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Full Length Research Article

Therapeutic Potential of Hesperidin in Parkinson's Disease with Dementia: Inhibition of Alpha Synuclein and Amyloid Beta in *Drosophila melanogaster*

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Summary: Neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD) share similar cellular and molecular mechanisms such as protein aggregation and inclusion body formation. Thus, we evaluated the action of hesperidin on α-synuclein and amyloid-β-induced neurodegeneration in *Drosophila melanogaster*. To model PD and Alzheimer's disease (AD), the bipartite system of GAL4 transcriptional activator was placed under a cell-type specific promoter; embryonic lethal abnormal visual system-GAL4 (ELAV-GAL4; pan-neuronally) or dopa decarboxylase (Ddc-GAL4; dopaminergic neurons) for the expression of amyloid-beta (Aβ42) or α-synuclein (α-syn), respectively, under the control of the upstream activating sequence (UAS) in Drosophila melanogaster.. Flies were either grown on food media supplemented with or without hesperidin (HSD) (1, 5, or 10 mM). Behavioral assays were carried to investigate the effect of treatment on fecundity, larval motility, climbing ability, and lifespan. UAS-$A\beta_{42}$>Elav-GAL_4 or UAS-α-synuclein>DDC-GAL_4 caused significant decrease in fecundity, larva motility, survival rate, and climbing activities in flies showing neurodegenerative phenotype. However, supplementation of flies' media with hesperidin (1, 5 and 10 mM) showed a dose-dependent increase in the number of flies' egg-laying ability, larva motility and adult climbing activity in comparison with flies grown on food media only. Conversely, supplementation of fly feed with HSD caused no significant change in lifespan. Findings from this experiment showed that hesperidin could be a potential neuroprotective agent in the amelioration of PD and AD pathogenesis.

Keywords: Alzheimer's disease; amyloid-beta; alpha-synuclein; Drosophila melanogaster; negative geotaxis assay; Parkinson's disease

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INTRODUCTION

Neurodegenerative diseases are known as a group of neurological disorders characterized by progressive loss of brain and spinal cord cells, motor response impairment (ataxia) and sensory dysfunction (dementia) (Jeong, 2017, Minter et al., 2016, Wyss-Coray, 2016). The etiology of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (AD) are multifactorial including genetic mutation, environmental factors and brain aging which is the most important cause of neurodegenerative diseases, cellular and molecular factors such as potent production of oxidative stress, mutation in mitochondrial DNA, inflammatory responses, defective regulation of apoptosis etc. also play significant role in the pathogenesis of neurodegenerative diseases (Bredesen et al., 2006; Minter et al., 2016). AD and PD have been reported to be the leading causes of all reported cases of neurodegenerative disease accounting for over 10% causes of neurological disorders and over 60% causes of dementia in Nigeria (Adeloye et al., 2019, WHO, 2019). Most common features of AD and PD are misfolding and aggregation of amyloid-β42 and α-synuclein proteins into seeds which further distort similar proteins causing them to aggregate and produce pathogenic assemblies (Jucker and Walker, 2013).

Hesperidin (C28H34O15) is a flavanone glycoside, richly found in the citrus fruits such as lemon, sweet orange (*Citrus sinensis*), and grapefruits. Its antioxidant and neuroprotective actions have been reported (Kesh et al., 2021). We have also reported the potential of hesperidin in the enhancement of antioxidant defense, cholinergic/BDNF signaling and spatial learning in mice (Ishola et al., 2019). Due to the strong neuroprotective, memory enhancing and antioxidant effects of hesperidin (Ishola et al., 2019; Kesh et al., 2021).

The fruitfly Drosophila melanogaster is widely used to model neurodegenerative disease evidence in its potential in the identification of genes that are required to maintain the structural integrity of the brain, defined by recessive mutations that cause adult onset neurodegeneration (Lessing and Bonini, 2009). Moreover, flies genetics are instrumental in the analysis of neurodegenerative disease with better understanding of AD and PD. In addition, *Drosophila* has a short lifecycle of ~10 days to the adult with complex central nervous system with neurons and glia cells protected by a blood-brain barrier, and shares striking similarities with the vertebrate brain. The smaller size of the *Drosophila* genome (1.2×10^8 base pairs) compared to human genome

