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Neuroprotective Effects of the Anthranilamide-Pyrazolo[1,5-a]Pyrimidine Derivative C2 in a *Drosophila* Model of Parkinson's Disease

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ABSTRACT

Parkinson's disease (PD), characterized by progressive dopaminergic neuron loss in the substantia nigra pars compacta (SNPc), lacks therapies that halt neurodegeneration. We evaluated the neuroprotective potential of C2, an Anthranilamide-Pyrazolo[1, 5-a]Pyrimidine (CDK inhibitor), in a rotenone-induced *Drosophila melanogaster* PD model (w^{1118}) . C2 administration significantly improved locomotor activity and survival while mitigating dopaminergic neurodegeneration. Molecular analysis revealed upregulation of mitochondrial quality control genes (*PINK-1*, *parkin* and *DJ-1*), indicating enhanced mitophagy. Further increased levels of TH show the improved survivability of the SNpc neurons. These findings suggest C2 exerts neuroprotection by rescuing mitochondrial dysfunction, a hallmark of PD pathogenesis. Overall, this study highlights the promising therapeutic potential of C2 as a treating neurodegenerative diseases such as PD.

KEY WORDS

PD (Parkinson's Disease), Anthranilamide-Pyrazolo[1,5-a]Pyrimidine derivatives, Rotenone, *Drosophila*, *Parkin*, Dopaminergic neuron, Survivability.

I. INTRODUCTION

Parkinson's disease (PD), the second most prevalent neurodegenerative disorder after Alzheimer's disease, is pathologically defined by the progressive and selective loss of dopaminergic (DA) neurons within the substantia nigra pars compacta (SNpc), resulting in debilitating motor dysfunction and non-motor comorbidities. The pathogenesis of PD is strongly correlated with advancing age, which remains the primary non-genetic risk factor for disease onset. Geriatric population (≥60 years) exhibit a 5-to-10-fold elevated incidence rate compared to adult under 60 younger individuals. Globally, PD affects approximately 1% of the population over the age of 60 [1, 2]. Notably, the disease exhibits a pronounced sex-related disparity, with men being affected nearly twice as often as women across most populations [3].

Emerging evidence over the past two decades has established mitochondrial dysfunction as a central mechanistic contributor to PD pathogenesis. Notably, multiple PD-associated susceptibility genes encodes proteins critical for mitochondrial homeostasis, including those regulating mitophagy, fission-fusion dynamics, and oxidative stress responses. Exogenous neurotoxicants such as rotenone, MPTP, and its bioactive metabolite MPP^+ have been shown to recapitulate parkinsonian phenotypes in experimental models. Among these, rotenone- lipophilic isoflavonoid derived from tropical plant species (the roots and stems of *Derris* and *Lonchocarpus*)-exerts selective neurotoxicity species via mitochondrial targeting [4]. As a potent competitive inhibitor of mitochondrial complex I, rotenone disrupts NADH dehydrogenase activity, including bioenergetics collapse through ATP synthesis failure and pathological reactive oxygen species (ROS) overproduction. Subsequent oxidative damage to mitochondrial lipids, proteins, and DNA activates intrinsic apoptotic pathway, driving neuronal degeneration [5]. Chronic rotenone administration in preclinical models reliably reproduce hallmark PD features, including selective dopaminergic neurons loss in SNpc, α-synuclein aggregation, and motor deficits, thereby serving as a robust platform for investigating disease mechanisms and evaluating neuroprotective therapeutics [6]. In our study, we utilized a rotenone-induced *Drosophila melanogaster* PD model.

Drosophila serves as a highly accessible and effective *in vivo* model for investigating neurodegenerative disorders, as it unexpectedly recapitulates many aspects of human disease pathophysiology [7]. This model also exhibits complex behavioral phenotypes, including motor impairments, learning deficits, and memory loss under disease conditions, thereby offering a comprehensive system for studying neurodegeneration [8]. It was shown that mitochondrial impairment, muscle degeneration, and dopaminergic neuron loss result from loss-of-function mutations in *Drosophila PINK1*. Notably, overexpression of *parkin* rescues these phenotypes, supporting the model in which *PINK1* acts upstream of *parkin* in a shared pathway that maintains mitochondrial integrity [9]. Consistent with this, both *PINK1* and *parkin* mutants show similar abnormalities, including disrupted mitochondrial morphology and reduced lifespan, while Parkin overexpression compensates for *PINK1* deficiency [10].

