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Synergistic Enhancement of Motor Function by Curcumin and Piperine in Alpha-Synuclein Expressing Drosophila Models

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease characterized by Lewy body formation mainly phosphorylated and aggregated α-synuclein, with subsequent neurotoxicity and death of dopaminergic neurons in the basal ganglia. The neuroprotective action of curcumin and piperine has been reported. We used the GAL4/UAS bipartite system for targeted expression of α-synuclein pan-neuronal and dopamine neurons to create genetically induced PD. Hence, in this study we assessed the ameliorative effect of curcumin and piperine on α-synuclein-induced dopaminergic neuron degeneration and photoreceptor degeneration in the *Drosophila melanogaster* model of PD. Male Drosophila melanogaster (upstream activating sequence (UAS) linked to synuclein (SNCA) flies were crossed with virgin wild-type Canton-S, DDC-GAL4 or GMR-GAL4 female flies) cultured on either medium containing graded concentrations of curcumin and/or piperine (5, 10, and 50 μM) or vehicle. The parameters measured included fecundity, larval motility, negative geotaxis, and lifespan. Vehicle, curcumin, or piperine supplementation in SNCA>Cs, SNCA>DDC, or SNCA>GMR flies did not affect fecundity or larval motility, but piperine (5 μM and 10 μM but not 50 μM) supplementation reduced fecundity. SNCA>DDC flies significantly reduced climbing performance and lifespan, peaked in week 4, ameliorated by the curcumin and piperine combination. Moreover, the increased aggregation of synuclein, as evidenced in ommatidial degeneration in SNCA>GMR flies' eyes, was attenuated by the curcumin and piperine combination. Findings from the present study further reinforced the potential benefit of curcumin and piperine in the management of PD.

Keywords

α-synuclein; Curcumin; Climbing activity; Drosophila melanogaster; Parkinson disease; Piperine

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INTRODUCTION

Parkinson's disease (PD) is the second most popular neurodegenerative disease after Alzheimer's disease (Váradi, 2020), which affects 1-2% of the global population (DeMaagd and Philip, 2015). It commonly affects people 60 years and older but has also been observed in younger populations. The hallmark of PD pathology is the irreversible and progressive loss of neuromelanin-containing dopaminergic neurons in the substantia nigra (Miller and O'Callaghan, 2015), with a corresponding decline in brain dopamine levels.

The clinical features of PD include motor symptoms usually presented in the form of bradykinesia, resting tremor, rigidity, and postural deficits (Kouli *et al*., 2018), which occur when 70% of the dopaminergic neuron population has been lost (Rana *et al*., 2015; Grosch *et al*., 2016). Oxidative stress plays a crucial role in the development of PD. The damage can ultimately lead to death or neuroinflammation, which contributes further to the progression and development of the disease (Lee *et al*., 2019). The current treatment for PD involves enhancing dopamine via administering levodopa and dopamine agonists (Kempuraj *et al*., 2016; Kujawska and Jodynis-Liebert, 2018), but not without drawbacks and adverse effects such as variability in responses, worsening of cognitive functions, fluctuations due to short duration of actions, diminishing long-term efficacy, and incomplete symptom control. While existing Parkinson's medications offer valuable relief, treatment options need to be improved to enhance patients' overall well-being. Thus, natural products could be a viable option in managing PD.

Curcumin is a polyphenol and a bioactive compound derived from the roots of *Curcuma longa* (Zingiberaceae). It has antioxidant, anti-inflammatory, anti-ageing, neuroprotective, and anti-cancer effects (Aggarwal and Harikumar, 2009; Mythri and Srinivas, 2012; Gupta *et al*., 2013). Similarly, piperine (1-piperodylpiperidine) is a polyphenol extracted from black pepper. It is known for its pungency and action on transient receptor potential vanilloid (TRPV) ion channels (McNamara *et al*., 2005). Its antioxidant, anti-inflammatory, and anti-ageing properties have been documented (Sharma *et al*., 2014; Mazumder *et al*., 2021). Moreover, the protective action of piperine on MPTP- and 6-hydroxydopamine-induced PD has been reported through its anti-apoptotic, antioxidant, and anti-inflammatory mechanisms (Vaibhav *et al*., 2012; Shrivastava *et al*., 2013).