(3.3*10^9 base pairs) and smaller number of genes in *Drosophila* (~14,000 *vs*. ~20,000-25,000 protein-encoding genes) have an important implication. In *Drosophila*, the reduced genome complexity allows easier interpretation of loss-of-function studies. The UAS-GAL4 system is an efficient bipartite approach in the activation of gene expression in *Drosophila* (Duffy, 2002). It is widely used to drive gene expression in a multitude of cell- and tissue-specific patterns. The UAS, together with a specific gene of interest, is kept in one fly lines, and GAL4 with tissue-specific promoter is kept in another. When flies of these lines are crossed, the GAL4 protein will activate the UAS-linked gene in specific tissue. One advantages of this system is that toxic genes will only be expressed when bound to the GAL4 protein. This allows flies carrying the inactivated form of a toxic protein to survive normally. Thus, this study sought to evaluate the potential neuroprotective effect of hesperidin on neurodegenerative disease in *Drosophila melanogaster*.

MATERIALS AND METHODS

Hesperidin, malt-agar (Sigma Aldrich, St. Loius MO, USA), diethyl-ether (GuangdonngGuangbua Sci. Tech CO. Ltd. China), sugar, corn flour (Latyf food and beverages Ventures LTD, Ogun state, Nigeria), yeast (STK Industries Ltd, China), agar (Himedia Laboratories Pvt. Ltd, Mumbai, India), methyl-p-hydroxy benzoate, propanoic acid (LOBA Chemie Laboratory Reagents & fine Chemicals, Mumbai, India), orthophosphoric acid (Thermo Fischer Scientific, Mumbai, India), phosphate buffered saline (Gibco Technologies, USA).

***Drosophila Melanogaster* Fly Stock and Culture:** *Drosophila melanogaster* strains; UAS-Syn/Cyo, UAS-Aβ42/TM3, Elav-GAL4/FM, Ddc-GAL4/TM3, Canton-Special (CS), and Wild type (W1118), were kind gifts from Dr. Rakesh Mishra Drosophila Laboratory, Centre of Cellular & Molecular Biology, Hydrabad, India, for the purpose of this study. The flies were maintained at an optimal temperature of 23ºC (±2ºC) and 60% humidity. Flies were allowed to develop on a standard diet (31.5% Sugar, 29.7% Corn flour, 9.5% Yeast,7.1% Agar, 24.6% Malt, 0.045% Methyl-p-hydroxyBenzoic, 0.045% Propanoic acid and 0.01% Orthophosphoric Acid) in 50 ml plastic vials (15-20 flies per vial) and cultured under 12:12 hours day/night cycle. Flies were transferred every 4 days into another vial containing the standard diet (Ishola et al., 2021).

Collection of Female Virgins and Crossing: Virgin female flies were collected within 6 hours of eclosion at 23ºC (±2ºC) after confirming the presence of meconium at the upper quadrant of the ventro-lateral part of abdomen under a stereomicroscope (AmScope, MO, Nigeria). Ten virgin female flies each expressing Ddc-GAL4/TM3 and Elav-GAL4/FM were crossed with males carrying UAS-Syn/Cyo and UAS-Aβ42/TM3, respectively. Similarly, ten virgin female flies each expressing Ddc-GAL4/TM3 and Elav-GAL4/FM were crossed with males of W1118 as negative control.

Fecundity Assay: Ten female virgins and 5 male Canton-special (CS) strains were crossed and placed on the appropriate diets for fecundity assay. The numbers of eggs laid were counted at intervals of 24 hours for 3 days with the use of a stereomicroscope. This assay was carried out on three separate vials per experimental diets. Simultaneously, total numbers of dark pupa and freshly eclosed flies were recorded (Chattopadhyay et al., 2015).

Larval Motility Assay: At day 6 after crossing, larvae were assessed for larva motility assay. Viable (second instar) larvae were carefully extracted from the fly vials, washed in phosphate buffered saline and transferred onto 15 cm diameter petri dish, half-filled with freshly prepared, solidified 2% agarose solution. The petri dish was placed over a graph paper with a $0.1cm^2$ grid. The larvae were allowed to acclimatize for 10 sec, after which the numbers of grid lines crossed in 60 sec were counted and recorded (Nichols et al., 2012).

