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Research Article

Neuroprotective Effects of Calycosin Against Fenpropathrin-Induced Dopaminergic Neurodegeneration in *Drosophila melanogaster*

Saba Afsheen[1], Mohammad Mumtaz Alam[2], Suhel Parvez[1*]

[1]*Department of Toxicology, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India.*
[2]*Department of Pharmaceutical Chemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India.*

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ABSTRACT

The loss of dopaminergic neurones in the substantia nigra pars compacta is a hallmark of Parkinson's disease (PD), a neurodegenerative condition. Environmental pesticide exposure, particularly to pyrethroids such as fenpropathrin (Fen), represents a significant risk factor for PD pathogenesis alongside genetic predispositions. Recently, studies have indicated that exposure to Fen may also increase the likelihood of developing PD. In this study, we validated a *Drosophila melanogaster* model of neurotoxicity by exposing 2 to 5-day-old male flied to 5 mg/L fenpropethrin (Fen) for 96 hours. Motor functions were assessed using climbing, jumping, and crawling assays, along with survival and pupal development studies. To investigate underlying molecular changes, protein analysis was performed through western blot to evaluate the expression of the dopaminergic neuron marker tyrosine hydroxylase and the apoptotic marker caspase-3. Subsequently, calycosin, an isoflavone phytoestrogen obtained from Astragalus membranaceus that has anti-inflammatory, antioxidant, and anti-apoptotic qualities, was evaluated for its neuroprotective effectiveness. Calycosin was tested at concentrations of 50, 100, and 200 µM, with 50 µM identified as the optimal dose for improved survival rates and locomotor performance. Previous studies suggest calycosin's neuroprotective effects, but its role in fenpropathrin (Fen)-induced neurotoxicity remains unexplored. This study evaluates calycosin's efficacy against Fen-induced neurodegeneration in *Drosophila melanogaster*. Calycosin significantly improved motor function, including a 48% increase in crawling behaviour and a 20% rise in survival rates. It reduced cleaved caspase-3 levels by 16.7%, alleviated developmental delays by 18.9%, and restored tyrosine hydroxylase expression by 44.1%, indicating protection of dopaminergic neurons. These findings demonstrate that calycosin provides significant neuroprotection against Fen-induced neurodegeneration in the *Drosophila* PD-like model, suggesting its potential as a therapeutic intervention for pesticide-related neurodegenerative diseases.

Introduction

Approximately 3.9% of people aged 60 to 65 worldwide suffer from Parkinson's disease (PD), the second most prevalent age-related movement illness. Tremors, stiffness, motor dysfunction, and cognitive impairment are its clinical hallmarks.[1,2] The development of Lewy bodies, α-synuclein aggregation, and the gradual loss of dopaminergic neurones in the midbrain's substantia nigra pars compacta are the hallmark pathological features of Parkinson's disease.[3,4] The intricate pathophysiology of idiopathic Parkinson's disease remains poorly understood despite scientific advancements, and current therapies such as dopamine precursors, dopamine agonists, anticholinergic drugs, and L-DOPA only temporarily alleviate symptoms and do not stop the illness's development. [5] While the exact cause of PD is not yet completely understood, environmental toxicants are believed to be a risk factor, with pesticides being one of the most common environmental exposures associated with the disease.[2,6] Several neurotoxic compounds, such as paraquat (PQ), rotenone, MPTP, and other neurotoxic chemicals, have been connected to an increased risk of developing PD.[7] More recently, the

***Corresponding Author:** Prof. Suhel Parvez

Address: Department of Toxicology, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India.

Email ✉**:** sparvez@jamiahamdard.ac.in

Tel.: +91-9811992124

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synthetic pyrethroid insecticide fenpropathrin (Fen) has also emerged as a potential contributor, as it is known to increase the risk of Parkinsonism. A notable case study from China reported Parkinsonism in a man following prolonged consumption of Fen-contaminated fish. [8] Recent animal studies have further demonstrated that Fen exposure is linked to PD, as it induces the loss of dopaminergic neurons and mimics some pathological features of the disease. Previous research in cell lines and rats has confirmed that Fen can cause neurodegeneration. [8,9]

Researchers have employed various animal models to better understand PD pathogenesis and screen potential therapeutics. These include vertebrates like mice and zebrafish, as well as invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans*. [10–12]

D. melanogaster has become a valuable model for studying PD pathogenesis and serves as an efficient platform for early-stage drug discovery before validation in mammalian models. Its robust phenotypes and multifunctional characteristics have enabled extensive genetic and pharmacological screenings, facilitating the identification of potential therapies for PD and other age-related neurodegenerative disorders. [13]

A plant-based diet, especially one high in flavonoids, has been shown in epidemiological studies to be associated with decreased mortality and a lower risk of age-related chronic illnesses. [14,15] L-dopa, the most commonly prescribed drug for Parkinson's disease, was first isolated from the Vicia faba bean[16], highlighting the relevance of natural products in PD treatment.