Several causative factors are linked with PD pathology, including environmental and genetic risk factors. Thus, there is a need to assess the beneficial effect of curcumin and/or piperine in treating PD with genetic aetiology. To the best of our knowledge, the protective effect of curcumin and/or piperine in the context of specific disease-causing genetic and mechanistic pathways is yet to be examined. In this study, we use Drosophila as a veritable tool to assess the effect of curcumin and/or piperine on α-synuclein aggregation related to neuronal and photoreceptor degeneration using the bipartite UAS/GAL4 system for targeted expression of the protein in dopamine neurons (using the UAS>SNCA/DDC-GAL4 flies) and photoreceptors in ommatidia (UAS>SNCA/GMR-GAL4 flies) (Feany and Bender, 2000).

Interestingly, the overexpression of mutant synuclein alpha (SNCA) in Drosophila dopamine neurons leads to the formation of Lewy bodies like synuclein-containing inclusions, resulting in the loss of dopaminergic neurons as well as behavioural abnormality (Feany and Bender, 2000). Thus, this study was carried out to further evaluate the potential benefits of the curcumin and piperine combination on α-synuclein aggregation-induced dopamine neuron and photoreceptor degeneration in Drosophila, as well as α-synuclein aggregation-induced locomotor deficit and mortality in the *Drosophila melanogaster* model of PD.

MATERIALS AND METHODS

Fly Husbandry and Crosses

The *Drosophila melanogaster* strains used include transgenic strains: the UAS-Synuclein (SNCA), Dopa decarboxylase-GAL4 (DDC-GAL4) (7010), and Glass multiple reporter-GAL4 (GMR-GAL4) (9146), while the wild-type strain was the Canton-Special (CS). Fly stocks were obtained from Bloomington Drosophila Stock Centre at Indiana University, USA, and Dr. Rakesh Mishra Laboratory, Centre of Cellular & Molecular Biology, India. Flies were maintained at 18°C -25° C and on a standard cultured medium containing malt/agar-sugar yeast. The males from the UAS (Upstream Activation Sequence) synuclein (SNCA) were crossed with virgin DDC-GAL4 or GMR-GAL4, respectively. Progenies from the CS flies serving as the non-PD-expressing flies were used as controls.

Fly Diet

Flies were housed in vials containing a malt/agar-sugar-yeast diet at 25°C at regular intervals of the light-dark cycles (12 hours light/12 hours dark). They were transferred to new vials containing fresh meals every two to three weeks. Flies were grown on 5 μM, 10 μM, or 50 μM of curcumin and piperine (Sigma Aldrich, St. Loius, MO, USA), respectively.

Fecundity

Five male and ten female virgin flies were observed using a dissecting microscope to identify the presence of sex combs on the forelegs and darker pigmentation at the posterior end of the abdomen for males; in contrast, females were identified as virgins between 8 and 10 h post-eclosion with the presence of one darker band at the bottom. The flies were introduced into a vial of fresh medium supplemented with vehicle, curcumin, or piperine (5 μM, 10 μM, or 50 μM) and allowed to mate for 48 h, after which they were removed. The number of eggs laid was counted under a light microscope and recorded for each vial; three vials per group were used (Ishola *et al*., 2021b).

Assessment of Larval Motility

A larva was placed in a petri dish containing 2% agar and allowed to acclimatise to its new environment for one minute. It was then allowed to explore the petri dish for one minute. The number of squares crossed (1 cm x 1 cm grid) underneath was recorded. This procedure was repeated for 10 larvae from each vial, three vials per group (Ishola *et al*., 2021c).