Negative Geotaxis Assay: Three separate vials containing 20 adult flies per group were used for the negative geotaxis assay (climbing assay) at the end of the experimental treatment days. The flies were anaesthetized and placed at the bottom of a clean measuring cylinder 15 cm tall. After recovery time of 15 mins, the measuring cylinder housing the flies was gently tapped to allow the flies settle at the bottom, and then the numbers of flies that crossed an height of 8cm within 8 sec was recorded. This was repeated periodically for 28 days at interval of 7day for each group (Poetini et al., 2018b, Shaltiel-Karyo et al., 2012).

Longevity Assay: Twenty (20) male/female flies per experimental groups were placed on standard fly diet at regulated temperature (18ºC - 23ºC). The flies were counted every 4 days and transferred to new diet vials. At each time point, the numbers of dead flies were counted until the entire flies were dead (Chattopadhyay et al., 2015).

Statistical Analysis

Data analyses were performed using GraphPad Prism software version 7 (GraphPad Software, Inc, CA, USA). Results were expressed as mean±standard deviation and analyzed using one or two-way analysis of variance (ANOVA) followed by Turkey's *Post hoc* multiple comparison tests.

RESULTS

Larva Motility Assay: First filial generation (F1) Larvae harvested from UAS-Aβ > elav-Gal4 or UAS-syn< DDC-Gal4 placed on the standard diet showed no significant change in locomotion activities (number of lines crossed) compared to W1118>elav-Gal4 (Fig. 1) or W1118 > DDC-Gal4, respectively (Fig. 2) (normal control). However, UAS-Aβ> elav-Gal4 [$F = 6.726$; $P= 0.002$] and UAS-syn>DDC-Gal4 [$F = 7.507$; $P< 0.05$] produced significant increase in locomotion activities of F1 larvae harvested from diet containing 5 or 10 mM of hesperidin compared to those placed on standard diet, respectively (Fig. 1 and 2). Conversely, F1 larvae harvested from 1mM of hesperidin diet showed no significant changes in locomotion activities compared to larvae of W1118>elav-Gal4 or W1118>DDC-Gal4.

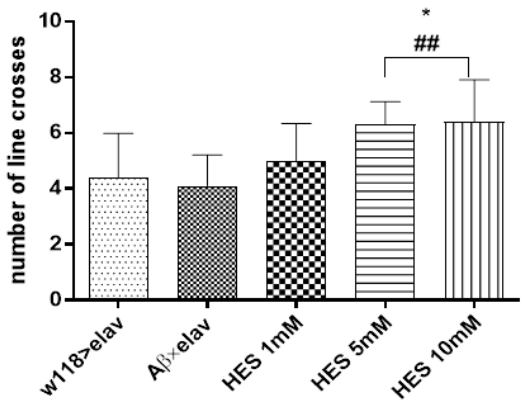

Figure 1:
Effect of hesperidin on F1 larva motility (number of line crosses in UAS-Aβ>elav-Gal4). (*****P < 0.05 compared to W1118> elav-Gal4, #P < 0.05, ##P < 0.01 compared to UAS-Aβ < elav-Gal4)

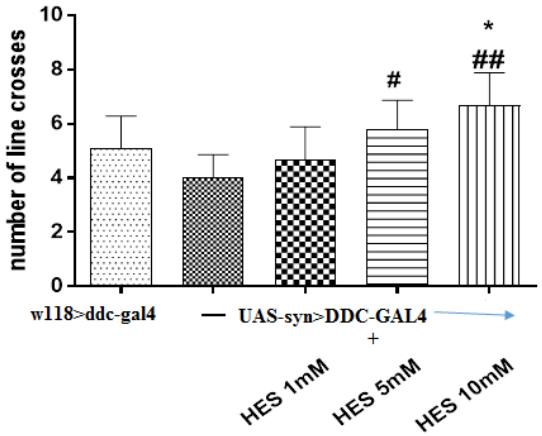

Figure 2:
Effect of hesperidin on UAS-syn < DDC-Gal4 F1 larvae motility (number of lines crossed). (***:** P < 0.05 compared to W1118 < DDC-Gal4, **#:** P < 0.05, **##:** P < 0.01 compared to UAS-syn < DDC)

