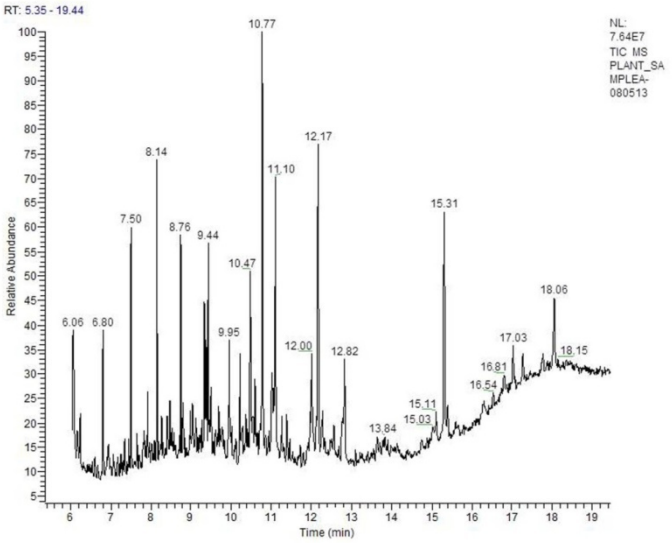


Figure 1. GC chromatogram of acetone extract of leaves of *Withania somnifera*.

The GC-MS chromatogram shows the presence of 2 major compounds with highest concentrations. Their retention times (RT), molecular formula and molecular weight (MW) in the leaves of *W. somnifera* are presented in Table 1.

Table 1. The active principles in the leaf extract of *Withania somnifera* with their retention time (RT), molecular formula and molecular weight.

No.	RT	Name of the compound	Molecular formula	MW
1	**7.65**	**Benzene, 1,1'-oxybis**	$C_{14}H_{14}O$	**198.26**
2	**10.22**	**Ethanol, 2-(9-octadecenyloxy)-**	$C_{20}H_{40}O_2$	**312.53**

The GC-MS analysis revealed that the acetone extract is mainly composed by dibenzyl ether and arachidonic acid or phytanic acid. Figures 2 A and B show the fragmentation pattern of mass spectrum and structures of the compounds extracted from the leaf extract and confirmed through NIST library of GC-MS. Those peaks matching similarity index (SI) greater than 70% in NIST library were assigned. Some of the major peaks are either column bleeding or impurities in plant extract. Mass spectra of two compounds extracted from plant leaf with similarity index (SI) greater that 70% confirmed by NIST library.

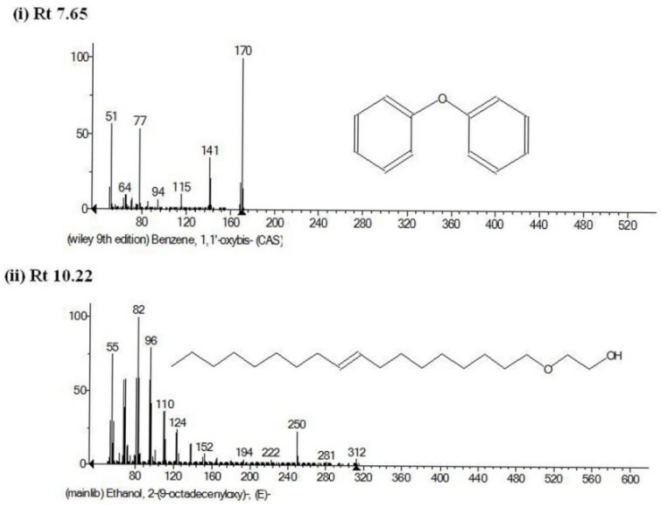


Figure 2. (i and ii) Mass spectra of acetone extract with its products having the high concentration compared with NIST library.

The climbing response of control flies did not change over 24 days in a time course evaluation (Figure 3).

