Original Research Article

Human *tauE14* mutation induced neurodegeneration in *Drosophila melanogaster* is ameliorated by developmental supplement of Ashwagandha root extract

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ABSTRACT

*Withaniasomnifera(L.)*Dunal (WS) known as Ashwagandha, has been used for centuries in Ayurvedic medicine to promote longevity and vitality. The use of herbal plant extract in treating several diseases has been documented from the very beginning in Ayurveda. The ethnopharmacological properties of this ''Indian Ginseng'' plant include adaptogenic, hypnotic, sedative, and diuretic. The root extract of WS has shown the properties of neuronal regeneration by stimulating axon and dendrite outgrowth in neurons in culture. Hence, in the recent decade Ashwagandha has been widely studied for its neuroprotective properties in many rodent models and cell lines. Here, we have used transgenic *Drosophila* model carrying human *tauE14*.The mutant protein codes for pseudophosphorylated Tau protein which is sepecifically expressed in isexpressed in photoreceptor neurons using GMR-Gal4 driver to induce photoreceptor neuronal degeneration. We treated these tauopathy mimicking flies with different concentrations of Ashwagandha to evaluate the neuroprotective/remedial effect of Ashwagandha at different stages of fly development. Our results demonstrated that Ashwagandha can rescue the neurodegeneration phenotype in *Drosophila* TauE14 disease model only when administered duringdevelopmenta.

Keywords: *Drosophila* disease model, Tauopathy, Ashwagandha / *Withaniasomnifera*, *htauE14*, Photoreceptor degeneration, Neurodegeneration, Developmental deformity

1. INTRODUCTION

In Ayurvedic System of Indian medicine, *Withaniasomnifera (L.) Dunal or* Ashwagandha (ASH) is classified as a Medhya-rasayana, a group of plant derived drugs considered to promote physical and mental health [1]. As an adoptogen, Ashwagandha can augment resistance of the body against the disease and adverse environmental factors, revitalizing the body and increase longevity [2]. Several *in vivo* and *in vitro* research studies support its polypharmaceutical uses, confirming antioxidant, anti-inflammatory, immune-modulating, and anti-stress as well as neuroprotective properties [3]. As an antioxidant, ASH as the whole plant extract and several separate constituents and active constituents like sitoindosides VII-X and withaferin-A have proven to be increasing the levels of endogenous superoxide dismutase, catalase, and ascorbic acid, while decreasing lipid peroxidation [4,5,6,7]. It has been demonstrated that the antioxidant property of ASH have a substantial role in symptomatic management of neurodegenerative diseases Parkinson's (PD) and Alzheimer's Disease (AD) [8,9,6]. The root extract of WS contains withanolides, which include steroidal alkaloids and lactones as active constituents [10]. The present investigation is designed to look into the role of Ashwagandha aqueous root extract in neuroprotection using in transgenic *Drosophila* tauopathymodel carrying *tauE14* mutant gene. Tau, a microtubule-associated protein (MAPT) [11,12], is an impotant protein that is widely present in the neurons (abundant in axons) and stabilises microtubule (MT) bundles and bring about axonal transport [13,14]. By regulating MT assembly and its dynamic behaviour, and spatial organization, Tau, along with other destabilising MAPs like stathmin, plays a crucial part in MT assembly and integrity [13].*Drosophila melanogaster* with its lesser lifespan, fully sequenced genome, gene manipulation tools, homology with human genes has proven to be a potential experimental model. *Drosophila* provides a flexible platform for identifying the cellular mechanisms underlying neuronal loss, in human genetic associated inherited neuronal disorders [15].A *tau* homolog exists in *Drosophila* [16] which has 46% identity and 66% similarity with the human Tau protein [17].The potential of Tau to assemble into beta-sheets is necessary for toxicity [18] In *Drosophila* Tau- induced neurodegeneration may not require the formation of large filaments, however small Tau assemblies are sufficient to cause neurodegeneration [19,20]. We used transgenic flies expressing human Phosphomimetic*tauE14*. The fourteen Serine (Ser) and Threonine(Thr)residuesin Tau protein, are replaced by Glutamate residues [21]. This isoform of human Tau protein has been showed to form filamentous aggregates and fail to bind to microtubules and cause

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cellular toxicity. In our study, we have induced degeneration of photoreceptor neurons of *Drosophila* by expressing the mutant *tauE14* gene in *Drosophila* eye, using GMR-Gal4 (a specific eye GAL4). We have assessed the ameliorative effect of Ashwagandha in rescuing the Tau toxicity mediated eye degeneration by following temporally regulated treatment regimes.