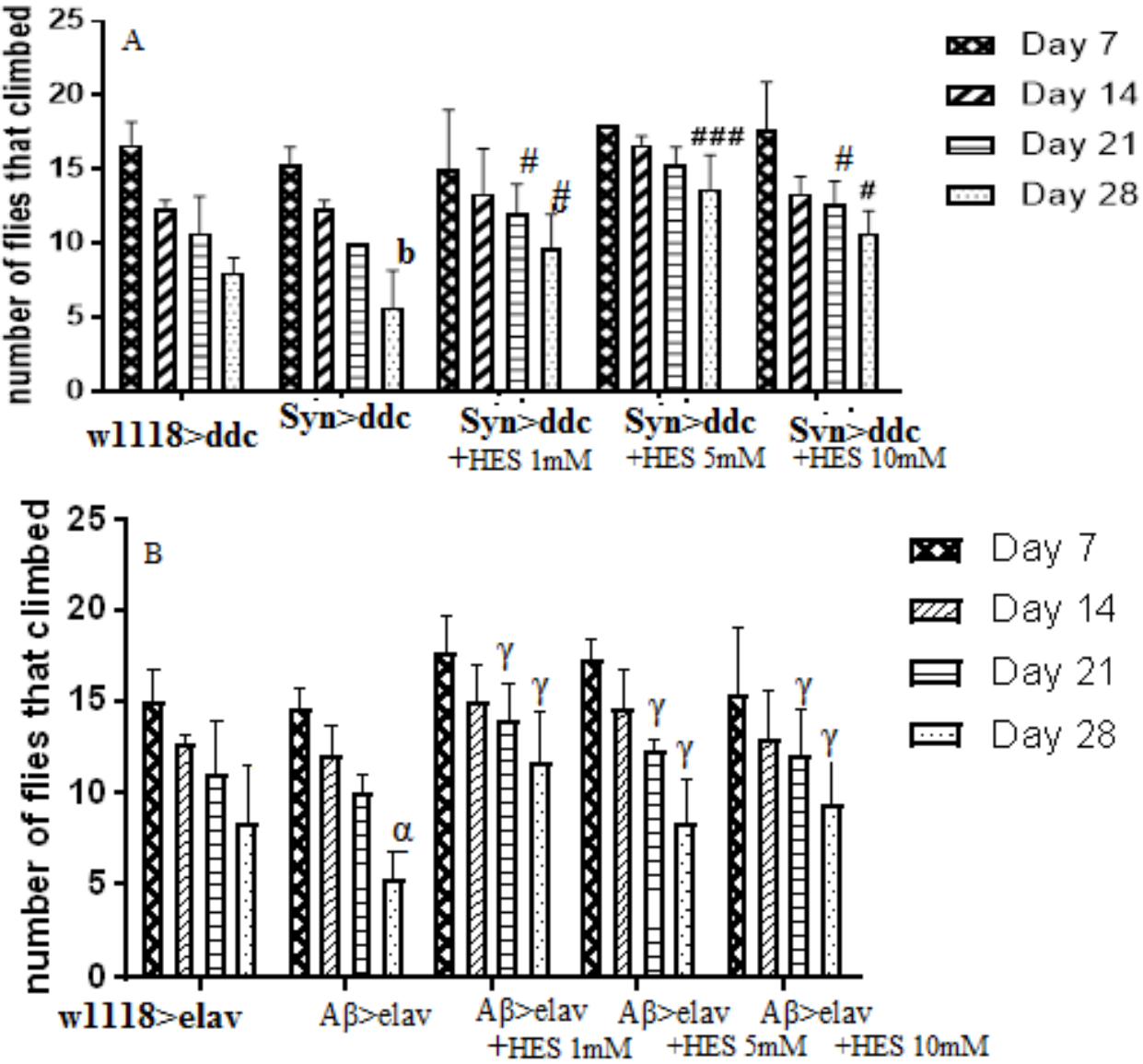

Figure 3A-B:
αEffect of hesperidin on climbing ability in (a) syn>ddc and (b) Aβ>elav adult flies. $^{b}p<0.01$ versus w1118>ddc; $^{\#}P< 0.05$,$^{\#\#\#}p<0.001$ versus syn>ddc; $^{\alpha}p<0.01$ versus w1118>elav; $^{\gamma}p<0.001$ versus Aβ>elav, statistical level of significance analysis by two way ANOVA followed by Tukey *post hoc* test.