Several plant-derived compounds, such as flavonoids and isoflavones, have shown significant neuroprotective effects in various models of neurodegenerative illnesses attributed to their antioxidant, anti-inflammatory, and anti-apoptotic characteristics. [17–20]

Several plant-derived compounds, such as flavonoids and isoflavones, have shown significant neuroprotective effects in various models of neurodegenerative diseases due to their antioxidant, anti-inflammatory, and anti-apoptotic properties. Among these, calycosin—a major isoflavone found in *Astragalus membranaceus*—has gained attention for its potent bioactivity, including neuroprotective potential. Previous research has shown that calycosin can protect against neurological damage induced by cerebral ischemia and reperfusion, high-glucose-induced oxidative stress, neuroinflammation, and neuronal apoptosis. [21,22]

However, while its antioxidant potential is well documented, the broader pathways through which calycosin may exert anti-neurodegenerative effects remain unclear. [23] In this study, we investigated the neuroprotective efficacy of calycosin in a *D. melanogaster* model of PD induced by exposure to the pyrethroid pesticide fenpropathrin.

There is a pressing need for disease-modifying drugs with increased safety and bioavailability in cases where PD therapies are restricted to managing symptoms and frequently linked to adverse effects, including dyskinesia and tolerance. In a *D. melanogaster* model of Parkinson's disease (PD) brought on by exposure to fenpropathrin, the study examines the neuroprotective effectiveness of calycosin, which has several pharmacological benefits. Calycosin, a naturally occurring isoflavone, offers a scalable and affordable substitute for manufactured medications. It may also have the potential to increase bioavailability through formulation techniques like nanoencapsulation. Its complex systems may be able to halt the onset of Parkinson's disease and lessen the neurotoxicity caused by pesticides. This study advances personalised therapy for neurodegenerative disorders by confirming calycosin in an invertebrate model, which lays the groundwork for translational development, including adjuvant medicines that target environmental risk factors in high-exposure populations.

Materials And Methods

Materials

Calycosin (purity ≥ 99%, Cat# HY-N0519), Fenpropathrin (> 98% pure Sigma-Aldrich), dimethyl sulfoxide (DMSO), Protease inhibitor (Abbkine), anti-TH (AB 152 Merck) and cleaved Caspase-3 antibody (9661 Cell Signaling), secondary antibodies (anti-rabbit Invitrogen), RIPA buffer (Cell Signaling Technology).

Methods

Drosophila culture and stock maintenance

D. melanogaster flies (CantonS; BDSC #64349) were raised at 24 ± 1°C on a standard medium. The flies were purchased from the Bloomington Stock Center. Fenpropathrin (> 98% pure Sigma-Aldrich) was dissolved in acetone and thoroughly mixed to reach the required concentration. Calycosin (purity ≥ 99%, Cat# HY-N0519) was obtained from Med Chem Express (Monmouth Junction, NJ, USA) and dissolved in 0.1% dimethyl sulfoxide (DMSO). [24]

Drosophila Feeding Regimen and Determination of Calycosin Concentration

Male flies aged 2 to 5 days were given 5 mg/l of Fen every day for the duration of this investigation. (based on LC_{50} results from previous work) [25,26] with different calycosin concentrations (50, 100, 200 µM) for 96 hours to determine the appropriate calycosin concentration. [27] Based on the surviving flies' locomotor activity and jumping distance, 50 µM was determined to be the optimal concentration for 96-hour exposure to calycosin.

Locomotor Behavioural Assays in a *Drosophila* for Assessing Fenpropathrin Toxicity and the Therapeutic Efficacy of Calycosin

Climbing assay

A climbing assay, also known as a negative geotaxis assay, was conducted following the method outlined by[28] with

minor modifications. Ten male flies were selected, briefly anesthetized with ether, and placed in an empty glass vial or 100 mL glass cylinder. A horizontal line was drawn 10 cm from the base of the cylinder. After recovery, *Drosophila* were allowed to acclimate for 1-minute. The cylinder was tapped against a foam pad, and the number of flies that climbed above the 10 cm line (ntop) within 10 seconds was recorded, while those remaining below the line (nbot) were also noted. Using the formula, the locomotor performance index (PI) was determined. (1/2 [(ntot + ntop – nbot)/ntot]). Ten trials were conducted separately for each control and treated group, and the experiment was repeated independently three times at 25°C.

Jumping assay

Flight capability was assessed using a jumping assay.[29] The wings of male flies were removed, and after a rest period, each fly was placed on a 10 cm high platform. The horizontal distance the fly could jump was measured, and the average distance of the six longest jumps was recorded. Ten trials were conducted for each fly in both the control and treated groups.

Crawling assay

Larval crawling is an easy-to-conduct assay that helps study rhythmic movements and detect neural abnormalities.[30,31] We calculated larval movement by adapting the approach.[32] with some adjustments. The behavior of third instar larvae was assessed by transferring them from both treated and control groups onto a PBS-washed petri plate and then placing them in a glass dish with diluted yeast solution. After a five-minute adjustment period, the total peristaltic contractions were counted, with each contraction representing a full anterior-to-posterior movement for a minute.