Similarly, *DJ-1* mutants in Drosophila display heightened sensitivity to environmental toxins associated with PD, such as rotenone, underscoring *DJ-1's* role in oxidative stress defense [11]. *DJ-1* is proposed to act independently of the *PINK1*/*parkin* pathway in regulating mitochondrial function and autophagy. Tyrosine hydroxylase (TH), a critical enzyme in dopamine biosynthesis, is widely used as a marker for dopaminergic neurons. In *Drosophila melanogaster*, TH has been extensively employed to elucidate PD mechanisms. Genetic or pharmacological modulation of TH activity provides valuable insights into potential therapeutic strategies for PD [12].

Accumulating evidence underscore the involvement of cyclic-dependent kinases (CDKs)-serine/threonine kinases classically linked to cell cycle control-in the molecular cascades driving neurodegenerative disorders, including PD [13]. Beyond proliferation, CDKs modulate diverse processes such as neuronal differentiation, metabolic regulation, and apoptotic signalling. Recent study highlight Anthranilamide-Pyrazolo[1,5-a]Pyrimidine derivatives as potent CDK inhibitors with neuroprotective efficacy in *in vitro* PD model [14]. In prior work, we demonstrated that the lead compound C2 attenuates PD-associated pathology by reducing intracellular ROS, restoring mitochondrial membrane potential, and suppressing caspase-mediated apoptosis. Expanding these findings, we evaluated C2 (**Fig 1**), a structurally optimized Anthranilamide-Pyrazolo[1,5-a]Pyrimidine derivative synthesized via established method [15], in a *Drosophila melanogaster* PD model. This system employs rotenone, a mitochondrial complex I inhibitor, to recapitulate key features of PD, including dopaminergic neuron degeneration and locomotor deficits. Our study investigates C2's capacity to mitigate rotenone-induced neurotoxicity, with a focus on mitochondrial rescue pathways and survival of nigral-analogous neurons.

2. MATERIALS AND METHODS:

2.1 *Drosophila* Stock Collection and Maintenance:

Mutant w^{1118} flies were obtained from Bloomington *Drosophila* Stock Centre and reared in standard conditions typically at 24°±1°C. The compound was added into the standard diet for the flies to ingest. The fly diet consists of the autoclaved corn flour, agar, yeast, sugar, distilled water along with propionic acid and sodium benzoate as an enhancer and preservative. Further fly food was placed into bottles and vials for subsequent utilization.

2.2 Longevity Assay:

In the survival ship assay, a cohort of 60 adult F1 progeny flies were employed. The lifespan of these flies was assessed starting from the first day following eclosion. Every fifth day, the flies were transferred to fresh food vials until they reached 11-12 weeks of their age. The percentage of surviving flies was determined by subtracting the number of deceased flies from the total count of flies (n=60) during each vial

change. This experiment was conducted three times, and the resulting data were analyzed using GraphPad Prism software version 9.5.1.

2.3 Negative Geotaxis Assay (Climbing Activity):

A group of 20-30 flies from control and treated groups were analyzed in every trial. For our experiments, the flies were tapped into a 15 cm long vial. After acclimatization, flies were left undisturbed in the measuring vial and gently tapped every 10 sec to take the reading [16]. The adult flies climbing above the median line within 30 seconds were considered healthy flies. The climbing activity was measured using 14^{th} and 21^{th} day old flies. Data was analyzed using GraphPad Prism 9.5.1 with $P<0.05$ level of significance.

2.4 RNA Isolation and Semi-Quantitative RT-PCR Analysis

Total RNA was extracted from w^{1118} flies treated with rotenone (75 µM), C2 (60 µM), and rotenone + C2, starting from day 1 post-eclosion and maintained under the same treatment for 14 days, using the Trizol reagent (Life Technologies) as described in our previous study [15]. cDNA synthesis was performed using Superscript II reverse transcriptase (Life Technologies). Semi-quantitative RT-PCR was carried out using specific primers for 18s RNA (used as a gel loading control), *PINK1*, *DJ-1*, and *parkin* in an Eppendorf PCR thermal cycler **(Table.1)**. The amplified products were electrophoresed on a 1% agarose gel containing ethidium bromide and visualized under a UV gel documentation system (BIO-RAD).

