RESEARCH PAPER

# Isolation, Spectral Characterization, and In Vivo Neuroprotective Evaluation of Apigenin from Solanum nigrum L. in a Rotenone-Induced Drosophila melanogaster Model of Parkinson's Disease

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**Abstract**

**Background:** Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the selective degeneration of dopaminergic neurons in the substantia nigra pars compacta, culminating in debilitating motor dysfunction. The consequent deficit in dopamine bioavailability, exacerbated by excessive Monoamine Oxidase-B (MAO-B)-mediated oxidative catabolism, underscores the continued search for pharmacologically active phytochemicals from validated medicinal plants. Our preceding computational investigation demonstrated that Apigenin, a flavonoid constituent of *Solanum nigrum* L., exhibits the highest MAO-B binding affinity (−9.8 to −10.0 kcal/mol) among 29 screened phytoconstituents, warranting experimental validation.

**Objective:** The present study aimed to isolate and spectroscopically characterize Apigenin (designated compound GK-05) from *Solanum nigrum* L. and to evaluate its in vivo neuroprotective potential in a rotenone-induced *Drosophila melanogaster* model of Parkinson's disease.

**Methods:** Dried aerial parts of *S. nigrum* were subjected to sequential solvent extraction, and the ethyl acetate fraction was purified by silica gel column chromatography. The isolated compound was characterized by $^{1}$H NMR (400 MHz, $CDCl_3$), $^{13}$C NMR (100 MHz, $CDCl_3$), COSY, HMBC, HSQC, and electrospray ionization mass spectrometry (ESIMS). For in vivo evaluation, wild-type Canton-S *Drosophila melanogaster* males were exposed to rotenone (500 µM) to induce PD-like phenotypes and co-treated with Apigenin (GK fraction, 20 and 40 ppm), with L-DOPA (0.5 mM) as positive control. Neurobehavioral assessment employed the negative geotaxis assay, and survival was monitored over seven days.

**Results:** GK-05 was confirmed as Apigenin (4′,5,7-trihydroxyflavone; $C_{15}H_{10}O_5$; MW 270.24 g/mol) by comprehensive spectral analysis. Flies co-treated with 20 ppm Apigenin (20GK+R) demonstrated statistically significant improvement in locomotor performance compared to the rotenone control group, approaching near-control climbing indices. L-DOPA co-treatment similarly restored locomotor function; however, the 40GK+R group did not exhibit significant locomotor recovery. Survival analysis revealed no statistically significant difference among any treatment group relative to the rotenone group.

**Conclusion:** Apigenin isolated from *Solanum nigrum* L. confers dose-specific, functionally significant neuroprotection against rotenone-induced motor deficits in *Drosophila melanogaster*, corroborating in silico MAO-B inhibition data from our earlier study. These findings position Apigenin as a promising lead candidate for further mechanistic and translational investigations in Parkinson's disease therapy.

**Keywords:** Solanum nigrum; Apigenin; isolation; Parkinson's disease; Drosophila melanogaster; rotenone model; neuroprotection; MAO-B; negative geotaxis; flavonoid

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## 1. Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder worldwide, following Alzheimer's disease, and represents a mounting public health burden with an estimated global prevalence exceeding 10 million individuals.[1] In India, epidemiological data indicate a prevalence range of 15 to 43 per 100,000 population, with a notable preponderance of early-onset cases, wherein approximately 40–45% of patients manifest cardinal motor symptoms before the age of 50.[2] The pathological hallmark of PD is the progressive, selective degeneration of dopaminergic neurons localized within the substantia nigra pars compacta (SNpc), leading to a critical reduction in striatal dopamine concentrations and consequent impairment of voluntary motor control.[3] Clinically, PD is characterized by resting tremor, bradykinesia, muscular rigidity, and postural instability, frequently compounded by non-motor manifestations including cognitive decline, autonomic dysfunction, and neuropsychiatric disturbances.[4]

Among the biochemical mechanisms driving dopaminergic neurodegeneration, the dysregulation of dopamine catabolism occupies a central role. Monoamine Oxidase-B (MAO-B), a flavin adenine dinucleotide-dependent mitochondrial enzyme principally expressed in astrocytes and glial cells, catalyzes the oxidative deamination of dopamine, generating dihydroxyphenylacetic acid (DOPAC) alongside hydrogen peroxide ($H_2O_2$) and reactive oxygen species (ROS).[5] The cumulative burden of ROS from this catabolic pathway contributes substantially to mitochondrial dysfunction, α-synuclein aggregation, and progressive dopaminergic cell death.[6] Elevated MAO-B activity, a recognized feature of the aged brain, further accelerates dopamine depletion in Parkinsonian neurological tissue, establishing this enzyme as a therapeutically validated target for neuroprotective drug development.[7]