Survival and Development assays in a *Drosophila* for Assessing Fenpropathrin Toxicity and the Therapeutic Efficacy of Calycosin

Survival assay

Male flies that had recently emerged were collected and allocated to various feeding conditions in groups of 10 males per vial. [33] The flies were checked every three days to count the dead, and the remaining flies were moved to new food until all were dead.

Assay of preadult development period/ Egg-to-adult development time

The newly emerged flies were placed in petri dishes to lay eggs, and the synchronized eggs were collected from the food plates and moved to the treated and control groups, where they were observed daily.[34] The duration from egg to prepupa, prepupa to fly, and egg to fly was individually recorded. Five vials, each containing 20 eggs, were used per group, making a total of 100 eggs per group. This experiment was conducted three times.

Protein Biomarkers in the *Drosophila* for Fenpropathrin Toxicity Assessment and Therapeutic Efficacy of Calycosin

Western blotting

Sample preparation involved freezing 200 *Drosophila* heads in liquid nitrogen, followed by homogenization in Protease inhibitor (Abbkine) combined with RIPA lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2 EDTA, and 1 mM EGTA). The samples were then centrifuged for 10 minutes at 4°C at 10,000 rpm. Following centrifugation, the particle was disposed of and the supernatant was shifted to a new Eppendorf tube. The Bradford technique test was used to quantify the proteins in the supernatant. An equal amount of protein (30–50 µg per well) was separated using 8 to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. Additionally, the membrane was incubated in a 5% skimmed milk solution (Millipore) at room temperature for 1 hour to block the protein blots. After blocking, the blot was incubated overnight at 4°C with primary anti-TH (AB 152 Merck) and cleaved Caspase-3 antibody (9661 Cell signaling) at 1:1000 dilutions. After washing the blot with PBST the following day, incubate it with secondary antibodies (anti-rabbit Invitrogen) at a 1:1000 dilution for 1 hour at 24 to 25°C. Finally, protein expression was detected using an enhanced chemiluminescence reagent on a ChemiDoc MP imaging system (BIO-RAD), and ImageJ software was used to conduct densitometric analysis.

Statistical Analysis

The GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) was used to analyze the data. The data from all experiments were expressed in the form of mean ± SEM and analyzed using ANOVA, followed by Tukey's post hoc test for comparison. Values with $p < 0.05$ were regarded as significant.

Results

Calycosin Mitigates Fen-induced Locomotor Dysfunction and Jumping Deficit in CantonS Flies

Research conducted on cell lines and rats has previously shown that Fen is capable of causing neurodegeneration[8,9] in our lab showed that Fen exposure significantly reduces survival and impairs. Consequently, we chose Fen, an environmental neurotoxin, to induce a neurodegenerative model in *Drosophila* that mimics employing the locomotor behaviours and other pathological signs of Parkinson's disease in humans as a screening tool to find nutraceuticals that have neuroprotective qualities against the neurotoxicity caused by Fen. We chose the maximum dosage of Fen (5 mg/l) for 96 hours as a working dose after a comparable exposure schedule (based on LC_{50}).[25,26] In this study, we excluded female flies and focused solely

on males because female *Drosophila* exhibit feeding behaviors that are not only inherently variable but also significantly influenced by their mating status.[35] To find out if calycosin could restore the locomotor function that Fen exposure had damaged in CantonS flies, male flies that were 2–5 days old were treated to 5 mg/l of Fen either by itself or with varying doses of calycosin (50, 100, and 200 µM) for 96 hours. (Fig.1 A). The results of the climbing assay indicated a reduction in the locomotory performance index in Fen-treated adults. Fen exposure caused a substantial drop in the climbing ability of flies compared to the control group ($p < 0.001$). Interestingly, calycosin co-therapy (50 µM) significantly restores locomotor abilities compared to the 5mg/l of Fen treated group ($p < 0.001$). However, further increasing the dose of calycosin does not result in significant restoration against Fen-induced locomotor impairment. Since DMSO treatment did not cause significant changes compared to the control, only the control group was included for comparison. Additionally, we conducted a jumping assay to examine the effect of calycosin by assessing the jumping performance of adult male flies. We followed a similar exposure protocol, where 2–5-day-old CantonS male flies were exposed to 5 mg/l of Fen alone or in combination with varying calycosin concentrations (50, 100, 200 µM) for 96 hours. The jumping performance was then analyzed (Fig. 1B). A significant reduction in the jumping performance was observed in the 5 mg/l Fen-treated vials compared to the control group ($p < 0.001$). Interestingly, further calycosin co-treatment (50 µM) demonstrated a significant improvement in the jumping ability compared to the 5mg/l of Fen treated group ($p < 0.001$). In line with previous observations, a similar pattern was observed in the jumping performance, where further increasing the calycosin dose did not lead to any significant enhancement in the jumping ability of adult male flies affected by Fen-induced behavioral deficits. Therefore, the 50 µM calycosin concentration was selected for our study, as it demonstrated the greatest protection ($p < 0.001$) against Fen-induced toxicity. In this study, we excluded female flies and focused solely on males because of the differences in feeding behaviors observed in the females.