Assessment of Negative Geotaxis

A total of 25 adult flies were randomly selected and placed in an empty vial with 8 cm mark. They were allowed to acclimatise for one minute. The vial was tapped until all the flies dropped to the bottom, and a stopwatch was started for 8 s. The number of flies passing the 8 cm mark was observed and recorded. The percentage of flies that crossed the 8 cm mark within 8 seconds was calculated and recorded. The negative geotaxis assay was conducted on Days 7, 14, 21, and 28 of the study (Ishola *et al*., 2021a).

Assessment of Longevity

Mortality was observed in these flies for 28 days. The number of flies that survived was recorded each week, and the remaining flies were euthanized with ethyl ether.

Eye Morphology

The adult flies were prepared for imaging by freezing them at −20°C for approximately 2 hours. Following this incubation, the flies were mounted and placed horizontally over a glass slide using a putty. Images were captured on a MrC5 colour camera mounted on an Amscope™ microscope.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism version 6 software. Data were expressed as mean ± SEM, and all experimental assays were conducted in triplicates per group. One-way ANOVA (for fecundity, larva motility and negative geotaxis assay) or two-way ANOVA (longevity assay) followed by Tukey post-hoc analyses were performed for multiple comparisons, and $P< 0.05$ was declared significant.

RESULTS

Effect of Curcumin and Piperine on the Fecundity of Flies

Findings show a significant reduction in fecundity following treatment with curcumin [F(4,10)=3.59, P=0.05] (Fig. 1a), piperine [F(4,10)=11.37, P=0.001] (Fig. 1b), and curcumin × piperine combination [F(4,10)=4.85, P=0.03] (Fig. 1c). However, post-hoc analysis showed no significant difference in eggs laid between curcumin and vehicle-treated SCNA>DDC flies. Moreover, piperine supplementation (5 μM and 10 μM) and curcumin-piperine (10 μM) combination caused a significant reduction in the mean number of eggs laid.

Effect of Curcumin and Piperine on Number of Crosses

There was a significant effect of piperine [F(4,10)=5.40, P=0.01] (Fig. 2b) and curcumin-piperine [F(3,8)=14.45, P=0.001] (Fig. 2c) supplementation but not curcumin [F(4,10)=0.19, P=0.93] on the number of crosses (Fig. 2a). Post-hoc analysis showed that curcumin administration did not affect the number of crosses compared to the vehicle-treated control (Fig. 2a). However, piperine (50 μM) and curcumin-piperine (10 μM) supplementation caused a significant increase in the number of crosses when compared with vehicle-treated control (Fig. 2b and 2c).

Effect of Curcumin and Piperine on Negative Geotaxis

There was a significant effect of curcumin [F(3,40) = 22.75, $P<0.001$] (Fig.3a), piperine [F(3,40)=16.13, $P<0.001$] (Fig.3b) and curcumin-piperine [F(3,40)=13.23, $P<0.001$] (Fig.3c) supplementation on climbing activity. Post-hoc analysis showed that over-expression of SNCA in dopamine neurons caused a statistically significant decline in climbing activity compared to vehicle-treated control at week 4. However, curcumin (5 μM) caused a significant increase in climbing activity when compared with the vehicle-treated group (Fig. 3a). Similarly, piperine supplementation caused a significant increase in climbing activity when compared with vehicle> SNCA-treated group with peak effect at week 4 (Fig. 3b). In addition, the curcumin-piperine combination produced a significant increase in climbing activity when compared with vehicle-treated control (Fig. 3c)