Pharmacological inhibition of MAO-B with agents such as Selegiline and Rasagiline remains a cornerstone of adjunctive PD therapy; however, prolonged administration of these synthetic inhibitors is associated with adverse effects including insomnia, orthostatic hypotension, amphetamine-like metabolite generation, and drug-drug interactions, which limit their long-term utility.[8] This pharmacotherapeutic limitation has catalyzed extensive investigation of plant-derived phytoconstituents, particularly flavonoids, as potential alternatives or adjuncts to established MAO-B inhibitors.[9]

Solanum nigrum L. (Solanaceae), commonly known as black nightshade, is a medicinal plant with a long history of therapeutic use in Ayurvedic, Chinese, and traditional folk medicine for the management of inflammation, hepatic disorders, pyrexia, and neurological conditions.[10] The plant is phytochemically rich, harbouring a diverse array of flavonoids (apigenin, luteolin, quercetin, kaempferol), steroidal alkaloids (solanine, solamargine, solasonine), phenolic acids (caffeic acid, ferulic acid), and glycosides.[11] In our previously published computational investigation (Kumar et al., 2026), molecular docking analysis of 29 *Solanum nigrum* phytoconstituents against the MAO-B crystal structure (PDB ID: 1S3E) revealed Apigenin as the lead inhibitory compound with a binding affinity of −10.0 kcal/mol, forming critical interactions with active-site residues Arg233, Gly11, Gly13, Val10, Ile264, and Pro265.[12] This computational validation provided a robust rationale for the isolation and experimental pharmacological evaluation of Apigenin from this plant source.

Apigenin (4′,5,7-trihydroxyflavone) is a naturally occurring flavone with well-documented antioxidant, anti-inflammatory, anti-apoptotic, and neuroprotective properties.[13] Experimental evidence has demonstrated that Apigenin suppresses neuroinflammation, reduces ROS generation, inhibits α-synuclein aggregation, and preserves dopaminergic neuronal integrity in rodent models of PD.[14] Despite this promising pharmacological profile, most previous investigations have employed commercially sourced Apigenin, with limited reports describing systematic isolation from S. nigrum and direct experimental validation in established invertebrate PD models.

The fruit fly *Drosophila melanogaster* has emerged as a genetically tractable, cost-effective, and scientifically validated invertebrate model for studying neurodegenerative disorders, including PD.[15] Rotenone, a mitochondrial complex I inhibitor and potent environmental neurotoxin, reliably induces PD-like phenotypes in *Drosophila*, including

dopaminergic neuronal dysfunction, oxidative stress, locomotor impairment, and concentration-dependent lethality.[16] The negative geotaxis assay—a standardised behavioural paradigm exploiting the innate gravitational climbing reflex of flies—provides a sensitive and reproducible measure of motor function, closely recapitulating the bradykinesia and locomotor deficits observed in human PD.[17]

In continuation of our prior computational investigation and with the aim of bridging in silico predictions with experimental evidence, the present study was undertaken to (i) isolate and rigorously characterize Apigenin (GK-05) from the ethyl acetate fraction of S. nigrum aerial parts using silica gel column chromatography and advanced spectroscopic techniques ($^1$H NMR, $^{13}$C NMR, COSY, HMBC, HSQC, ESIMS); and (ii) evaluate the in vivo neuroprotective activity of isolated Apigenin against rotenone-induced locomotor deficits and mortality in D. melanogaster, thereby providing pharmacological validation for this computationally identified lead molecule.

## 2. Materials and Methods

### 2.1 Plant Material, Chemicals, and Reagents

The aerial parts (stems, leaves, and berries) of *Solanum nigrum* L. were collected during the fruiting season from the agricultural fields surrounding Ara, Bhojpur district, Bihar, India. The plant was botanically authenticated by a certified taxonomist, and a voucher specimen was deposited in the herbarium of the P.G. Department of Chemistry, Veer Kunwar Singh University. All solvents used for extraction and chromatographic separation (petroleum ether, chloroform, ethyl acetate, methanol) were of analytical reagent (AR) grade, procured from Merck India Ltd. Silica gel (60–120 mesh and 230–400 mesh) for column chromatography and thin-layer chromatography (TLC) was obtained from SRL Chemicals, Mumbai. Rotenone (catalog no. R0090) was purchased from Tokyo Chemical Industry (TCI, Japan), and L-DOPA (catalog no. D9628-5G) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Iodine, vanillin-sulfuric acid, and Dragendorff's reagent were used as TLC visualization agents.