Canton S male flies (2–5 days old) were exposed to 5 mg/l fenpropathrin (Fen) for 96 hours, with varying concentrations of calycosin (50, 100, 200 µM). Based on the locomotor activity and jumping distance of the surviving flies, 50 µM was determined to be the optimal concentration for 96-hour exposure to calycosin. The number of flies that ascended 10 cm in 10 seconds was recorded. Statistical significance is indicated by *** $p < 0.001$ in comparison to the unexposed control group and ### $p < 0.001$ in relation to the Fen exposed group. About 10 separate biological replicates with 10 male flies per feeding condition are shown in the data. The data is presented as the mean ± SEM from three distinct studies, analyzed via one-way ANOVA and Tukey's multiple-comparison test.

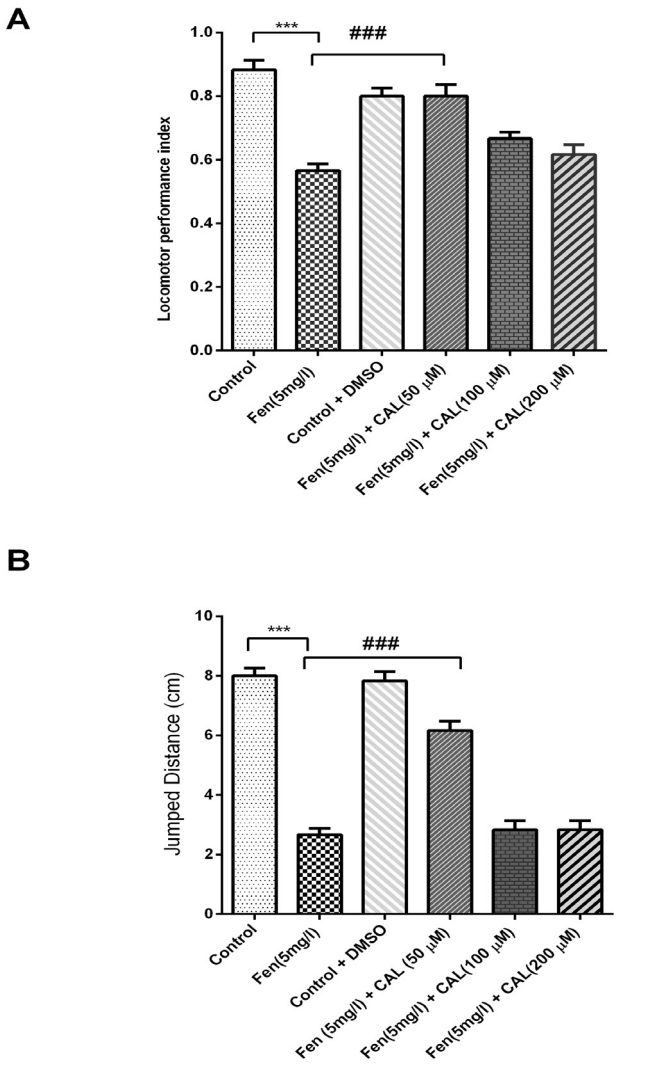

Fig. 1: Effects of the phytoestrogen, calycosin against Fen-induced toxicity on *D. melanogaster* CantonS adult male flies A) climbing B) jumping assay

Calycosin ameliorates Fen-induced larval crawling defects in the third larval crawling assay

The results in Fig. 2 show a significant decrease in body wall contractions in the group treated with 5 mg/l Fen ($p < 0.001$) compared to the control group. However, when calycosin was co-administered, there was a significant improvement in the crawling behavior of third instar larvae compared to those treated with 5 mg/l Fen alone ($p < 0.01$).

Third instar larvae from control and treated groups were subjected to a crawling assay. Larvae exposed to 5 mg/l Fen exhibited a significant reduction in body wall contractions compared to the control group ($p < 0.001$).

Calycosin co-treatment significantly improved crawling ability, with higher body wall contraction counts compared to the Fen-only group ($p < 0.01$). significance is ascribed as *** $p < 0.001$ vs unexposed control group and ## $p < 0.01$ vs Fen exposed group. The data is presented as mean ± SEM from three independent experiments and analyzed using one-way ANOVA, followed by Tukey's multiple-comparison test.