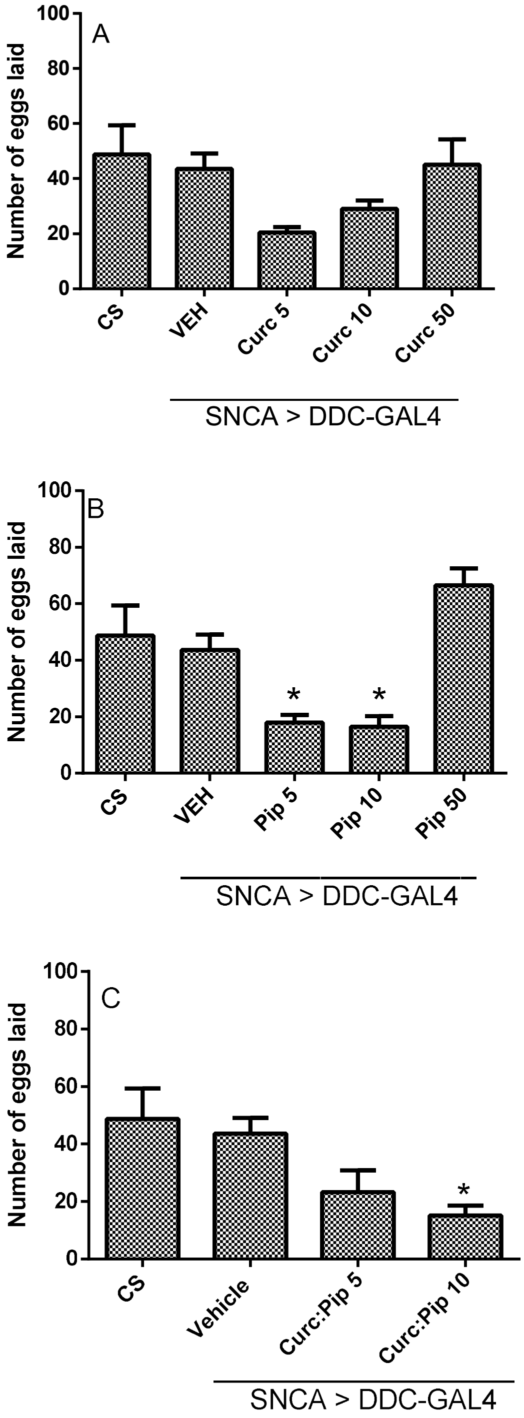

Fig. 1A-C: Effect of curcumin and piperine supplementation on the fecundity of flies. * $p < 0.05$ versus vehicle-treated control. {Cs: Canton-S (wild-type), Vehicle, Curc (Curcumin), Pip (Piperine)}

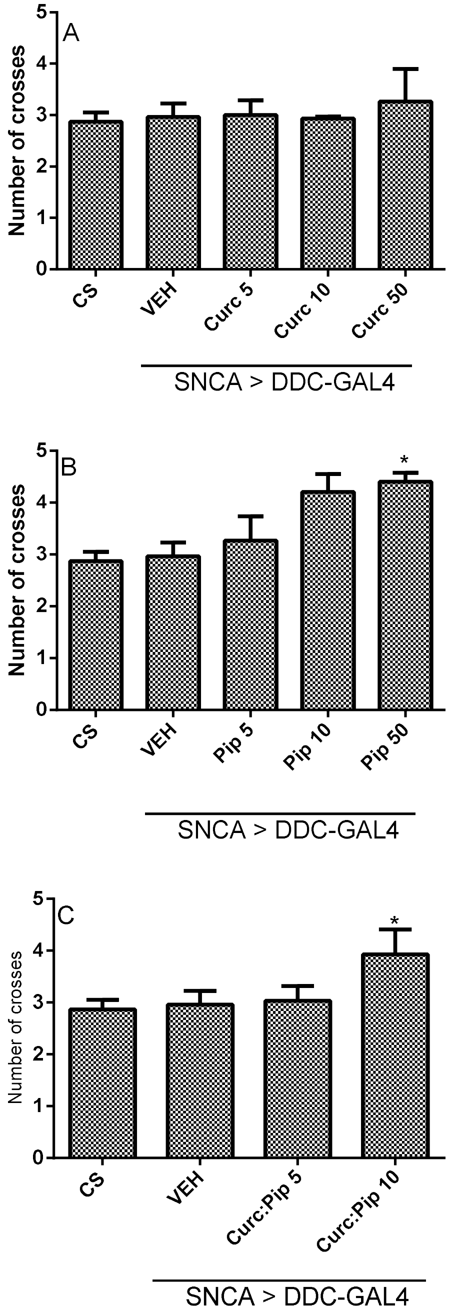

Fig. 2a-c: Effect of curcumin and piperine supplementation on number of crosses in flies. (*P < 0.05 versus vehicle-treated control). {Cs: Canton-S (wild-type), VEH (Vehicle), Curc (Curcumin), Pip (Piperine)}