2. Materials and Methods

2.1. Drosophila stocks and maintenance

Wild type Oregon-K was procured from Drosophila Stock Centre, University of Mysore, Mysuru. UAS-h*tauE14*(microtubule-associated protein tau-MAPT [21] and GMR-Gal4 *(*Glass Multimer Reporter)[22] flies were used, which were previously described. All *Drosophila melanogaster* stockswere cultured in bottles on standard wheat-cream-agar medium with yeast granules. 12 hr light/ 12 hr dark condition was maintained at 22±2 °C.

2.2. Generation of*Drosophila* Tauopathy model using GAL4/UAS system

The GAL4*/*UAS is the bipartite gene expression system in *Drosophila* developed byBrand and Perrimon [23]. We used this methodology to express the mutant human *tauE14* gene in the photoreceptor cells of developing eye to induce photoreceptor degeneration. Virgins UAS-h*tauE14* flies were crossed to GMR-Gal4 (which is a eye disc specific Gal4 line). F1 heterozygotes (GMR-Gal4>h*tauE14)*, which express mutant form of human TauE14 in the developing eye disc were collected for scoring the eye severity. GMR-Gal4 crossed to Oregon-K flies, and F1 heterozous flies (GMR-Gal4/+) served as control.

*2.3.*Aqueous extraction of Ashwagandha

Root powder of Ashwagandha *(Withaniasomnifera(L.)*Dunal)is commercially available and purchased from The Nikhila Karnataka Central Ayurvedic Pharmacy Ltd (NKCA), Mysuru. (Batch No. EM-4-09). To prepare aqueous extract of Ashwagandha, a simple boiling and filtration method was employed. 10g of powder was mixed with a 10ml of double distilled water to make a thick paste. This paste was placed in a

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clean wet muslin cloth of 10X10 sq.cm like a pouch and immersed into the 100ml of boiling double distilled water in a glass beaker.

When water boils, the soluble contents from the paste were released into water. About 300ml more water was added to the paste at intervals of 15 to 20 min and stirred slowly and continuously using a clean glass rod. The fine powder passes through the pores of muslin cloth leaving behind debris. The boiling was continued for about an hour. Later, the muslin cloth was allowed cool and removed carefully. The crude constituents of ASH thus obtained in the beaker was boiled for about one and an half hours to get a thick paste. Dry weight of Ashwagandha extract per 1g wet paste was calculated for each time of the extraction procedure. Based on the dry weight calculation, an appropriate amount of extract was used to prepare desired concentration of media. The obtained paste was stored in refrigerator for further use.

2.4. Ashwagandha treatment

We have used two different concentrations of ASH and three different treatment regimes(Table 1). Based on the dry weight per 1 g wet paste obtained, a pertinent amount of ASH root extract was mixed to freshly prepared *Drosophila* standard food medium, to obtain the desired concentration medium. The flies were grouped into untreated and 6 treatment groups with two different concentrations of Ashwagandha with three different treatment regimes.The untreated mutant flies served as positive control. F1 progenies from each group were selected, age and sex matched for scoring of eye size reduction. For photoreceptor studies, 0.3% and 1.2% (w/v of medium) were administered to experimental flies at a) larval and adult stage (L^+/A^+); b) only larval stage ($L^+/A^-$and c) only adult stage (L^-/A^+) Yeast was not added to treatment medium. In all experiments, normal wheat-cream-agar medium served as control medium. Experimental flies were reared in culture vials separately with respective treatment medium and changed to fresh medium once in every 2 days.

Treatment Medium	Treatment regime	Experiment Group	Genotype of the flies
Wheat cream agar medium	No Ashwagandha	Negative control	GMR-Gal4/+
Wheat cream agar medium	No Ashwagandha	Positive control (Mutant untreated)	GMR-Gal4>h*tauE14*

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Wheat cream agar medium+ 0.3% ASH	L+/A+	0.3% ASH-root extract treatment	GMR-Gal4>h*tauE14*
	L+/A-		
	L-/A+		
Wheat cream agar medium+ 1.2% ASH	L+/A+	1.2% ASH-root extract treatment	GMR-Gal4>h*tauE14*
	L+/A-		
	L-/A+		

Table 1: Ashwagandha-root extract treatment regimes supplemented to tauopathy model of *Drosophila melanogaster*.L+/A+ meaningAshwagandha supplanted at both larval and adult stage; L+/A- meaning Ashwagandha supplanted only at larval stage; L-/A+ meaning Ashwagandha supplanted only at adult stage.