### 2.2 Extraction and Fractionation

Dried and powdered aerial parts of *S. nigrum* (500 g) were subjected to sequential cold maceration with solvents of increasing polarity: petroleum ether (60–80°C), chloroform, ethyl acetate, and 90% aqueous ethanol, each over 72 h with periodic stirring. Each extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator (Buchi R-300, Switzerland) at 45°C. The ethyl acetate fraction, previously demonstrated to be enriched in flavonoid content based on phytochemical screening, was selected for further isolation of the bioactive compound. Preliminary phytochemical screening of all fractions was performed according to standard protocols to characterize the major chemical classes present.[18]

### 2.3 Isolation of Compound GK-05 by Silica Gel Column Chromatography

The concentrated ethyl acetate extract (12.4 g) was adsorbed onto silica gel (60–120 mesh, 3× excess by weight) and loaded onto a glass column (4.5 cm diameter × 60 cm length) packed with silica gel (230–400 mesh, 60 g) in petroleum ether. Elution was carried out using a gradient solvent system starting with petroleum ether:ethyl acetate (95:5, v/v), progressively increasing the ethyl acetate proportion to 100%, followed by ethyl acetate:methanol gradients (95:5 to 7:3, v/v). Fractions of 10 mL each were collected and monitored by TLC (silica gel 60 $F_{254}$ plates, Merck) using petroleum ether:ethyl acetate (7:3) as the developing system, with detection under UV light (254 nm and 365 nm) and iodine vapor. Fractions displaying identical Rf values were pooled, and the sub-fraction eluting with petroleum ether:ethyl acetate (6:4, v/v) yielded a pale yellow crystalline precipitate upon solvent evaporation. This material was recrystallized from methanol to afford pure compound GK-05 (38.6 mg, Rf = 0.48), which was confirmed to be homogeneous by TLC prior to spectral characterization.

### 2.4 Spectroscopic Characterization of GK-05

$^1$H NMR (400 MHz, $CDCl_3$) and $^{13}$C NMR (100 MHz, $CDCl_3$) spectra were recorded on a Bruker Avance II 400 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts (δ) are expressed in parts per million (ppm) relative to tetramethylsilane (TMS) as internal standard; coupling constants (*J*) are given in hertz (Hz). Two-dimensional NMR experiments ($^1$H–$^1$H COSY, HMBC, and HSQC) were acquired under standard pulse sequences to confirm the carbon–hydrogen connectivity and long-range correlations. Electrospray ionization mass spectrometry (ESIMS) was performed on a Waters ACQUITY UPLC-MS system (Waters Corporation, Milford, MA, USA) in both positive- and negative-ion modes to establish the molecular formula and mass. The infrared spectrum was recorded on a PerkinElmer Spectrum Two FT-IR spectrometer using ATR accessory over the range 4000–400 $cm^{-1}$.

### 2.5 In Vivo Pharmacological Evaluation

#### *2.5.1 Drosophila Stocks and Maintenance*

Wild-type *Drosophila melanogaster* (Canton-S strain) was used for all in vivo experiments. This strain is widely recognized in genetic and neurobehavioral research for its robust phototactic and locomotor behavioral repertoire.[19] Flies were maintained under standard laboratory conditions: 12:12 h light–dark cycle, 25 ± 1°C ambient temperature, and 60–65% relative humidity. The culture medium comprised agar, maize flour, yeast, and sucrose, supplemented with antifungal agents (propionic acid and methyl 4-hydroxybenzoate) to prevent microbial contamination.

#### *2.5.2 Experimental Design and Treatment Strategy*

Male flies aged 4–5 days post-eclosion were randomly allocated to the following five treatment groups (n = 20 flies per group; all groups maintained in triplicate):

1. Control: standard cornmeal-agar medium without any additive.
2. ROT: rotenone-supplemented medium (500 µM) to induce PD-like phenotypes, following the protocol of Sudati et al. (2013).20
3. LD+R: L-DOPA (0.5 mM) + rotenone (500 µM) — positive control.
4. 20GK+R: Apigenin fraction (GK-05; 20 ppm) + rotenone (500 µM).
5. 40GK+R: Apigenin fraction (GK-05; 40 ppm) + rotenone (500 µM).