Effect of Curcumin and Piperine on the Lifespan of Flies

There was no significant effect on the lifespan in the curcumin [F(4,40)=1.58, P=0.20] (Fig. 4a) and piperine [F(4,40)=1.34, P=0.20] (Fig. 4b) treated flies but showed a significant effect on the curcumin-piperine combination treated flies [F(3,40)=34.49, P<0.001] (Fig. 4c). Post-hoc analysis revealed that curcumin or piperine supplementation did not affect the flies' lifespan. Still, combining curcumin and piperine improved the flies' lifespan in the 4th week.

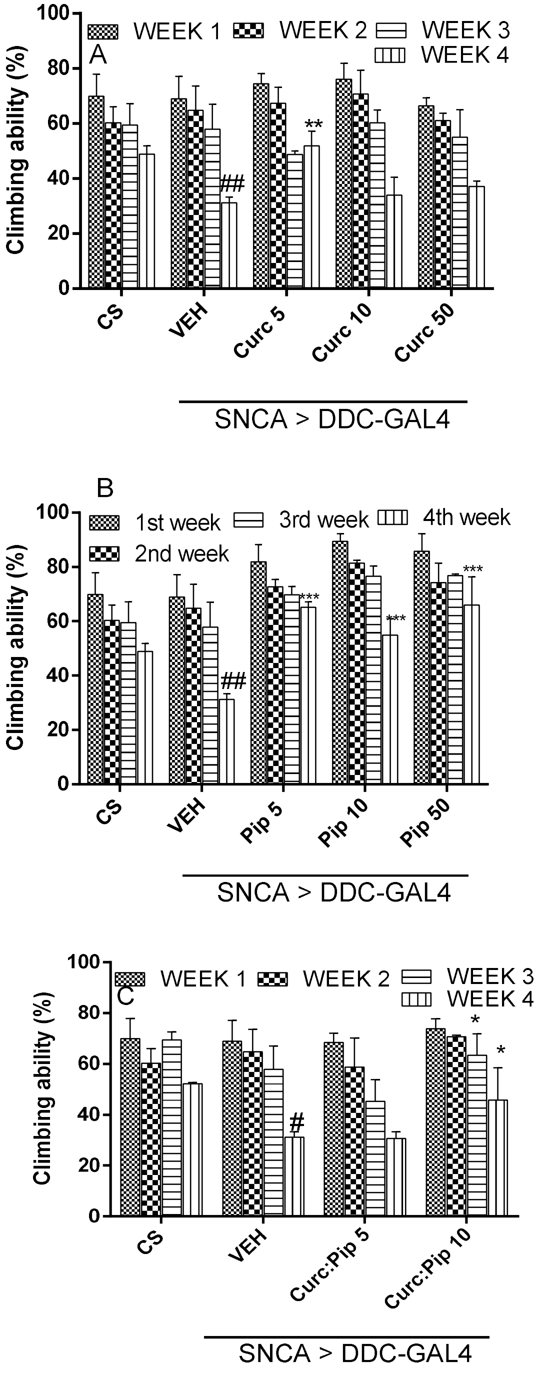

Fig. 3a-c: Effect of curcumin and piperine supplementation on climbing activity in flies. (#P < 0.05. ##P < 0.01 versus vehicle treated control week 1; *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle treated control week 4). {Cs: Canton-S (wild-type), VEH (Vehicle), Curc (Curcumin), Pip (Piperine)}