2.5. Drosophila eye morphology studies

Adult flies from control and treatment groups were anesthetized using mild anaesthetic ether and placed on a clean microscopic slide. Rough eye phenotype observations of eye were made with a stereo-zoom equipped with camera. Based on the reduced eye size, flies were grouped into (i)"less severe-S1" in which eye size reduced to 55%; (ii)"moderately severe-S2" where eye size is reduced to 35% and (iii)"most severe-S3" with eye size reduced to 15% of that of the control. The eye area is calculated using ImageJ software.

3. Results

3.1 Ashwagandha treatment rescues Tau-toxicity-induced photoreceptor degeneration in Drosophila model of tauopathy.

In our study we have used pseudo phosphorylated human *tauE14* transgenic *Drosophila* model to study the neuroprotective effect of Ashwagandha-root extract on Tau-toxicity-induced neurodegeneration. A crystal like arrangement of about 750-800 [24] ommatidial arrangement in the adult compound eye of *Drosophila* allows us to detect even a slight change in the external morphology. This provides an excellent and sensitive system to study morphostructural changes caused due to neurodegeneration and rescue mechanism by any medicinal extract supplementation at cellular level.

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Several studies on TauE14 suggested that, the phosphomimetic human TauE14 is found to be strongly toxicin the developing eye and in the mushroom body neuroblasts of *Drosophila* [25-27,21]. We have used, GMR-Gal4, which is specific to the developing eye disc GAL4 driver [22], to express the h*tauE14* by adopting GAL4/UAS mechanism [23].The F1 adult flies (GMR-Gal4>h*tauE14*) were screened for morphostuctrural variations of the eye. A total of 100 flies in each control, untreated and ASH-root extract tretment groups with different feeding regimes were screened to score the frequency of occurrence of ommatidial rough eye phenotype. The hTauE14 expressed in the developing eye disc exhibited a range of rough eye phenotypes with the loss of photoreceptor cells and consequently reduction in the adult eye size. Based on the reduced eye size, the phenotypes were classified into three severity groups in comparison with that of the control (GMR-Gal4/+). The severity groups are referred to as (i)"less severe-S1" in which eye size reduced to 55%; (ii)"moderately severe-S2" where eye size is reduced to 35% and (iii)"most severe-S3" with eye size reduced to 15% of that of the control.

In all the three groups of phenotypes, the crystal like ommatidial arrangement was lost resulting in "roughened" eye morphology. The GMR-Gal4>htauE14 individuals were fed with fly food supplemented with aqueous extraction of ASH-root powder at the concentrations of 0.3% and 1.2% w/v, at different feeding regimes (Table 1). The different eye phenotypes were scored from 1 d.p.e (day post eclosion) till 20 d.p.e. GMR-Gal4>h*tauE14*untreated flies served as positive control for comparison.