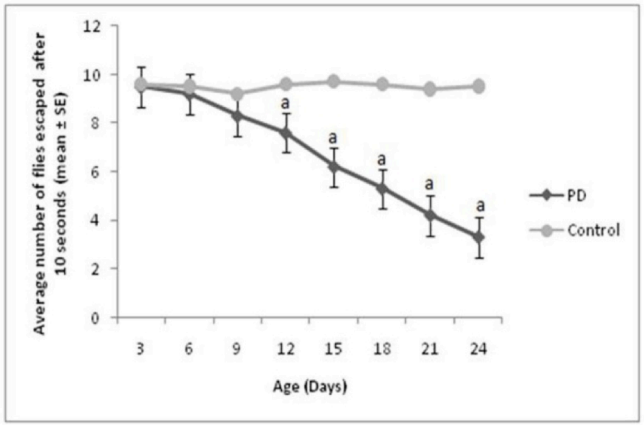


Figure 3. Climbing ability in Parkinson disease (PD) flies and control for a period of 24 days. The values are the mean of five assays ([a]Significant with respect to control $p < 0.05$).

From the day 12 on**,** however, the response of the PD flies were significantly lower as compared to the control ($p<0.05$). The climbing assay was performed after 24 days of the exposure to various doses of *W. somnifera*. The exposure of PD flies to 0.25, 0.50 and 1.0 µL/mL of *W. somnifera* did not show any significant delay in the loss of climbing ability as compared to untreated PD flies (Figure 4). The mean climbing ability of control flies was 9.6 ± 0.26 (Figure 4). The PD flies and the PD flies with L-Dopa were associated with the mean climbing ability of 3.2 ± 0.06 and 6.7 ± 0.17 respectively (Figure 4). The control flies treated with 0.25, 0.50, and 1.0µl/ml of *W. somnifera* leaf extract were associated with mean climbing ability of 9.4 ± 0.28, 9.2 ± 0.23 and 9.5 ± 0.32, respectively (Figure 4).

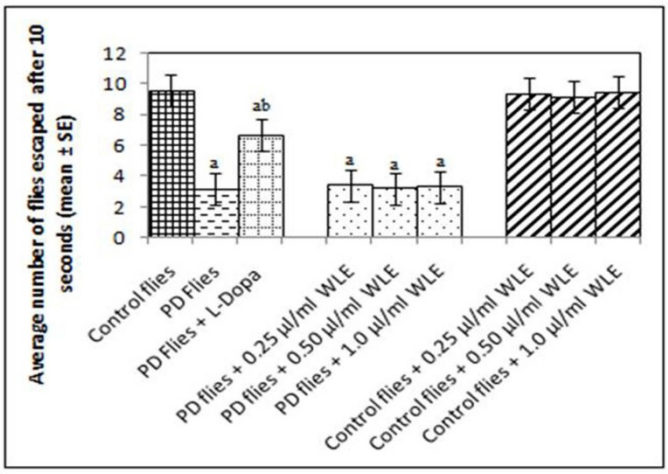


Figure 4. Effects of *Withania somnifera* on the climbing ability. The flies were allowed to feed on the diet supplemented with *Withania somnifera* for 24 days and then assayed for climbing ability. The values are the mean of five assays. ([a]significant with respect to control $p < 0.05$; [b]significant with respect to PD Flies $P < 0.05$). (PD – Parkinson disease model flies).

The mean absorbance values for the estimation of lipid peroxidation for the control, PD flies and PD flies with L-Dopa were, 0.10 ± 0.001, 0.98 ± 0.049 and 0.32 ± 0.029 respectively, (Figure 5). The treatments of 0.25, 0.50 and 1.0 µL/mL of *W. somnifera* extract to PD were associated with the mean absorbance values of 0.94 ± 0.063, 0.99 ± 0.073 and 0.94 ± 0.076 and**,** with control**,** the values were 0.11 ± 0.001, 0.12 ± 0.003 and 0.10 ± 0.004, respectively (Figure 5).

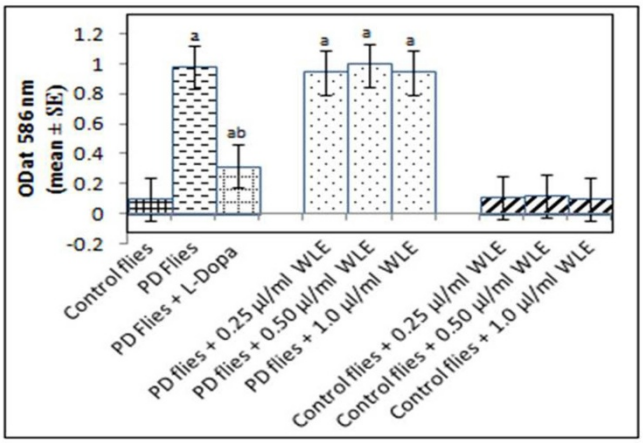


Figure 5. Effect of Withania somnifera on lipid peroxidation measured in the brain of transgenic Drosophila melanogaster after 24 days in various treated groups. [a) insignificant with respect to control $p<0.05$; b) significant with respect to PD model flies $p<0.05$].