Table.1: Specific primers for 18s RNA (gel loading control), *PINK1*, *DJ-1*, and *parkin*

Primer Name	Primer Sequence (5'-3')
PINK 1-FP	TTGCGCAGCTATTGTAAACG
PINK 1-RP	AAGATTCCACTGCTGCTGGT
parkin -FP	ACCACAATTGGGACTTCAGC
parkin -RP	GCTAAGCGAAGGTTCCTCCT
DJ1-FP	ATCATTTCCAAATGGGGTCA
DJ1-RP	CGGTTCACAATCATGCAAAC
18s rRNA-FP	CCTTATGGGACGTGTGC TT
18s rRNA-RP	CCTGCTGCCTTCCTTAGATG

2.5 Western Blot Analysis:

For protein isolation, flies head (n= 80-100) were decapitated and homogenised with RIPA lysis buffer (Sigma) with protease inhibitor cocktail (Sigma) at 3,000 rpm speed for 3 minutes, two-3 times in a dounce homogenizer setup in cold conditions. The homogenised lysate was then centrifuged at 12,000 rpm for 20 minutes at 4 ˚C. The clear supernatant was collected in the fresh tube and was later quantified by Bradford protein estimation assay. Western blotting was carried out by using an equal amount of protein as previously mentioned [17] and the blots were overnight probed with TH antibody (abcam; 1:1000). Next day, the blots were re-probed with HRP-tagged secondary antibody and developed the bolts using luminol reagent in ChemiDoc system (BioRad) [18].

2.6 Compound Synthesis:

The Anthranilamide Pyrazolo[1,5-a]Pyrimidine derivative C2 was synthesized following previously reported protocols [15]. C2 is an Anthranilamide–Pyrazolo[1,5-a]Pyrimidine conjugate which is a selective combination of Pyrazolo[1,5-a] pyrimidine derivative and an anthranilamide moiety both known for their unique functions (Figure 1). In the current study we check whether C2 has neuroprotective effects against w^{118} rotenone-induced *Drosophila* Parkinson's model [15]. The structure is drawn using ChemDraw 18.0

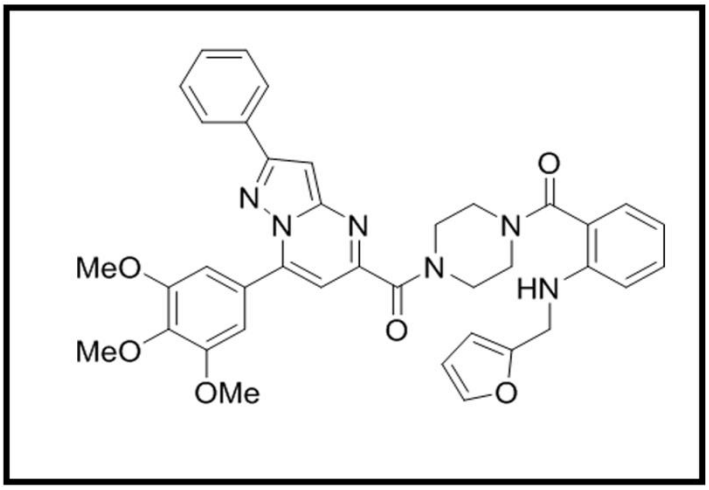

Figure.1: Chemical structure of C2 compound [IUPAC name: (4-(2-(furan-2ylmethylamino)benzoyl) piperazin-1-yl)(2-phenyl-7-(3,4,5trimethoxyphenyl) pyrazolo[1,5-a]pyrimidin-5-yl) methanone]

2.7 Statistical Analysis:

Statistical analysis was performed using Graph Pad prism version 9.0. Data is represented as mean ± SD of three independent experiments in each group.