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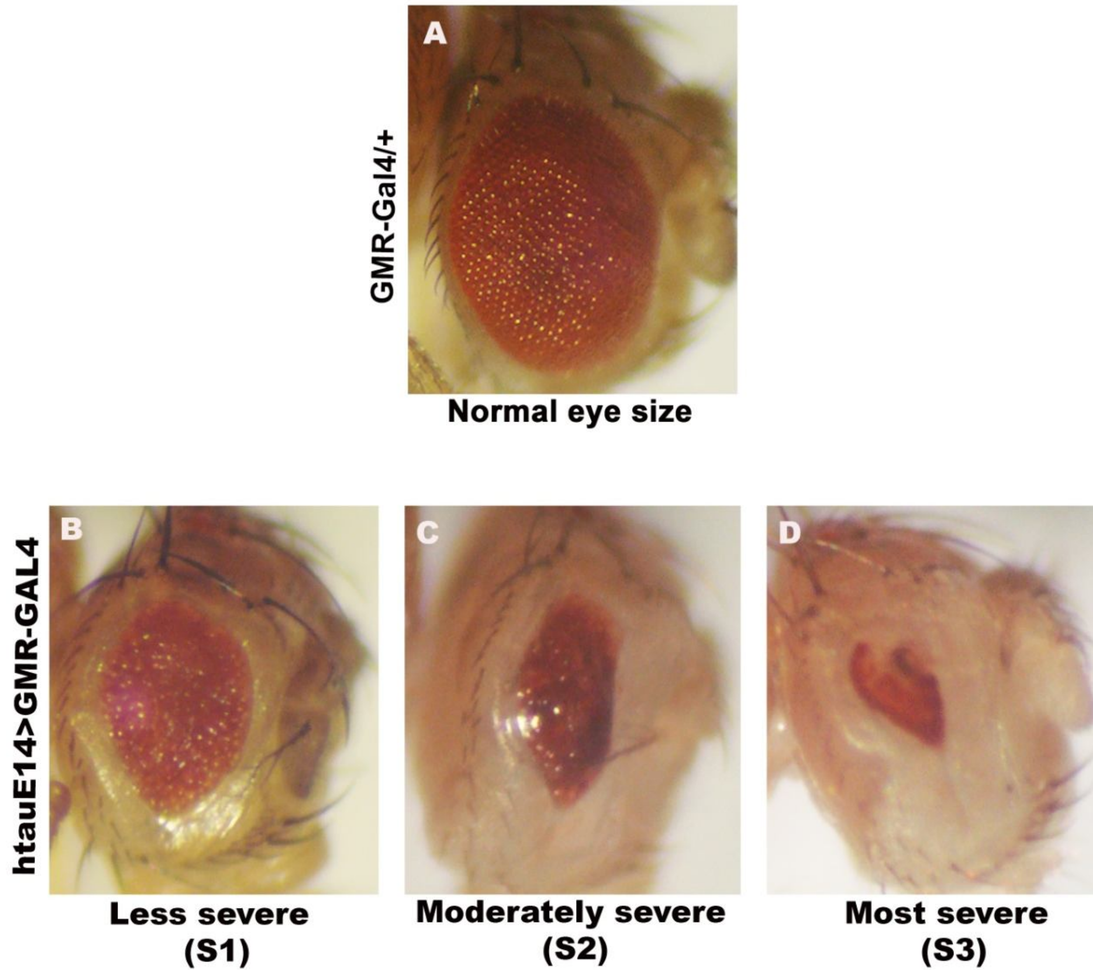
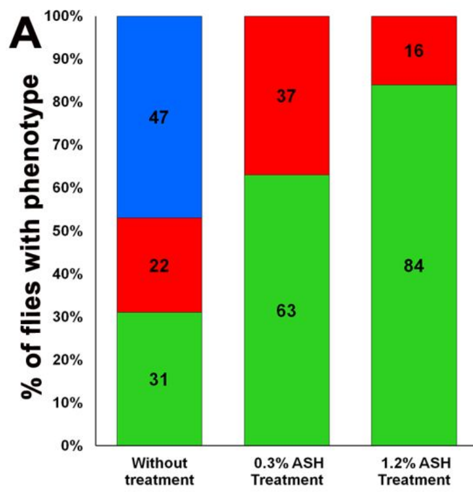
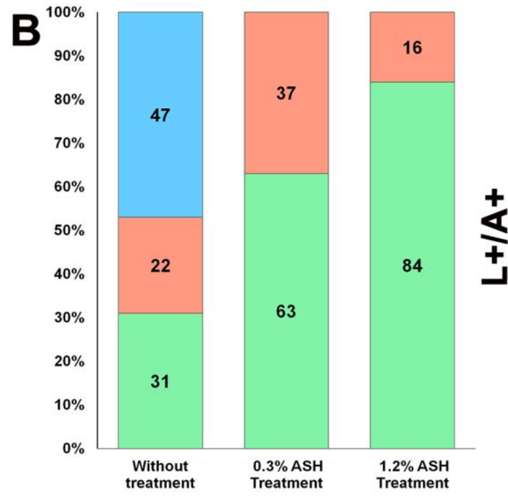


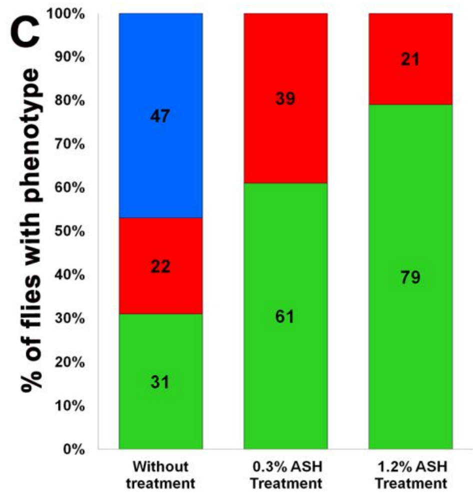
Figure 1: The stereo micrographic representations of *Drosophila* eye phenotypes. GMR-Gal4/+ was considered as negative control phenotype with normal eye size (A). Based on the reduced eye size, the phenotypes are classified as less severe-S1 (B); moderately severe-S2 (C) and most severe-S3 (D).