Calycosin Enhances Survival and Decreases Mortality in Flies Exposed to Fen: Insights from a Survival Assay

We carried out a survival assay to assess whether calycosin can mitigate Fen-induced mortality. Reflecting earlier findings, all control male flies survived for the entire 15-day duration. In contrast, males exposed to various Fen doses experienced a marked drop in survival compared to the untreated group. Notably, the 5 mg/l Fen-treated group displayed just 40% survival by day six (Fig. 3) compared to the control group. Interestingly, most flies in this group survived almost nine days. Remarkably, calycosin treatment markedly improved survival rates, with 60% of flies in the 5 mg/l Fen in combination with the calycosin group surviving by day six, compared to the 40% survival rate in the 5 mg/l Fen group.

CantonS adult male flies. As previously mentioned, survival experiments were conducted using adult male flies that were two days old (10 flies per vial) in several treatment and control groups. The number of living flies was counted until all of the flies perished. Every other day, the food vial was replaced. *** $p < 0.001$ is the significance level when compared to the unexposed control. This data is displayed as mean ± SEM and is subjected to one-way ANOVA and Tukey's multiple-comparison test.

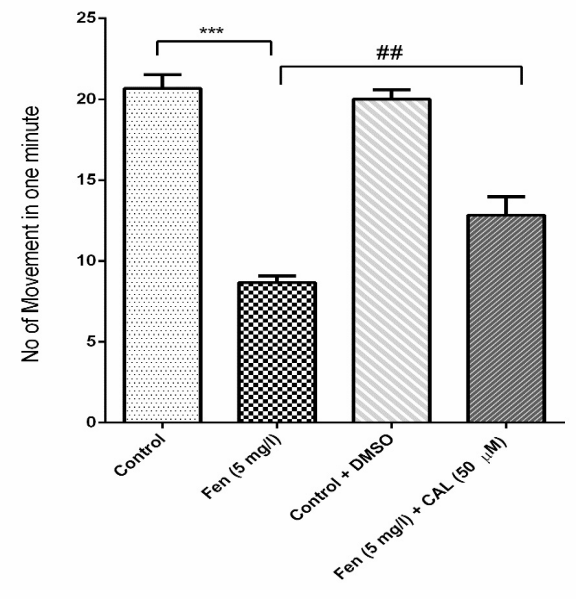

Fig. 2: Protection of Drosophila crawling impairments caused by Fen by calycosin larvae

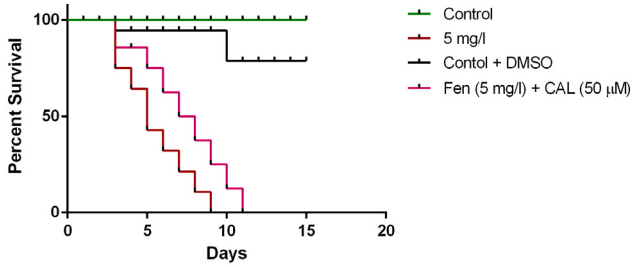

Fig. 3: Calycosin Reduces Fen-Induced Mortality and Extends Lifespan in Drosophila

Effect of Calycosin on the developmental time period

To further assess calycosin's impact on *Drosophila's* developmental timeline, we treated the eggs with 5 mg/l Fen either alone or in combination with calycosin. The findings indicated a notable extension in the egg-to-prepupa developing duration in groups administered 5 mg/l Fen relative to the control group ($p < 0.001$), as illustrated in Fig. 4. Additionally, there was no progression from prepupa to adult fly in the 5 mg/l Fen-treated group, indicating that development stalled before reaching the egg-to-fly stage. However, when calycosin was administered in combination with 5 mg/l Fen, there was a noticeable reduction in the developmental time from egg to prepupa ($p < 0.05$).

Treatment with 5 mg/l Fen significantly extended the egg-to-prepupa developmental period, with no transition from prepupa to adult flies ($p < 0.001$). In contrast, co-treatment with calycosin significantly shortened the egg-to-prepupa developmental time compared to the Fen-only group ($p < 0.05$). This implies that calycosin may be able to reverse the developmental deficits brought on by Fen. The significance level is established as ### $p < 0.05$ *vs.* the Fen-exposed group and ### $p < 0.001$ vs. the unexposed control group. The data is shown as the mean ± SEM of three experiments, analyzed using one-way ANOVA and Tukey's multiple-comparison test.

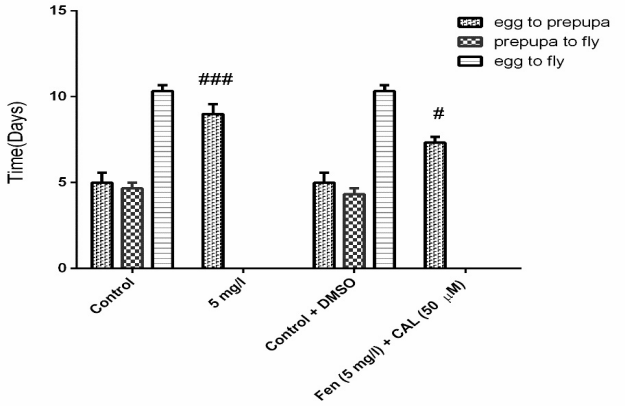

Fig. 4: Effect of calycosin on Fen-induced developmental time delay in different stages of *D. melanogaster* CantonS