3. RESULTS:

3.1 Rotenone-Induced *Drosophila* PD Model Generation:

Systemic administration of rotenone is a well-established method for inducing Parkinson's disease (PD)-like phenotypes in Drosophila melanogaster, including selective dopaminergic neuron degeneration and associated behavioral deficits [19]. In the present study, rotenone-induced PD models were generated using w^{1118} flies by supplementing the fly food with increasing concentrations of rotenone (5, 25, 75, and 125 μM) starting from day one post-eclosion and continuing throughout the lifespan. A concentration-dependent increase in lethality was observed in rotenone-treated flies compared to untreated controls. Notably, approximately 45–50% of flies exposed to 75 μM rotenone survived up to 7 weeks (Fig. 2A), and this concentration was selected for subsequent experiments as the effective dose for modeling PD-related phenotypes. To assess motor function, a negative geotaxis assay (climbing test) was performed following exposure to varying doses of rotenone. Groups of flies were transferred to vertical glass vials (15 cm in

height, 1.5 cm in diameter), tapped to the bottom, and allowed to climb for 30 seconds after a brief acclimatization period. The number of flies that crossed a predefined median line was recorded as a measure of locomotor ability. In untreated control groups, 90% of flies successfully climbed above the median line within 30 seconds. In contrast, rotenone-treated flies exhibited a dose-dependent decline in climbing ability, with a significant reduction in motor performance observed at higher concentrations (Fig. 2B), indicating a clear locomotor dysfunction consistent with PD-like symptoms. These findings confirm that chronic rotenone exposure effectively induces PD-like motor and survival phenotypes in Drosophila, establishing a robust model for further neuroprotective studies.

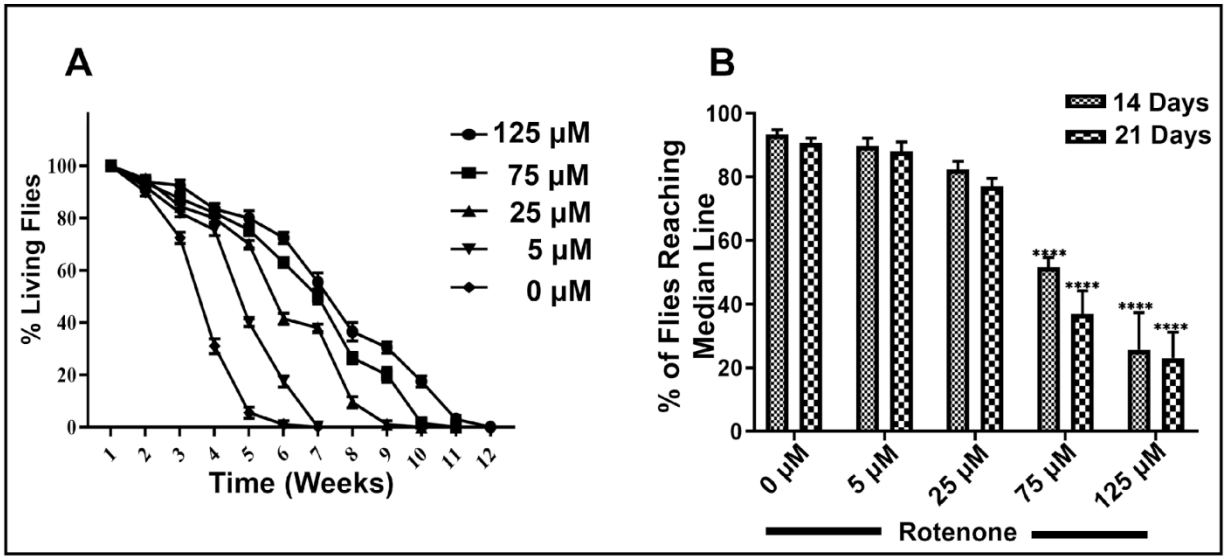

Figure 2: **Rotenone-induced PD-like phenotypes in *Drosophila*.** (**A)** Fly survival ratios were scored weekly with 60 flies used for the data analyzing. (**B**) Climbing activity was monitored by performing flight assay at different rotenone concentration of 14 day and 21 days aged flies.

3.2 Treatment with C2 Ameliorated Rotenone-Induced PD-Like Symptoms in *Drosophila*

To evaluate the *in vivo* neuroprotective potential of the compound C2, it was administered throughout the lifespan of *Drosophila melanogaster* via dietary supplementation. C2 was incorporated into fly food at concentrations of 20, 40, 60, and 80 µM beginning from day one post-eclosion. All treatment groups were concurrently exposed to 75 µM rotenone to induce Parkinson's disease (PD)-like phenotypes. Fly survival was monitored weekly, and locomotor performance was assessed using the negative geotaxis (climbing) assay at 14 and 21 days post-treatment. Among the tested doses, C2 at 60 µM produced the most pronounced protective effect. Specifically, this concentration significantly improved survival rates in rotenone-treated flies compared to the rotenone-only group (Fig. 3A). Additionally, C2 treatment markedly attenuated the rotenone-induced locomotor deficits, as demonstrated by enhanced climbing ability in both 14-day and 21-day-old flies (Fig. 3B). Increasing the concentration of C2 beyond 60 µM did not result in further improvement in either survival or locomotor function, suggesting a plateau in its therapeutic efficacy at this dose. Overall, these results indicate that C2, at an optimal concentration of 60 µM, significantly