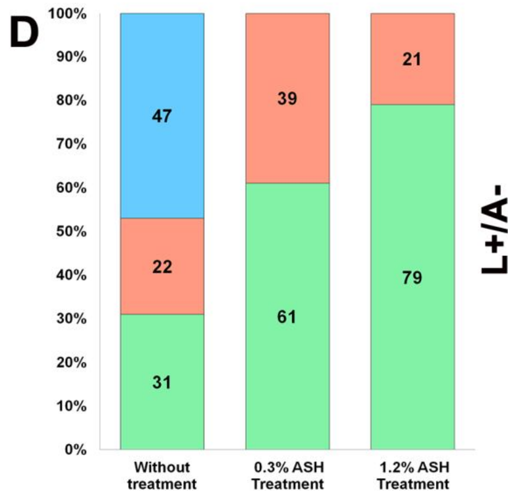
Stereomicroscopic eye images of representative phenotypes are presented in Figure 1. The phenotypes scored in the negative control GMR-Gal4/+ (figure 1 A) and GMR- GMR-Gal4>h*tauE14*individuals exhibiting S1, S2 and S3 phenotypes are depicted in figure1 B-D respectively.The different severity levels in the untreated GMR-Gal4>h*tauE14*flies exhibited less severe -S1=31%, moderately severe-S2=22% and most severe-S3=47% indicating the toxicity of TauE14 in the photoreceptor cells.



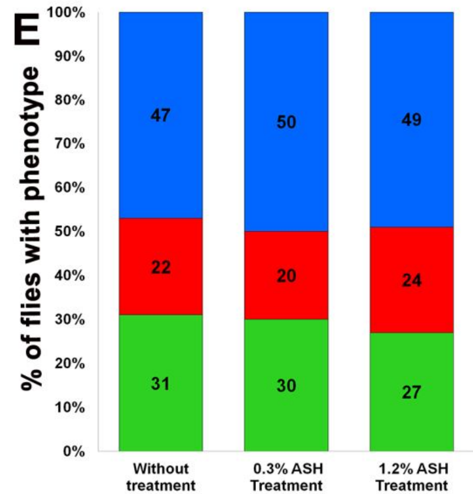


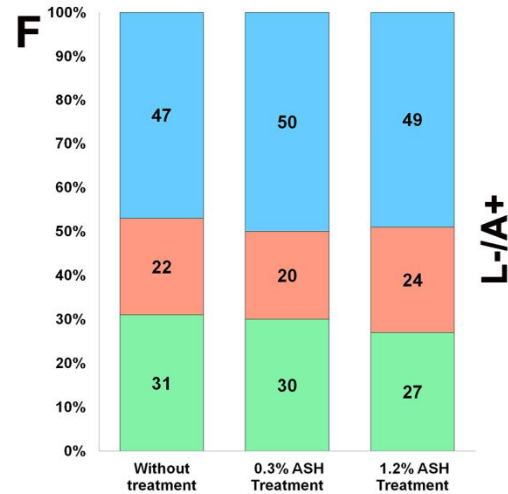
L+/A+





L+/A-





L-/A+

Less severe (Day1)
Moderately severe (Day1)
Most severe (Day1)

Less severe (Day20)
Moderately severe (Day20)
Most severe (Day20)

Figure 2: The percentage of distribution of rough-eye phenotype. GMR-Gal4>h*tauE14*flies expressing less severe-S1, moderately severe-S2 and most severe-S3 groups in untreated and ASH-root treatment groups (0.3% and 1.2% w/v) with different treatment regimes L+/A+ , L+/A- and L-/A+ on day 1 and day 20 are represented.GMR-Gal4>h*tauE14*are the experimental flies.

In consideration with the feeding regime L+/A+ (larval and adult stage), in both 0.3% and 1.2% ASH-root extract treated GMR-Gal4>h*tauE14*flies showed improvement in the eye size from 1 d.p.e. In 0.3%, only S1 and S2 groups were present with the occurrence of S1 and S2 63% and 37% respectively. These treated groups were devoid of S3 category phenotype. There is a prominent increase in the number of individuals having S1 phenotype in 1.2%, in comparison with untreated and 0.3% treated groups. The 1.2% ASH-root extract fed GMR-Gal4>h*tauE14*flies showed 84% of S1 and 15% of S2 respectively. We did not find any changes in the eye phenotype till 20 d.p.e (Figure 2 A and B).

L+/A- (only larval fed), both 0.3% and 1.2% ASH-root extract treated GMR-Gal4>h*tauE14*flies showed improvement in the eye size. 0.3% ASH-root extract fed flies had only S1 and S2 eye morphology with the occurrence of 61% and 39% respectively. The 1.2% Ashwagandha administered flies exhibited 79% and 21% of S1 and S2 respectively. There was no difference in the eye phenotype from what was seen on 1 d.p.e till 20 d.p.e (Figure 2 C and D).