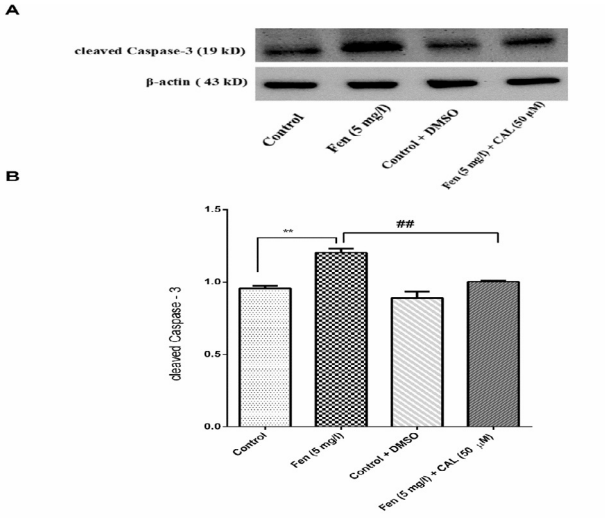

Fig. 5: Calycosin-supplemented flies showed increased resistance to neuronal cell death following Fen exposure

Calycosin Mitigates Fen-Induced Apoptosis by Reducing cleaved Caspase-3 Activation in adult male Flies

Increased expression of caspase-3- 3 protein has been related to apoptosis. We found a significant increase in cleaved caspase-3 expression in Drosophila brain tissues exposed to 5 mg/l Fen compared to the control group ($p < 0.01$). This suggests that exposure to Fen triggers apoptosis, namely through the activation of cleaved caspase-3 (Fig. 5). However, as compared to the Fen-only group, co-administering calycosin with Fen at a dosage of 5 mg/l substantially decreased cleaved caspase-3 levels ($p < 0.01$).

Representative immunoblots showing expression of cleaved caspase-3 were analyzed by western blotting (A). Densitometric data presented are shown after normalization with loading control β-Actin (B) Graph shows quantitative analysis of protein-band intensity; significance is assigned ** $p < 0.01$ vs unexposed control and ## $p < 0.01$ vs Fen exposed group. Data from three distinct experiments are provided as mean ± SEM.

Calycosin Protects Dopaminergic Neurons from Fen-Induced Toxicity by Preserving Tyrosine Hydroxylase Expression

Pesticides such as paraquat (PQ) have been demonstrated to cause damage to dopaminergic neurons, resulting in motor impairments across various laboratory models. [36–38] Similarly, Fen, a commonly used pesticide, has been linked to the degeneration of dopaminergic neurons in several studies.[39] Dopaminergic neurons rely on the enzymatic activity of tyrosine hydroxylase to convert L-dopa into dopamine, which is crucial for proper neuronal function. [40] Given these effects, we aimed to investigate whether calycosin could counteract the neurotoxic effects of Fen on dopaminergic neurons. To evaluate this, we conducted a Western blot analysis to measure the brain tissue's tyrosine hydroxylase (TH) expression levels.

Our results demonstrated that TH levels were significantly reduced following exposure to 5 mg/l of Fen compared to controls ($p < 0.001$) (Fig. 6). However, when calycosin was co-administered with Fen at the same dose (5 mg/l), TH expression levels were substantially greater compared to the Fen-only group ($p < 0.01$).

Expression of tyrosine hydroxylase was assessed using the western blot technique (A). Data from densitometric analysis are displayed following normalization to the loading control β-actin (B). Statistical significance is shown by *** $p < 0.001$ versus the control group and ## $p < 0.01$ when compared to the Fen-exposed group. Results are presented as mean ± SEM from three separate studies.

Discussion

This research attempts to assess the neuroprotective effects of calycosin, a phytoestrogen, against Fen-induced neurodegeneration in *Drosophila*. Fen, a widely used pesticide, has been consistently linked to Parkinson's disease-like symptoms in various models, including *Drosophila* [8,39,41], highlighting its relevance in investigating neuroprotective agents. While L-DOPA is considered the most effective treatment for Parkinson's PD symptoms, its long-term use can lead to motor fluctuations