No significant decrease in the mean absorbance values for lipid peroxidation was observed after the exposure to various doses of *W. somnifera* extract as compared to untreated PD flies (Figure 5).

The mean absorbance values for the estimation of protein carbonyl content are shown in figure 6. The control flies, PD flies and PD flies + L-Dopa were associated with 0.114 ± 0.0017, 0.181 ± 0.0015 and 0.136 ± 0.0019 respectively (Figure 6). The PD flies treated with 0.25, 0.50, and 1.0 µl/ml of *W. somnifera* leaf extract were associated with the mean values of 0.180 ± 0.0019, 0.183 ± 0.0020 and 0.181 ± 0.002, respectively (Figure 6). The control flies treated with. 0.25, 0.50, and 1.0µl/ml of *W. somnifera* leaf extract were associated with the mean values of 0.112 ± 0.0011, 0.113± 0.0016 and 0.112 ± 0.001, respectively. No significant decrease in the mean absorbance value was observed as compared to PD flies, at the exposure to 0.25, 0.50 and 1.0 µL/mL of *W. somnifera* extract (Figure 6).

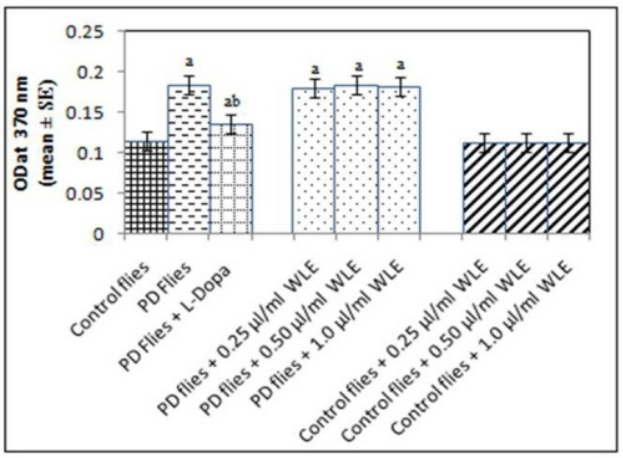


Figure 6. Effect of *Withania somnifera* on Protein Carbonyl content measured in the brain of transgenic *Drosophila melanogaster* after 24 days in various treated groups.[[a]significant with respect to control $p<0.05$; [b]significant with respect to PD model flies $p<0.05$].

The results of the present study reveals that the leaf extract of *W. somnifera* is not potent in reducing the PD symptoms in the transgenic flies expressing human α-synuclein. The accumulation of Lewy bodies together with the loss of dopaminergic neurons and the loss of climbing ability has been reported in the transgenic flies.[22]