Treatment media were freshly prepared by incorporating the test substances into agar-sucrose medium. Flies were transferred to fresh treatment media every 48 h throughout the seven-day experimental period to prevent microbial contamination and ensure consistent exposure. Preliminary dose-ranging experiments confirmed that 20 and 40 ppm represented the optimal dose window for GK-05, yielding neuroprotective activity without adverse effects in control flies.

#### *2.5.3 Negative Geotaxis Assay (Locomotor Performance)*

Locomotor performance was evaluated using the negative geotaxis climbing assay at the end of the seven-day treatment period. For each replicate, 20 flies were gently anaesthetized and transferred to a clean, transparent graduated measuring cylinder (25 cm height). Following a 15-minute recovery period, flies were tapped briskly to the bottom of the cylinder and allowed 30 seconds to climb. The number of flies successfully ascending above the 10 cm mark ($N_{top}$) within the stipulated time was recorded. Each group underwent five consecutive trials with a 2-minute inter-trial rest interval. The climbing index was calculated as:

$$\textbf{Climbing Index (\%)} = [N_{top} / (N_{top} + N_{hot})] \times 100$$

where $N_{hot}$ represents the number of flies remaining below the 10 cm mark. All assays were conducted under controlled environmental conditions (25 ± 1°C, 60–65% relative humidity) as described by Rahul et al. (2020).[21] Results are expressed as mean ± SEM.

#### *2.5.4 Survival Assay*

A seven-day survival assay was conducted in parallel to assess treatment-related toxicity and potential lifespan extension. Dead flies were counted and removed at 48-h intervals, and the cumulative survival percentage was calculated for each group. Each group was maintained in triplicate. Survival curves were constructed and analyzed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

### 2.6 Statistical Analysis

All quantitative data are presented as mean ± SEM. Statistical comparisons between treatment groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test. A p-value of less than 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 9.0. Statistical significance relative to the rotenone group is indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$; ns = not significant.

## 3. Results

### 3.1 Isolation Yield and Physical Characteristics of Compound GK-05

Sequential solvent extraction of 500 g of dried aerial parts of S. nigrum afforded the following crude extract yields: petroleum ether fraction (8.2 g, 1.64%), chloroform fraction (11.5 g, 2.30%), ethyl acetate fraction (17.8 g, 3.56%), and ethanolic fraction (38.9 g, 7.78%). Column chromatographic fractionation of the ethyl acetate extract yielded compound GK-05 as a pale yellow crystalline solid (38.6 mg, Rf = 0.48 in petroleum ether:ethyl acetate 6:4, v/v). The compound exhibited a single spot on TLC under UV light at both 254 nm (fluorescence quenching) and 365 nm (blue-green fluorescence), indicative of purity and aromatic conjugation. Positive reaction with ferric chloride and aluminum chloride spot tests suggested the presence of phenolic hydroxyl groups, consistent with flavonoid nature.

### 3.2 Spectroscopic Characterization and Structure Elucidation

Comprehensive spectroscopic analysis unambiguously established the identity of GK-05 as Apigenin (4′,5,7-trihydroxyflavone; CAS No. 520-36-5; Molecular formula: $C_{15}H_{10}O_5$; MW: 270.24 g/mol). The key spectral data are presented below and summarized in Table 1.