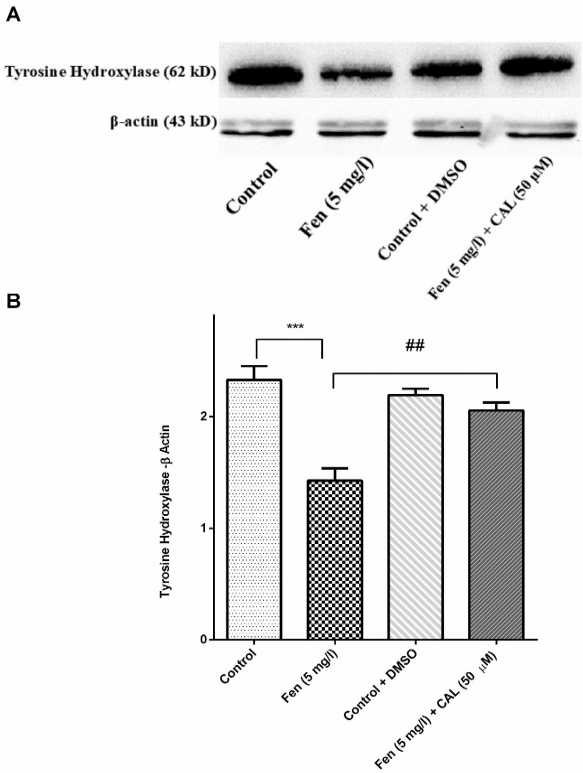

Fig. 6: Effect of calycosin on tyrosine hydroxylase expression in brain tissue of *D. melanogaster* CantonS male adults

and dyskinesia.[42,43] Current therapies for PD mainly offer symptomatic relief and do not address the underlying disease mechanisms. [44] Therefore, there is a dire need for new therapeutic approaches that target the root causes of PD and provide more effective management. Flavonoids, made up of plant polyphenols, have demonstrated the ability to diminish the risk of various chronic diseases, including neurological disorders. [45–47] Given their potential, exploring new pharmacological agents derived from natural plant products with minimal side effects is essential for advancing PD treatment. This *in-vivo* study evaluated the protective efficacy of Calycosin against Fen-induced neurotoxicity in Drosophila. Earlier studies showed a neuroprotective impact of calycosin against PQ using the MPTP-induced PD model.[48,49] Therefore, several aspects of neurotoxicity were assessed, including locomotor dysfunction, developmental delays, dopaminergic abnormalities, and apoptotic markers, all of which have been linked to PD in both human and experimental models.[50]

The results of our study indicate that calycosin positively influenced treatment outcomes in FEN-exposed flies, leading to significant improvements in neurobehavioral deficits, such as locomotor and jumping performance. This observation is consistent with previous studies highlighting calycosin's neuroprotective capabilities, particularly its role in enhancing motor performance in PQ-exposed flies.[48]

Next, we performed larval crawling assays, which provide insights into the integrity of the neuromuscular system, with defects in this behavior indicative of impaired neuronal function. Our study demonstrated that Fen-treated larvae exhibited a marked reduction in body wall contractions compared to the control group, indicating that Fen damages the neurons of larvae. The observed defects likely result from an impact on dopamine, which is crucial for insect locomotion. However, co-treatment with calycosin significantly improved larval crawling behavior by approximately 48% compared to the Fen-treated flies, suggesting that calycosin exerts a protective effect even during early developmental stages. His improvement is crucial as it indicates that calycosin may preserve neuromuscular function, reducing the neurodevelopmental deficits induced by Fen. This finding is consistent with previous research on *Drosophila* models of ALS and rotenone exposure, which also reported similar crawling defects. [51,52]

Additionally, the study examined the developmental impact of Fen exposure, as previous research has shown that insecticides and pesticides can disrupt key developmental stages such as hatch rates and emergence in *D. melanogaster*[53–55] Similar developmental delays have been observed with other pesticides, including chlorpyrifos and captafol [53] Interestingly, calycosin treatment led to a notable reduction of 18.9% in the developmental delays caused by Fen, indicating its potential protective role in development. While it didn't entirely reverse the Fen-induced defects, the slight recovery in the egg-to-prepupa stage suggests calycosin mitigates some developmental toxicity caused by Fen. This effect might be linked to its anti-inflammatory and antioxidant properties, critical in neutralizing oxidative stress, a major driver of developmental toxicity in pesticide-exposed organisms.

Regarding survival and mortality, flavonoids like fisetin, hesperidin, and xanthohumol have been shown to improve survival rates in Drosophila exposed to paraquat, highlighting their protective effects.[56] Similarly, research has indicated that combining paraquat with polyphenols, such as propyl gallate and epigallocatechin gallate, results in significantly increased lifespan and enhanced locomotor activity in flies compared to untreated controls [57] We conducted a survival assay to evaluate whether calycosin could mitigate Fen-induced mortality.

Similarly, our findings revealed that calycosin treatment resulted in a 20% increase in the survival rate of flies, likely due to its ability to mitigate oxidative stress and enhance neuronal survival under Fen-induced toxicity. This improvement is consistent with previous studies demonstrating calycosin's ability to enhance survival in the context of paraquat-induced toxicity. However, further studies need to be conducted on the post-treatment (rescue) approach to enhance our findings' translational value. Interestingly, in a related study, calycosin significantly improved survival in Drosophila exposed to paraquat during co-treatment and when administered 24 to 72 hours after PQ exposure, confirming its rescue potential even in pre-intoxicated flies. This supports the therapeutic relevance of calycosin beyond possible interference with pesticide uptake. [48]

In addition, the triggering of caspases, particularly caspase-3, and the subsequent apoptosis of dopaminergic neurons play an important role in the progression of PD. Caspase-3, the key executioner in the apoptotic cascade, has been found in its active form in the substantia nigra of PD patients.[58] Flavonoids are widely recognized for their ability to modulate apoptotic pathways in neurodegenerative disease models, both in vitro and in vivo. [59,60] Previous studies have demonstrated calycosin's neuroprotective effects against paraquat-induced neuronal death. In light of this, our study focused on investigating the protective potential of calycosin in mitigating Fen-induced neuronal apoptosis.