In our present study the PD flies showed a progressive loss in the climbing ability as the age of the flies progresses. At 24th day the climbing ability of the flies were at the lowest**,** hence the duration of the treatments was selected for 24 days. The dietary supplementation of *W. somnifera* leaf extract did not show any significant delay in the loss of climbing ability**,** indicating that the extract is not potent in delaying the PD symptoms. The accumulation of the Lewy bodies leads to the toxicity and oxidative stress[6]. The damaging neurons as result of PD have been reported to generate hydrogen peroxide and free radicals[7]. Various studies on the experimental models suggest that the oxidative stress plays an important role in neurodegenerative diseases. Hence**,** the lipid peroxidation (LPO) and protein carbonyl content (PC) was measured in the *Drosophila* brains as a marker of oxidative stress. LPO represents a reliable marker of free radical generation and indicates the membrane damage[4]. Oxidative stress leads to the damage of lipid, protein and DNA[27]. The present method used for the estimation of lipid peroxidation is based on the reaction of malondialdehyde (MDA) with 1-methyl-2-phenylindole at 45°C. Two molecules of 1-methyl-2-phenylindole react with one molecule of MDA to form a stable chromophore**,** having a maximal absorbance at 586nm[28]. The results obtained for lipid peroxidation showed no reduction in the PD model flies treated with *W. somnifera* extract. PC content indicates the protein oxidation by the free radicals or ROS. The oxidation of protein results in the generation of carbonyl (CO) groups on protein side chains and is widely used as a marker of oxidative stress. The treatment of *W. somnifera* did not show any reduction in the oxidative stress markers used in the present study, hence suggesting that *W. somnifera* leaf extract has no effect on the oxidative stress. The results of our present study support the study carried out by Kaur et al[29]. The leaf extract was reported to be anti- proliferative but not anti- oxidative. The therapeutic intervention that could effectively decelerate the rate of degeneration within the substantia nigra pars compacta could add years of mobility and reduce morbidity associated with PD[7]. Hence the attention has been directed towards the identification of the inhibitors of αS aggregations or free radicals scavengers[30]. In this regard the leaf extract of *W. somnifera* did not show any protective effect against the PD symptoms in the transgenic model flies expressing h- αS. In mouse model of Parkinson's disease the leaf extract has shown to increase the levels of superoxide dismutase, catalase and malondialdehyde, reduced levels of glutathione and glutathione peroxidase in the mid brain and corpus striatum thus suggesting it as an antioxidative and protective against PD symptoms[13]. There are various neurotoxins that have provided valuable information about the pathophysiology of PD but paraquat and rotenone failed to produce any obvious signs of dopaminergic damage[31]. The process of damaging the dopaminergic neurons by the various neurotoxins and α-Synuclein aggregation is entirely different[31]. However**,** the roots of *W. somnifera* contain steroid lactones (Withanolides), phytosterols and alkaloids (ashwagandhine, ashwaghandhinine, withasomine, visamine, somniferine, somniferinine)[8, 32, 33]. Our GC-MS analysis reveals the absence of these compounds in the leaf extract of *W. somnifera*. This may be the possible reason of not showing any protective effects against the PD symptoms in the flies. The above compounds have been shown to have neuroprotective and neuro regenerative potential[32]. Our earlier study with *Eucalyptus citriodora* leaf extract showed a protective effect in the PD model flies expressing h- αS[34]. The natural antioxidants such as apigenin,[35] curcumin,[28] nordihydroguaiaretic acid,[23] ascorbic acid,[34] and grape extract,[36] are potent in delaying the loss of climbing ability and reduction in the oxidative stress in the brains of PD model flies. The dietary supplementation of the flavonoids showed improvement in cognitive function possibly by protecting vulnerable neurons, enhancing existing neuronal function or by stimulating neuronal regeneration.[30] The complex brain of *Drosophila* is capable of learning and memory. Almost all the major classes of ion channels, receptors and neurotransmitters similar to humans composed of specialized cell types are found in *Drosophila* brain[22]. There are reports for the presence of novel and known withanolides from *W. somnifera* leaf extracts[16, 37]. Various pharmacological studies have attributed linkage of therapeutic actions to one or the other type of withanolides. A significant qualitative as well as quantitative difference

between the leaf and root tissue, particularly with respect to secondary metabolites, has been reported in the GC-MS analysis[37]. However, in our GC-MS analysis**,** no peaks corresponding to the withanolides were observed according to the NIST library. $C_{12}H_{10}O$ is arachidic acid, a saturated fatty acid[38] and $C_{20}H_{40}O_2$ is phytanic acid, a branched fatty acid**,** and its metabolites have been reported to bind or activate transcription factors[39].

The antioxidant and free radical scavenging potential have been reported due to the presence of the withanolides/withanone. But there are reports of having no antioxidant potential in the leaf extract of *W. somnifera*[29]. No one scientific approach is likely to single handedly solve all the mysteries of neurodegenerative disease and hence multidisciplinary research approaches utilizing many model systems will be required for elucidating the neuropathogenesis with the disease[40]. The complex brain of *Drosophila* consists of all major classes of ion channels, receptors**,** and neurotransmitters found in humans[40]. The neural complexity and proteomic analysis have revealed that more than 70% of the disease related loci in humans have a clear ortholog in *Drosophila*[41].

Hence**,** the *Drosophila* is the most ideal organism for studying the neurodegenerative diseases. The variation in the compounds in the GC-MS gram may be due to the type of solvent used in the preparation of extract or part from where the leaves were collected. Age of the leaves may also have some effects. In our present study with leaf extract of *W. somnifera*, the extract was not found to be potent in reducing the PD symptoms, in the transgenic flies expressing h- αS.

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