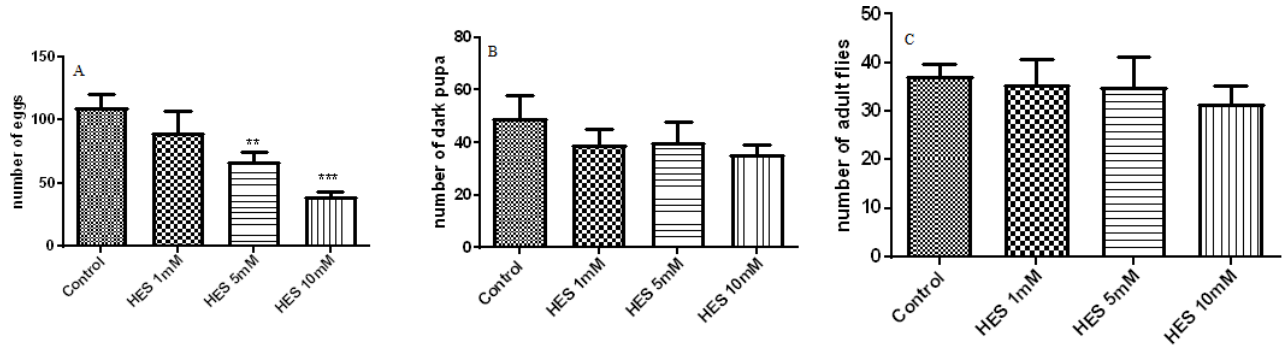

Figure 4a-c:
Effect of hesperidin on fecundity parameters in Cs flies (a) numbers of eggs laid, (b) number of dark pupa and (c) number of adult flies. Statistical level of significance analysis by one way ANOVA followed by Tukey post hoc test. ****P*<0.01, *****P*< 0.001 compared to control)

Climbing Assay

Effect of hesperidin on negative geotaxis in synuclein expressing flies: Figure 3a shows time course decrease in climbing activity across treatment groups. Syn>DDC showed significant decrease in negative geotaxis with peak deficit on day 28 when compared with w1118>ddc. α-syn aggregation in dopamine neuron induced motor deficit were significantly reversed in Syn>DDC flies placed on hesperidin 1, 5 and 10mM supplemented media. Post hoc analysis showed significant increase in number of flies that crossed the 8 cm mark at days 21 and 28. Post hoc analysis showed that adult flies expressing $A\beta_{42}$ pan-neuronally (elav) displayed significant time course decrease in climbing activity when compared with w1118>elav (Fig. 3b). however, neuronal aggregation of $A\beta_{42}$-induced motor deficits were reversed by supplementation of flies media with HES (1, 5 or 10mM).

Effect of hesperidin treatment on fecundity wild flies: Supplementation of flies' media with HES (5 or 10mM) caused statistically significant decrease in the numbers of eggs laid by CS flies when compared with control on standard diet (Fig. 4a). However, despite the decrease in number of eggs laid, we observed no significant change in number of dark pupa (Fig. 4b) and enclosed flies (Fig. 4c) when compared to the standard diet.

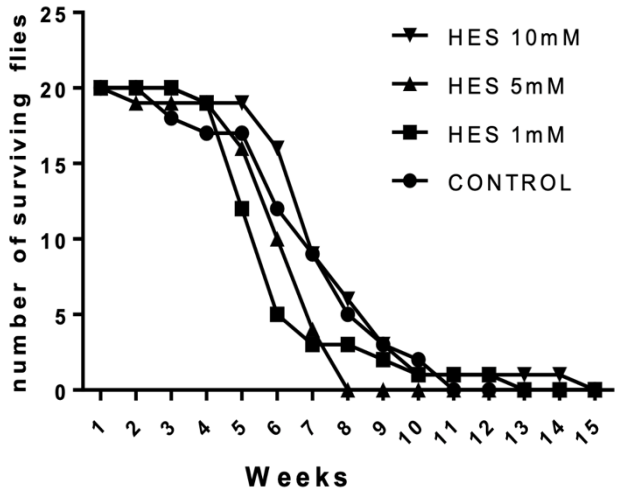

Figure 5:
Effect of hesperidin on Cs lifespan. Values are expressed as mean±SD. Statistical level of significance analysis by two way ANOVA followed by Dunnett's multiple comparison tests; p>0.05.

Lifespan Assay: The supplementation of fly media with hesperidin failed to confer significant change in lifespan in Cs flies (Fig. 5). No significant change in number of surviving flies when compared with the control on standard diet. Interestingly, flies cultured on HES 10mM media showed slight increase longevity when compared with control at the end of the 15th week.