mitigates behavioral and PD-like symptoms in Drosophila, supporting its potential as a therapeutic candidate for further investigation.

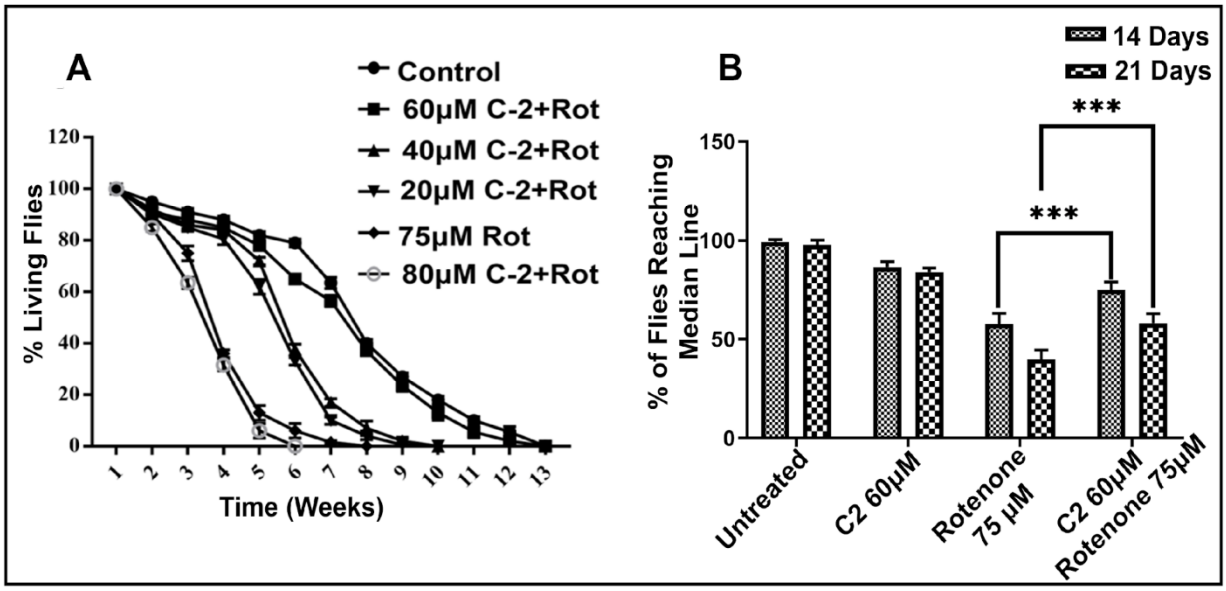

Figure 3: C2 treatment alleviated rotenone-induced PD symptoms in *Drosophila*. (**A**). Flies were exposed to (75 µM) rotenone in the presence or absence of **C2** at various concentrations. The fly survival ratio was scored weekly. (**B**). Flies were exposed with (75 µM) Rotenone in the presence or absence of 60uM **C2** for 2 and 3weeks. Locomotor function was monitored at 14 and 21 days using climbing assay.

3.3 Rotenone-Induced Alterations in *PINK1*, *parkin*, *DJ-1* Expression and Tyrosine Hydroxylase Restoration

To assess the impact of rotenone exposure on the expression of key genes implicated in mitochondrial function and neuroprotection—*PINK1* (PTEN-induced kinase 1), Parkin, and *DJ*-1 and to determine whether treatment with C2 could modulate their expression, transcriptional analyses were performed on adult fly brains. Fourteen-day-old flies, treated with 75 µM rotenone with or without C2 (60 µM), were decapitated and their brain tissues subjected to semi-quantitative reverse transcription PCR (RT-PCR). Consistent with previous reports which demonstrated the neuroprotective role of *DJ-1* in rotenone-induced dopaminergic cell damage and its association with increased TH-positive cells in the rat substantia nigra [20]. Our results showed that both *PINK1* and *DJ-1* transcript levels were significantly downregulated in rotenone-treated *Drosophila* compared to untreated controls (Fig. 4A, Fig.4B). Parkin expression was also significantly altered, showing a similar trend of reduction under rotenone stress conditions.