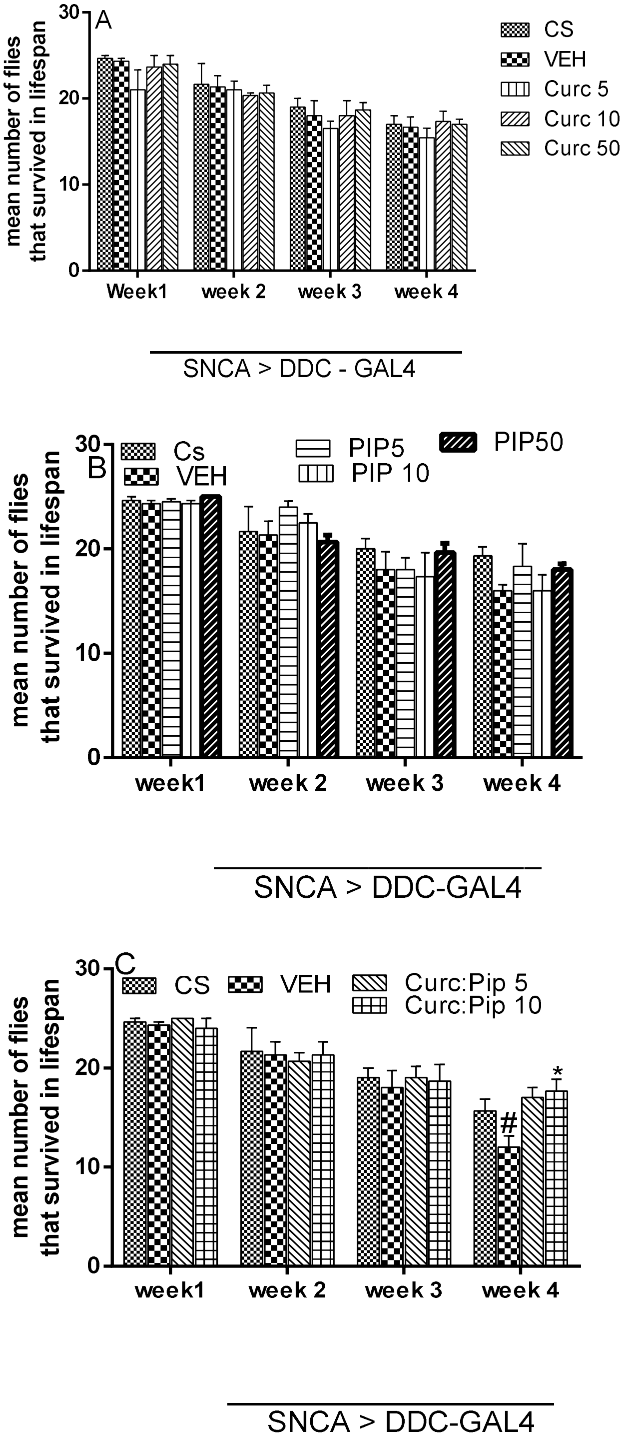

Fig. 4a-c: Effect of curcumin and piperine supplementation on the lifespan of flies. # $p < 0.05$ versus vehicle-treated control week 1; *$p < 0.05$ versus vehicle treated control week 4. {Cs: Canton-S (wild-type), Veh (Vehicle), Curc (Curcumin), Pip (Piperine)}

Effect of Curcumin α-syn Overexpression in the Eye in SNCA>GMR Flies

Wild-type Cs flies showed normal ommatidia, while overexpression of α-synuclein in GMR flies' eyes caused observable degeneration of ommatidia indicative of α-syn aggregation. Curcumin administration reduced the aggregation (Fig. 5).

DISCUSSION

Drosophila melanogaster is widely used to study patterns in genetic behaviour, diseases, and disorders. Parkinson's disease has a vital genetic component that contributes to its pathophysiology, including genetic mutations, defective gene expression, and a compromised protein quality system that regulates α-synuclein (Guo *et al.*, 2022). Feany and Bender (2000) first recorded the use of these genetic components in the overexpression of α-synuclein in *Drosophila melanogaster*, thus recapitulating a key trait in the pathogenesis of PD in clinical patients. α-synuclein was observed to aggregate under pathological conditions, particularly in oxidative stress and inflammatory cascades, where it acquired a toxic gain of function, leading to motor and non-motor symptoms (Sulzer and Edwards, 2019).