The flies with L-/A+ (only adult fed) treatment, interestingly all the three severity phenotypes were present in both 0.3% and 1.2% ASH-root extract fed GMR-Gal4>h*tauE14*flies which is similar to untreated group. On 1 d.p.e, 0.3% ASH-root extract fed flies showed 30%, 20% and 50% of S1, S2 and S3 eye morphology respectively. Comparably, the 1.2% Ashwagandha administered flies also exhibited 27%, 24% and 49% of S1, S2 and S3 respectively. Surprisingly in both the concentrations, the percentage of distribution of flies resemble closely to untreated group. There was no difference in the eye phenotype till 20 d.p.e in both the Ashwagandha concentrations (Figure2 E and F).

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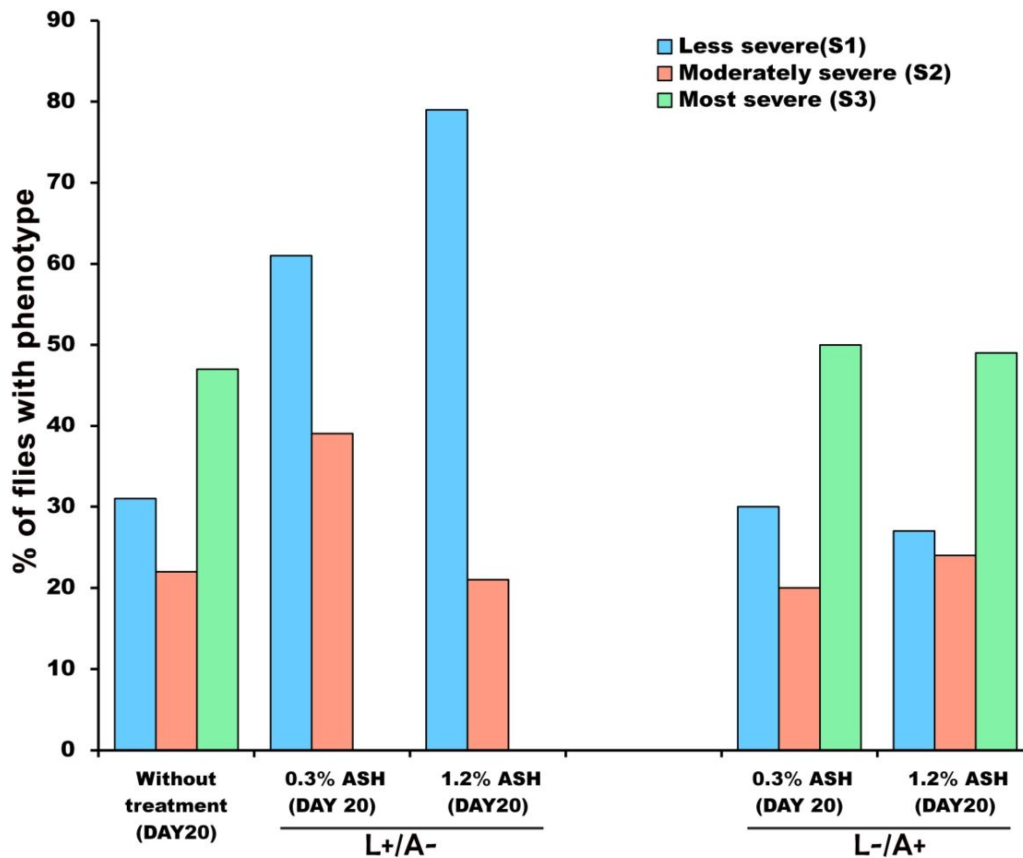

Figure 3: The percentage of flies with reduced eye phenotype in Larval only (L+/A-) and Adult only (L-/A+) Ashwagandha root extract (0.3% and 1.2% w/v) fed flies. GMR-Gal4>h*tauE14* are the experimental flies.

This finding strongly proposes that the treatment of ASH-root extract can protect the photoreceptor neurons from hTauE14-induced neurotoxicity. We can observe the shifting of phenotype from S3 to S1 or S2 types in the treatment groups fed L+/A+ and L+/A- treatment regimes. Between 0.3% and 1.2% ASH-extract treatment, the most effective protection was seen in L+/A+ 1.2% ASH-extract treatment as seen by the highest occurrence (84%) of S1 phenotype in this group indicating the protective effect of ashwagandha is dose dependant. Continued observation of eye phenotype for 20 days in each group did not reveal any further progressive degeneration or treatment dependent improvement.