Interestingly, co-treatment with C2 resulted in a marked upregulation of all three genes (*PINK1*, *parkin*, and *DJ-1*) in rotenone-exposed flies, suggesting that C2 exerts a neuroprotective effect at the transcriptional level. This transcriptional rescue corresponds with the previously observed improvement in locomotor function following C2 treatment (as shown in the climbing assay), supporting the functional relevance of these gene expression changes. Moreover, tyrosine hydroxylase (TH), a well-established marker of dopaminergic neuron integrity, was also assessed. Rotenone treatment led to a reduction in TH expression, indicative of dopaminergic neuron loss. However, C2 supplementation significantly restored TH levels in

rotenone-exposed flies, suggesting improved dopaminergic neuron survival in the substantia nigra pars compacta (SNpc) equivalent regions of the fly brain(Fig. 4C). Together, these findings demonstrate that C2 not only improves behavioral outcomes but also reverses rotenone-induced transcriptional dysregulation of genes critical to mitochondrial health and oxidative stress defense, supporting its therapeutic potential in PD models.

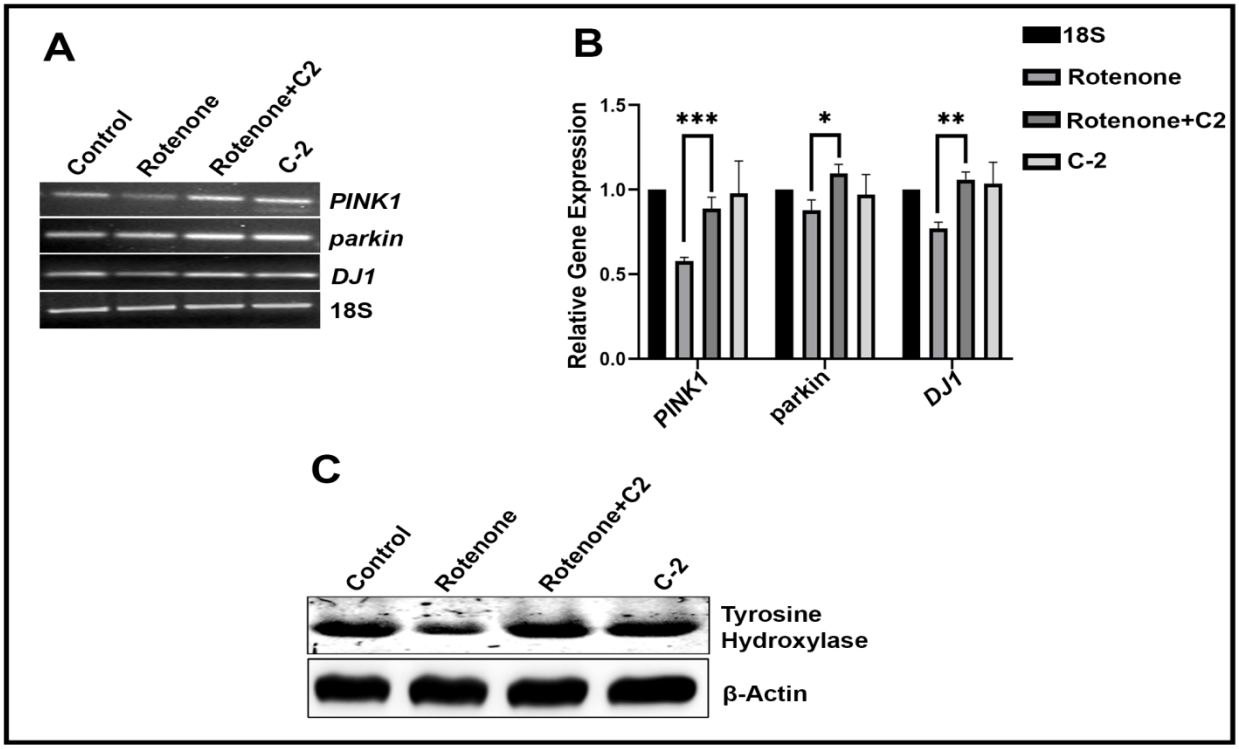

Figure 4: C2 treatment reversed transcriptional alterations in PD candidate genes and restored TH levels. (**A**) Semi-quantitative RT-PCR analysis of *PINK1*, *parkin*, and *DJ-1*. (**B**) The densitometric based semi-quantitative RT-PCR analysis of the gene expression changes for *PINK1*, *parkin*, and *DJ-1*. (**C**) Western blot analysis of tyrosine hydroxylase (TH) expression in Rotenone and C2 compound treated flies.