Findings from this study showed no significant relationship between the fecundity rate following curcumin and piperine treatment alone or in combination. However, the fecundity rate was lower in curcumin-treated and piperine-treated groups than in flies cultured on the regular media for α-synuclein-expressing DDC-GAL4 flies. The pattern of fecundity rate measured in the DDC-GAL4 fly strain treated with curcumin was more erratic. Therefore, the complexity of the influence of the treatments employed in this study concerning the fecundity rate may be dependent on the presence/absence of chemical stimuli, as well as the concentration of chemical stimuli present in dietary media, interfering with behaviours that ensure successful reproductive survival and development of offspring (Esquivel *et al.*, 2020; Klepsatel *et al.*, 2020).

Larva motility assays help characterise pathological changes in the nervous system as Drosophila larvae expressing mutant α-synuclein develop motor deficits characteristic of PD. The pathological model group of this study did not show any significant difference in the number of crosses compared to the wild-type (CS) flies or flies treated with curcumin and piperine. Normal wild-type larvae are reported to explore at a typical speed of approximately three centimetres per minute (Nichols *et al.*, 2012), which agrees with our findings. However, there was an observable increase in the number of crosses in larvae treated with curcumin in combination with piperine at 10 µM. For the DDC-GAL4 strain, there was a concentration-dependent decrease in the average number of crosses made by larvae cultured on curcumin. This decreae may be due to the hormetic nature of curcumin in the modulation of cellular processes that contribute to the overall integrity of the nervous system (Concetta Scuto *et al.*, 2019; El-Saadony *et al.*, 2022; Kim *et al.*, 2024; Wan *et al.*, 2024).

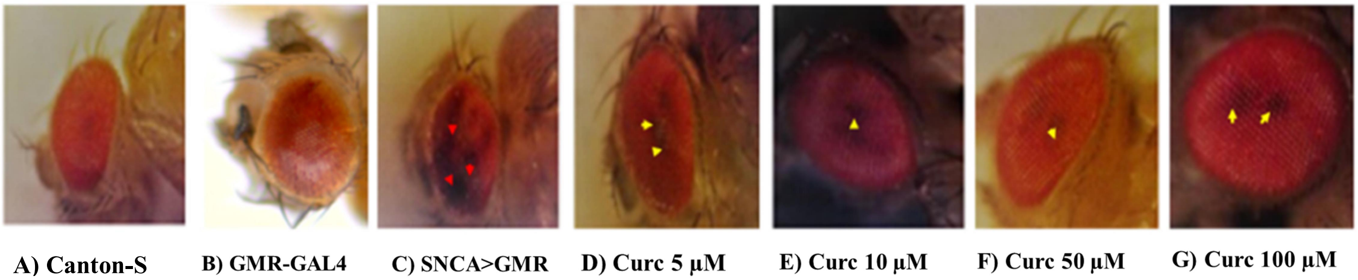

Fig. 5: External morphology of adult drosophila eyes demonstrating GMR-GAL4 targeted expression of α-syn, with (A) showing normal eye and (B) showing a stereotypic array of ommatidia separated by the interommatidial bristles. (C) shows black spreading pigmentation, disrupting the stereotypic arrangement of the eye, denoting aggregation of α-syn (red arrowheads). (D)-(F) showing curcumin improved the structural architecture of the compound eyes of flies expressing α-syn with fewer deposits (yellow arrowheads).

Adult flies exhibited a marked instinctive and natural preference for vertical movement against gravity, mainly when a startled response, perceived as a threat, is applied (Ali *et al*., 2011). This behaviour has been reproducible for generations (Rodan and Rothenfluh, 2010). Thus, it is helpful for the study of nervous system diseases and disorders, including PD (Aggarwal *et al*., 2019). Molina-Mateo *et al*. (2017) showed that the locomotor deficits of *Drosophila melanogaster* only started to emerge in the 3rd week after eclosion, followed by a significant exacerbation in subsequent weeks. Similarly, our findings showed that locomotor deficits in climbing ability were not significant until the fourth week. However, an age-dependent decline in climbing activity was observed from week one (Day 7) to week four (Day 28), possibly due to compensatory mechanisms that delayed the onset of Parkinsonian-like symptoms.