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3.2 Discussion

*Withaniasomnifera (L.)*Dunal-Ashwagandha, a herbal medicinal plant belonging to the Solanaceae family plays an important role in Ayurveda in treating various disease conditions from simple to complex neurodegenerative diseases. ASH as the whole plant extract and several separate constituents like root or leaf extracts have different quantity of active constituents like sitoindosides VII-X and withaferin-A have proven to be increasing the levels of endogenous superoxide dismutase, catalase, and ascorbic acid, while decreasing lipid peroxidation [4,6]. Withaferin A, 12-deoxywithastromonoilide, and withanolide A were identified as the active compounds present in the aqueous extract of Ashwagandha [28]. A number of other alkaloids such as somnine, somniferinine, withananine, tropine, psuedo-withanine, withanine, isopelletierine, somniferine, anaferine and anahydrine, have also been shown to have medicinal properties [5-9]. Tau (MAPT) is a microtubule-associated protein [11,12] which is abundant in the neuronal axons where it stabilizes microtubule (MT) bundles. It is located in several cellular components, including axon; neurofibrillary tangle and nuclear lumen. The major functions of Tau including positive regulation of cellular component organization; protein-containing complex assembly; and regulation of protein localization[29]. MAPT tau is found to be a biomarker in many tauopathy induced neurodegenerative diseases like dementia, progressive supranuclear palsy, temporal lobe epilepsy, Alzheimer's disease, Creutzfeldt-Jakob disease [30].TauE14 is a pseudophosphorylated protein that mimics hyperphosphorylated Tau which when expressed brings about neuronal toxicity leading to cellular death.

Many studies in *Drosophila* demonstrated that Tau is an essential protein during development. Talmat-Amar [31]and others overexpressed the TauE14 using a motor neuron specific driver RapGAP1-OK6 Gal4 and observed the defects in larval segmental nerves and synaptic vesicle kinetics.Expression of TauE14 under the control of pan neural specific elav-Gal4 resulted in severe mushroom body (MB) abnormalities [25]. And also resulted in the neurodegeneration of both the cortex and the neuropil in 5-day old mutant flies, with these regions showing an increase in neuronal apoptosis, as compared to their controls[26]. Studies demonstrated that TauE14 when expressed under GMR-Gal4 in the developing eye, could induce disorganized ommatidial architecture, photoreceptors fusion and missing of

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mechanosensory bristles[21,32] occurs due to microtubular instability resulting in mitotic arrest during cell division [33]. Tau has proven to have an important role during development cellular mitosis. In our study, we demonstrated that the ameliorative effect of Ashwagandha can be seen in both the concentrations (0.3% and 1.2% w/v). But only in L+/A+ and L+/A- regimes, shifting of eye phenotype from most severe (S3) to less severe(S1) and moderately severe (S2) is observed.Here, TauE14 mutant flies predominantly obtained Ashwagandha as food supplement during larval stage which is the time window for development of photoreceptor. Flies fed in both larval and adult stage benefitted more when compared to ASH non-fed flies. The results of L+/A- flies almost resemble that of the L+/A+ flies, supporting the argument that larval feeding of ASH was more beneficial. Interestingly, in L-/A+ group of flies in both the concentrations did not show any rescue from the severity in eye phenotype. They exhibited all the three severity (S1, S2 and S3) in eye size as equivalent to that as seen in untreated group. In short, our study demonstrated that Tau-mediated toxicity in photoreceptor neurons is largely ameliorated by feeding of Ashwagandha root extract during development in comparison with the other treatment regimes when only adults are treated. We opine that, Ashwagandha could interfere with the formation of beta-sheets of neurofibrilary tangles or by inhibiting binding of abnormal Tau protein to microtubules.The ommatidial dysregulation is a developmental phenomenon unlike many other adult onset disorders. Hence, the treatment with Ashwagandha during development is critical, whereas the adult only (L-/A+) treatment has no effect on the TauE14 induced neurodegeneration.

4. CONCLUSION

In the light of this, we conclude that *Drosophila* tauopathy models have been proven to be great individuals to study Tau-mediated studies in *in vivo* paradigm using rough-eye readout phenotype as a neurodegenerative parameter. The human mutant Tau formwhen expressed in developing *Drosophila*eye have shown eye degeneration and it is greatly rescued by the cumulative effect of constituents of Ashwagandha root extract when fed at larval stage in a dose dependant manner. However, further investigation is needed with adult onset disease models of *Drosophila*to confirm the effect of Ashwagandha on age related progressive neurodegeneration. Further, the molecular targets of the

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Ashwagandha phytoconstituents, in cellular system for its neuroprotective function needs to be investigated.