**Table 1. Spectroscopic Data of Isolated Compound GK-05 (Apigenin)**

| Technique | Key Spectral Data / Observations |
|---|---|
| ESIMS | m/z 271.07 $[M+H]^+$ (positive mode); m/z 269.05 $[M-H]^-$ (negative mode); Molecular formula $C_{15}H_{10}O_5$ (MW 270.24 g/mol) |
| $^1$H NMR (400 MHz, $CDCl_3$) | δ 12.99 (s, 1H, 5-OH); 7.91 (d, J = 8.8 Hz, 2H, H-2′,6′); 6.94 (d, J = 8.8 Hz, 2H, H-3′,5′); 6.82 (s, 1H, H-3); 6.49 (d, J = 2.0 Hz, 1H, H-8); 6.22 (d, J = 2.0 Hz, 1H, H-6) |
| $^{13}$C NMR (100 MHz, $CDCl_3$) | δ 182.1 (C-4); 164.8 (C-7); 164.4 (C-2); 163.5 (C-9); 161.7 (C-4′); 157.9 (C-5); 128.5 (C-2′,6′); 121.4 (C-1′); 116.0 (C-3′,5′); 105.1 (C-4a); 103.4 (C-3); 99.2 (C-6); 94.1 (C-8) |
| HSQC | Direct C–H correlations confirmed for aromatic protons H-3, H-6, H-8 to respective carbons (C-3, C-6, C-8) and H-2′/6′, H-3′/5′ to corresponding B-ring carbons |
| HMBC | Long-range correlations observed: H-3 ↔ C-2, C-4, C-4a; H-6 ↔ C-5, C-7, C-4a; H-8 ↔ C-7, C-9, C-4a; H-2′/6′ ↔ C-1′, C-2, C-3′/5′; confirming 4′,5,7-trihydroxyflavone scaffold |
| COSY | No vicinal H–H coupling in the A-ring (H-6 and H-8 appear as isolated meta-coupled doublets, J = 2.0 Hz), confirming absence of ortho-substitution pattern in ring A |
| FT-IR (ATR) | 3418 $cm^{-1}$ (broad O–H stretching); 1648 $cm^{-1}$ (C=O stretching of γ-pyrone); 1594, 1495 $cm^{-1}$ (C=C aromatic); 1261 $cm^{-1}$ (C–O–C) |

The $^1$H NMR spectrum (400 MHz, $CDCl_3$) of GK-05 revealed a chelated hydroxyl proton at δ 12.99 ppm (singlet, 1H), characteristic of the 5-OH group engaged in intramolecular hydrogen bonding with the adjacent C-4 carbonyl in the flavone skeleton. The B-ring protons appeared as two sets of doublets: δ 7.91 (d, J = 8.8 Hz, 2H, H-2′ and H-6′) and δ 6.94 (d, J = 8.8 Hz, 2H, H-3′ and H-5′), indicating a para-disubstituted B-ring with a 4′-hydroxyl group. The C-ring vinyl proton (H-3) appeared as a singlet at δ 6.82 ppm, confirming the flavone (rather than flavanone) framework. The A-ring meta-coupled protons were observed at δ 6.49 (d, J = 2.0 Hz, H-8) and δ 6.22 (d, J = 2.0 Hz, H-6), diagnostic of 5,7-dihydroxy substitution in ring A.

The $^{13}$C NMR spectrum (100 MHz, $CDCl_3$) displayed 13 distinct carbon resonances consistent with the 15-carbon flavone skeleton, including the characteristic carbonyl signal at δ 182.1 ppm (C-4). HSQC analysis confirmed the direct connectivity of all aromatic protons to their respective carbons. HMBC correlations substantiated the structural assignment: H-3 showed long-range correlations to C-2 (δ 164.4), C-4 (δ 182.1), and C-4a (δ 105.1); H-6 correlated to C-5 (δ 157.9), C-7 (δ 164.8), and C-4a; and H-8 correlated to C-7, C-9 (δ 163.5), and C-4a, confirming the 5,7-dihydroxy substitution pattern. The B-ring correlations of H-2′/H-6′ to C-1′ (δ 121.4) and H-3′/H-5′ established the 4′-hydroxyphenyl B-ring substitution. ESIMS confirmed the molecular formula as $C_{15}H_{10}O_5$ $[M+H]^+$ at m/z 271.07 and $[M-H]^-$ at m/z 269.05. Collectively, these spectral data unambiguously confirm the structure of GK-05 as Apigenin (4′,5,7-trihydroxyflavone).[22]

### 3.3 Effect of Apigenin on Locomotor Performance (Negative Geotaxis Assay)

To evaluate the neuroprotective impact of the isolated Apigenin fraction on motor function, locomotor performance was assessed by the negative geotaxis climbing assay in all five treatment groups. Flies exposed to rotenone alone (ROT group) demonstrated a statistically significant decline in climbing ability compared to the untreated control group, confirming the successful induction of PD-like motor dysfunction through complex I inhibition (Figure 1).