Abolaji *et al*. (2020) showed that curcumin ameliorates copper-induced cytotoxicity in *Drosophila melanogaster* via its antioxidant properties. Also, piperine was shown to abrogate oxidative stress in the MPTP-induced toxin model of Drosophila due to its antioxidant and anti-inflammatory properties (Yang *et al*., 2019). In this study, curcumin and piperine improved the climbing ability of α-synuclein expressing DDC-GAL4 adult flies when used alone at single or combined concentrations compared with vehicle-treated ones. However, there was no significant improvement in motor deficits in curcumin-treated flies compared to pathological control flies. The inability of curcumin to improve motor coordination may be due to its poor oral availability, as studies have revealed its intestinal sulfation and glucuronidation conjugation reaction, which reduced its plasma levels in humans and animals (Singh and Kumar, 2017). In addition, Phom *et al*. (2014), stated that treatment with curcumin replenished depleted dopamine levels in the brain only during early life stages and inferred its limitations as a therapeutic agent for late-onset neurodegenerative disorders such as PD. This alludes to its life-phase-specific impact, especially when using life-stage-specific animals to screen genotropic drugs for their DAergic neuroprotective efficacy (Ayajuddin *et al*., 2022). The longevity of α-synuclein expressing DDC-GAL4 flies was documented as the number of flies that survived after each week of the study, as evidenced by the significant decline of the SNCA>GAL4 flies' lifespan at day 21 (Week 3), which is in agreement with the findings of Esquivel *et al*. (2020). They reported that in α-synuclein-expressing DDC-GAL4 flies, only flies treated with 10 µM of curcumin showed a substantial decrease in average lifespan at days 21 (week 3) and 28 (week 4), which implies that curcumin at this concentration could not clear the accumulation of α-syn which the flies' physiological process failed to manage effectively. Hence, 10 µM could not reverse the impact of the expression of α-syn on the lifespan of the flies. The improvement of motor coordination and the life span of flies by curcumin and piperine revealed the absorption and bio-enhancing properties of piperine when combined with curcumin, which has been shown in other studies (Islam *et al*., 2024; Thanawala *et al*., 2024).

Ommatidial disorganisation occurs when portions of the ommatidia become damaged due to the overexpression of α-synuclein in the eye (Iyer *et al*., 2016). The transgenic strain 9146 (Glass Multiple Reporter, GMR-GAL4) was used to study the effects of neurodegeneration on day 28 (week 4) with curcumin as the dietary intervention. In the SNCA>GMR, ommatidial disorganisation was characterised by extensive scarring, a black spreading pigmentation denoting accumulation of Lewy bodies, and the disruption of the stereotypic array due to the presence of the Lewy bodies in the eye of α-synuclein-expressing flies. However, curcumin effectively rescued the eye from ommatidial degeneration by improving the structural architecture of the eyes by restoring the interommatidial bristles and reducing the black-coloured protein aggregates of the presence of Lewy bodies. Hence, keeping the ommatidial arrangement intact infers curcumin's ability to counteract the degenerative effects of α-synuclein and prevent further damage at specific concentrations.

Conclusion

Curcumin and piperine ameliorated α-synuclein overexpression-induced neurodegeneration, as evidenced by curcumin's improved locomotor activity, lifespan, and restoration of interommatidial bristles. A curcumin and piperine combination could be a potential therapeutic intervention in the treatment of PD.

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Conflict of Interest
None declared.

Authors' Contribution
OO - Conceptualized designed the experiment and drafted the manuscript and data interpretation; GO - Data analysis and manuscript preparation; MO - collected the data (sample preparation and imaging); IO - designed the experiment, data interpretation and final correction.

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