ETHICAL APPROVAL

Not applicable

COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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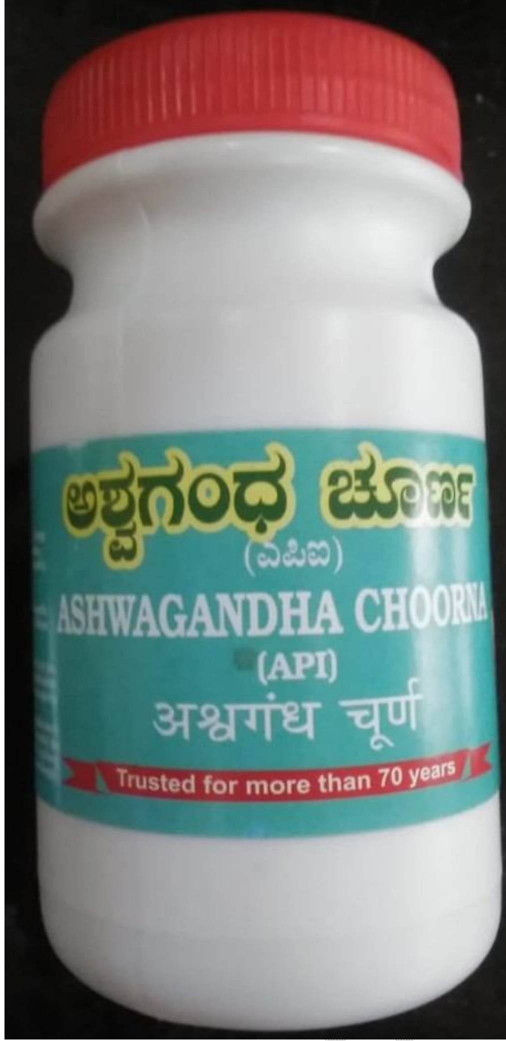
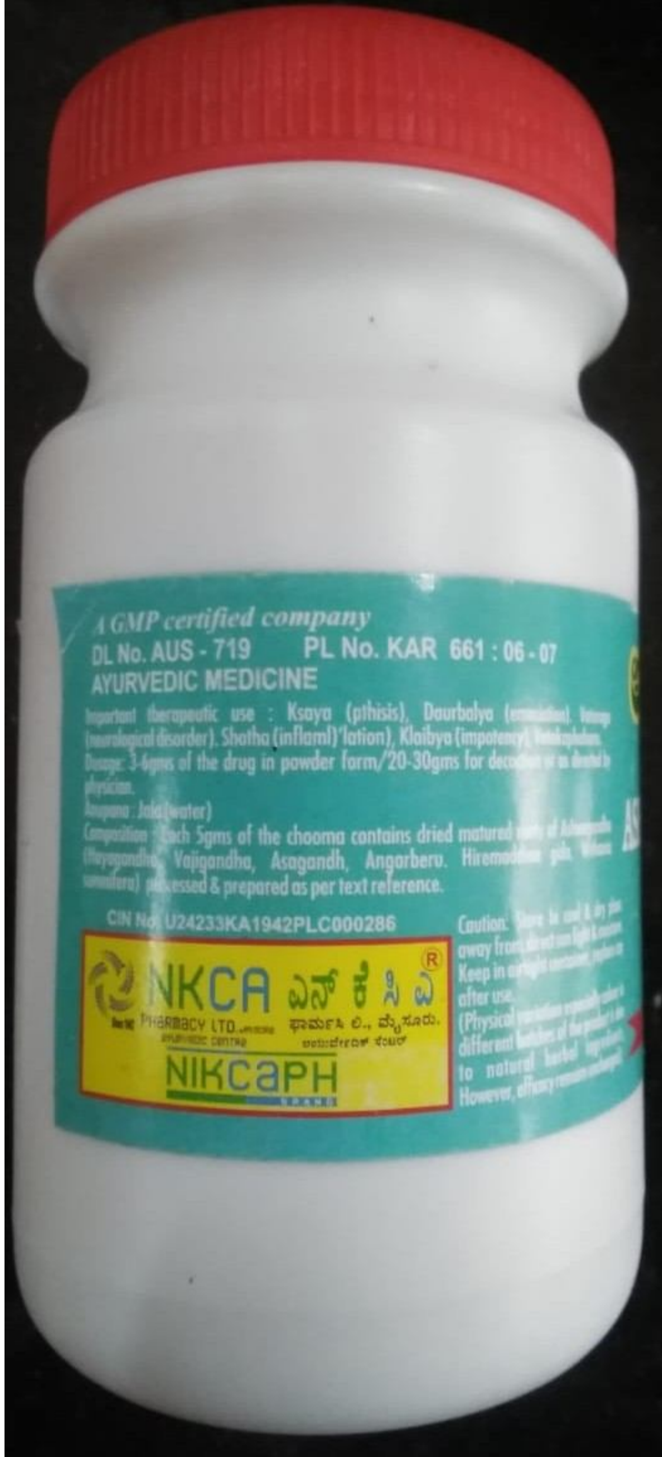


ASHWAGANDHA PRODUCT 1

UNDER PEER REVIEW



ASHWAGANDHA PRODUCT 2

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