4. DISCUSSION

This study provides compelling evidence that the compound C2 confers significant neuroprotection against rotenone-induced Parkinson's disease (PD)-like pathology in *Drosophila melanogaster*. Dietary administration of C2 improved key phenotypic outcomes in a rotenone-induced PD model, including increased lifespan, enhanced climbing ability, and attenuation of locomotor deficits. These behavioral improvements are indicative of preserved neuromuscular coordination and reduced dopaminergic neuronal loss, hallmark features of neurodegenerative rescue in PD models. The protective effects of C2 were further substantiated at the molecular level. Rotenone exposure significantly downregulated the expression of critical PD-associated genes, *PINK1*, *parkin*, and *DJ-1*, all of which play essential roles in mitochondrial quality control, oxidative stress regulation, and neuronal survival [21] Following C2 treatment, transcript levels of all three genes were markedly restored, indicating that C2 may exert its neuroprotective effects, at least in part, through transcriptional modulation of pathways involved in mitochondrial maintenance and

antioxidative defense. Consistent with the transcriptional data, restoration of tyrosine hydroxylase (TH), a key enzyme in dopamine biosynthesis [22] and a marker of dopaminergic neuron integrity, was observed in brain tissues of C2-treated flies, as confirmed by western blot analysis. The recovery of TH expression further supports the conclusion that C2 mitigates rotenone-induced dopaminergic neuronal degeneration.

These findings align with previous studies implicating the *PINK1*/*parkin* pathway in maintaining mitochondrial homeostasis, where *PINK1* functions upstream to recruit and activate *parkin* in response to mitochondrial stress [23]. Loss of function in either gene leads to defective mitophagy and has been associated with familial forms of PD. Similarly, *DJ-1* has been shown to protect against oxidative damage and complements the *PINK1*/*parkin* axis in regulating mitochondrial dynamics and autophagy[24]. The concurrent restoration of these three genes by C2 suggests that the compound may target multiple arms of the mitochondrial stress response network. Although PD has long been regarded as a sporadic disorder with environmental and aging-related triggers, an increasing body of evidence indicates that 5–10% of cases have monogenic origins. The genes studied here, *PINK1*, *parkin*, and *DJ-1*, are among the most well-characterized genetic contributors to familial PD. The ability of C2 to modulate their expression opens avenues for exploring its potential not only in toxin-induced models but also in genetic models of PD. This dual relevance underscores C2's value as a candidate for disease-modifying therapies. Despite these promising findings, further studies are warranted to elucidate the precise molecular mechanisms by which C2 exerts its protective effects. Detailed investigations into its interaction with mitochondrial pathways, oxidative stress markers, and dopaminergic signaling networks are needed. Moreover, validation in higher model organisms and eventual translation to mammalian systems will be crucial to evaluate its therapeutic potential in humans.

5. CONCLUSION

In conclusion, C2 demonstrates robust neuroprotective activity in a rotenone-induced w ^{1118} *Drosophila* model of PD by rescuing both behavioral and molecular phenotypes. These findings provide a foundation for future studies aimed at developing C2 or its derivatives as novel therapeutics for Parkinson's disease.

6. CONFLICT OF INTEREST

Authors declare no conflict of interest.

7. FUNDING

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8. ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present study does not require any ethical approval and consent to participate.

9. AUTHOR CONTRIBUTION

Suresh Yerramsetty, Lavanya Ayyagari: Conceptualization, Methodology, Experimentation, Validation, Writing-original draft, Methodology, Validation, Data curation. Ahmed Kamal: Sourcing Compound. Manika Pal Bhadra, Smita C Pawar: Supervision, Conceptualization, Investigation, Project administration, funding acquisition.

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