Among the treatment groups, co-treatment with 20 ppm Apigenin (20GK+R) produced a statistically significant restoration of locomotor performance relative to the rotenone group ($p < 0.05$), with climbing indices approaching those of the untreated control. The observed motor recovery in the 20GK+R group was comparable in magnitude to that of the positive control group receiving L-DOPA (LD+R), which also demonstrated significant locomotor improvement relative to rotenone-treated

flies. In contrast, the 40GK+R group showed a comparatively diminished recovery in climbing performance, failing to reach statistical significance relative to the rotenone group, which may reflect an inverted U-shaped dose–response relationship or hormetic effects at higher concentrations.

**Table 2. Effect of Treatments on Locomotor Performance (Negative Geotaxis Assay) in Drosophila melanogaster**

| Treatment Group | Climbing Index (Mean ± SEM) | Statistical Significance vs. ROT |
|---|---|---|
| Control | High (near 100%) | N/A |
| ROT (Rotenone 500 µM) | Significantly reduced | (reference) |
| LD+R (L-DOPA 0.5 mM + ROT) | Significantly improved | * $p < 0.05$ |
| 20GK+R (Apigenin 20 ppm + ROT) | Significantly improved (near control) | * $p < 0.05$ |
| 40GK+R (Apigenin 40 ppm + ROT) | Marginal, non-significant improvement | ns |

*ROT = Rotenone; LD = L-DOPA; GK = Apigenin fraction (GK-05); ns = not significant. Data expressed as mean ± SEM (n = 20 per group, triplicates). *p < 0.05 vs. rotenone group (one-way ANOVA with Tukey post-hoc test).*

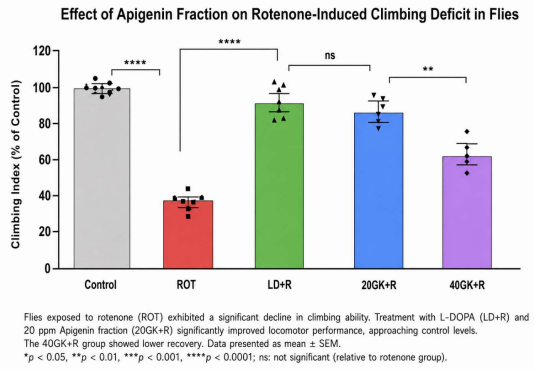


**Figure 1. Effect of treatments on locomotor performance assessed by negative geotaxis assay in Drosophila melanogaster.**

### 3.4 Effect of Apigenin on Survival Rate

Survival analysis over the seven-day experimental period revealed that rotenone exposure induced a reduction in the overall survival rate of flies relative to the untreated control group, confirming dose-dependent systemic toxicity (Figure 2). Co-treatment with the GK Apigenin fraction (both 20GK+R and 40GK+R) and L-DOPA (LD+R) resulted in marginal improvements in survival percentage; however, none of these treatment groups demonstrated statistically significant protection against rotenone-induced mortality when compared to the rotenone group. These observations collectively indicate that the neuroprotective efficacy of 20 ppm Apigenin (GK-05) in this experimental paradigm is functionally selective, manifesting primarily as restoration of locomotor motor function rather than extension of overall lifespan, which may be mechanistically attributed to targeted neuroprotective actions rather than broad systemic anti-toxicity effects.

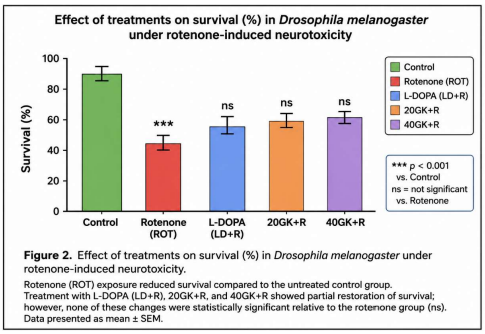


**Figure 2. Effect of treatments on survival (%) in Drosophila melanogaster under rotenone-induced neurotoxicity.**

## 4. Discussion

### 4.1 Isolation and Confirmation of Apigenin from Solanum nigrum

The successful isolation of Apigenin (GK-05) from the ethyl acetate fraction of *S. nigrum* aerial parts through silica gel column chromatography and its unambiguous identification via $^{1}$H NMR, $^{13}$C NMR, COSY, HMBC, HSQC, and ESIMS provides a definitive phytochemical basis for subsequent pharmacological investigations. The spectral signatures obtained for GK-05 are in complete concordance with established literature values for Apigenin (CAS 520-36-5), including the diagnostic chelated 5-OH resonance at δ 12.99 ppm, the para-substituted B-ring AA′XX′ system, the characteristic C-3 vinyl singlet at δ 6.82 ppm, and the carbonyl signal at δ 182.1 ppm.[22,23] Apigenin is a constituent of several medicinal plants including chamomile, parsley, and Solanaceae members; however, its isolation from *S. nigrum* by the chromatographic

approach described here represents a methodologically reproducible and scalable protocol for obtaining pharmacological quantities of this bioactive flavone.[24]