DISCUSSION

Findings from this study showed that the UAS-GAL4 bipartite system increased the expression of α-syn in dopamine neuron and $A\beta_{42}$ pan-neuronally leading to motor deficits as observed in climbing assay which were ameliorated by hesperidin supplementation. Conversely, the expression of α-syn in dopamine neuron and $A\beta_{42}$ pan-neuronally did not affect larva motility. Moreso, hesperidin did not produce adverse effect on fecundity parameters in the present study.

The incidence of neurodegenerative disorders due to aging has been on the increase in Nigeria and Africa, with Parkinson's and Alzheimer's disease reported as the commonest form of neurodegenerative disorders in Nigeria and Sub-Saharan Africa (Lekoubou et al., 2014, Olayinka and Mbuyi, 2014). The advents of synthetic therapy including levodopa and acetylcholinesterase inhibitors have revolutionized the treatment of PD and AD, respectively but not without adverse events after prolonged use such as dyskinesia and failure to modify the aetiopathogenesis. Moreover, incidences of neuropsychiatric side effect associated with these therapies have also been on the increase, thus, limiting their use (Smith et al., 2012; Hashimoto et al., 2000; dos Santos Moraes et al., 2006).

Findings from our study showed that the expression of α-synuclein in dopamine neurons using UAS-GAL4 system might have contributed to the reduction in larva motility and progressive deterioration in negative geotaxis performance of the adult flies. Interestingly, supplementation of flies' media with hesperidin did not affect fecundity but improved spontaneous motor activities in both the larva and adult flies. The ability of hesperidin to reduce the expression of α--synuclein and Leucine-rich repeat kinase 2 (LRRK2) enzymes as well as its potential ability to inhibit the depletion of dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) have been reported as possible mechanism for

the improved motor functions (Antunes et al., 2014; Santos et al., 2016; Hajialyani et al., 2019). Similar to PD, incidence of motor dysfunction have been strongly associated with elderly people living with AD (Härlein et al., 2009; Buchman and Bennett, 2011). Accumulations of amyloid beta in the extracellular matrix in the neurons of the cortical region of the brain have been linked with the incidence of motor dysfunction and cognition impairment associated with AD (Pedrinolla et al., 2018, Reitz and Mayeux, 2014, Albers et al., 2015). It is worthy of note that in the present study, accumulation of amyloid beta protein pan-neuronal induced motor dysfunction in the larva but not in the adult flies. This is in agreement with report that flies expressing the mutation of human APP gene alone showed no statistically significant difference in motor dysfunction (Mhatre et al., 2014, Chakraborty et al., 2011; Holschneider et al., 2011, Shinotoh et al., 1999, Zhou et al., 2016)

Reduction in female fecundity and male fertility have been associated with accumulation of amyloid beta and alpha synuclein (Oriá et al., 2020, Naghavi, 2018). Hence, adjuvant for the management of these neurodegenerative disorders should have the potency of improving fecundity. In this study, hesperidin maintained the metamorphosis of dark pupa into adult flies. Scavenging of reactive oxygen species (ROS) by hesperidin have been highlighted as a possible mechanism for the ameliorative function in both Drosophila and rodent models of neurodegenerative diseases (Poetini et al., 2018a, Jayapalan et al., 2020). Also, its activities in suppressing various inflammatory signaling markers including glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (Iba-1), NF-kB and TNF-α and improvement of sexual hormones (FSH and LH) by enhancing the pituitary gland of the CNS have been described as potential mechanism for enhancing fecundity and fertility in neurodegenerative disorders (Justin-Thenmozhi et al., 2018; Hozayen, 2012).

Reductions in life span due to neuronal death and loss of systemic coordination by the brain have been strongly associated with neurodegenerative disorders including AD and PD. Cellular lipid peroxidation have been strongly linked as possible mechanism encouraging cell death in neurodegenerative disorders. Over the decade, improvements in cellular function and protection by the use of hesperidin, an antioxidant and anti-inflammatory phytochemical present in citrus, have been linked to its ability to inhibit accumulation of lipid peroxides in the extracellular matrix of neurons (Abolaji et al., 2017; Arumugam et al., 2018; Jayapalan et al., 2020). This could be the possible mechanism employed by hesperidin in the present study towards enhancing life span of CS flies.

In conclusion, findings of this study suggest that hesperidin, a bioflavonoid, could be a potential natural product for improved treatment of neurodegenerative diseases evidenced in its ability to improve lifespan and motor activity.

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