Similarly, Western blot results from our study revealed a marked increase in cleaved caspase-3 expression in the brain tissues of flies treated with Fen compared to the control group. However, when calycosin was co-administered with Fen at the same concentration, cleaved caspase-3 levels were significantly reduced by 16.7% relative to the Fen-treated flies. This suggests that calycosin protects against Fen-induced apoptosis in

dopaminergic neurons. This investigation is supported by earlier studies that have shown plant extracts and flavonoids to be effective in inhibiting cleaved caspase-3 activity in PD models.[61–63]

Tyrosine hydroxylase, a crucial indicator for dopaminergic neurons, plays a significant role in PD.[64] A decline in dopamine (DA) levels is linked to several central nervous system disorders, including PD.[65] PD is characterized by a distinct loss of dopaminergic neurons and a decrease in dopamine levels within the striatum.

Numerous studies indicate that exposure to pesticides such as MPTP, rotenone, and paraquat diminishes TH expression by inducing the death of dopaminergic neurons in both Drosophila and mammalian models of Parkinson's disease.[37,61,66] Previous studies showed that Fen exposure led to a notable decrease in tyrosine hydroxylase levels and TH-positive cell counts in the SNpc and striatum of mice SNpc and striatum. [67]

In line with these findings, our results demonstrated that calycosin treatment significantly enhanced TH expression levels by 44.1% in Fen-exposed flies. This suggests that calycosin may exert neuroprotective effects by modulating the dopaminergic signaling pathway. The reduction in cleaved caspase-3 levels and the preservation of TH expression strongly suggest that calycosin's neuroprotective effects are mediated by its antioxidant properties and indirectly support the hypothesis that calycosin mitigates ROS formation and subsequent neuronal apoptosis via modulating the oxidative stress pathway.

Several studies further supported this; for instance, cellular assays demonstrated that calycosin exerted neuroprotective effects counteracting α-synuclein amyloid-induced neurotoxicity via its antioxidant capabilities.[68] In another study, calycosin was shown to upregulate endogenous antioxidant levels and reduce ROS generation and apoptosis, suggesting that its antioxidative activity plays a critical role in mitigating neurotoxicity. [69]

There are certain limitations in our investigation. Initially, the study focused on the immediate effects of calycosin (96-hour co-treatment period), highlighting the need for further research using a long-term post-treatment (rescue) approach. Furthermore, as the present study offers preliminary evidence in *D. melanogaster,* a more in-depth investigation is needed to understand its exact molecular mechanisms.

When combined, our results provide a coherent picture of a calycosin-induced multi-level neuroprotective cascade. As demonstrated by the 16.7% decrease in cleaved caspase-3, the noteworthy 44.1% restoration of tyrosine hydroxylase (TH) expression is not an isolated occurrence; rather, it seems to be the direct downstream result of reducing apoptosis. In summary, calycosin is essentially protecting the dopaminergic neurones against Fen-induced cell death in addition to encouraging dopamine production. The amazing functional improvement we witnessed has a clear biological foundation thanks to this cellular-level intervention. A healthier, more complete dopaminergic circuit is directly reflected in the improved motor performance in adult flies and the 48% improvement in crawling in larval flies. Additionally, the improvements in developmental time (18.9% reduction of delay) and survival (20% increase) imply that calycosin's advantages go beyond the neural system, promoting a comprehensive physiological resilience to Fen's systemic toxicity. This implies that calycosin functions as a strong modulator of cellular health rather than a specific, one-target agent. It can stop the toxic cascade at a crucial point, preventing cell death, which then spreads to restore intricate biological processes and enhance overall organismal fitness.

Conclusion

The present research highlights the neuroprotective potential of calycosin against Fen-induced Parkinson's-like phenotypes using *D. melanogaster* as a model organism. The results emphasize calycosin's ability to mitigate behavioral deficits by improving locomotor functions, including climbing and jumping abilities, and partially alleviating Fen-induced developmental delays and neuronal apoptosis while reducing caspase-3-mediated apoptosis. Furthermore, calycosin significantly restored tyrosine hydroxylase (TH) levels, suggesting its protective effect on dopaminergic neurons. These results position calycosin as a promising candidate for addressing pesticide-induced neurodegeneration and potentially extending its therapeutic relevance to PD. This is the first study to show that calycosin provides significant neuroprotection against fenpropathrin (Fen)-induced neurotoxicity in *D. melanogaster*. However, future research should further explore its therapeutic potential and underlying mechanisms across different models and conditions.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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