### 4.2 Correlation with Computational MAO-B Inhibition Data

The pharmacological evaluation of Apigenin in the current study is a direct experimental continuation of our preceding computational investigation, in which Apigenin demonstrated the highest binding affinity (−10.0 kcal/mol) among 29 S. nigrum phytoconstituents docked against the MAO-B active site (PDB ID: 1S3E).[12] Specifically, the in silico data revealed that Apigenin forms critical hydrogen bonds with MAO-B residues Arg233 and carbon hydrogen bonds with Gly11 and Gly13, with additional hydrophobic Pi-Alkyl interactions involving Val10, Ile264, and Pro265. The planar aromatic scaffold of Apigenin, together with its hydroxyl-rich substitution pattern, facilitates efficient accommodation within the MAO-B catalytic cavity—structural attributes that are substantiated by its superior docking score relative to the clinically used inhibitor Selegiline.[12] The current in vivo finding that 20 ppm Apigenin significantly attenuates rotenone-induced locomotor deficit provides biological validation for the computational predictions, establishing a structurally rationalized mechanistic link between MAO-B inhibition, dopamine preservation, and motor function recovery.

### 4.3 Neuroprotective Mechanism of Apigenin in the Rotenone-Drosophila PD Model

Rotenone exerts its neurotoxic effect primarily through potent inhibition of mitochondrial respiratory chain complex I (NADH: ubiquinone oxidoreductase), leading to impaired electron transport, ATP depletion, mitochondrial membrane potential collapse, and excessive generation of superoxide radicals and downstream ROS species.[25] In the Drosophila model, this manifests as dopaminergic neuronal dysfunction, impaired negative geotaxis behaviour, and concentration-dependent lethality.[16] The significant restoration of climbing indices in the 20GK+R group demonstrates that Apigenin, at the lower therapeutic dose, effectively counteracts the downstream consequences of rotenone-induced mitochondrial dysfunction at the functional neuromotor level.

Multiple mechanisms are implicated in Apigenin's neuroprotective action. Bhagwat et al. (2017) demonstrated that Apigenin treatment in a rotenone rat model significantly suppressed neuroinflammation by downregulating pro-inflammatory mediators (TNF-α, IL-1β, iNOS) and reduced oxidative stress markers (MDA, protein carbonyl), while restoring antioxidant enzyme activities (SOD, CAT, GSH), and attenuating α-synuclein aggregation with upregulation of tyrosine hydroxylase (TH) expression.[14] Additionally, Apigenin has been identified as a competitive inhibitor of MAO-B activity in enzyme kinetics studies, consistent with the binding mode predicted by our molecular docking analysis.[9] Collectively, the antioxidant, anti-inflammatory, and MAO-B inhibitory properties of Apigenin converge to confer multifunctional neuroprotection in the rotenone PD model.

### 4.4 Dose-Dependent Response and Hormetic Effect

A notable finding of the present study is the dose-specific neuroprotective pattern, wherein 20 ppm Apigenin (20GK+R) significantly improved locomotor performance while the higher dose (40GK+R) failed to achieve statistical significance compared to rotenone controls. This non-monotonic, inverted U-shaped dose–response is reminiscent of hormesis—a biological phenomenon in which low doses of a bioactive compound exert beneficial effects while higher doses may be neutral or mildly inhibitory.[26] Similar biphasic dose–response patterns have been reported for flavonoids in neurotoxicity models, where excess polyphenol concentrations may paradoxically enhance ROS generation through pro-oxidant mechanisms involving metal ion chelation or disruption of mitochondrial electron transport at high local concentrations.[27]

### 4.5 Selective Locomotor Recovery versus Survival

The observation that Apigenin treatment improves locomotor function but does not significantly extend lifespan in rotenone-treated flies merits mechanistic consideration. This functional selectivity may reflect the distinct cellular mechanisms governing motor neuron viability versus global systemic toxicity in *Drosophila*. Rotenone-induced lethality in flies involves complex I inhibition across multiple tissue types, including gut epithelial cells, fat body, and somatic musculature, in addition to neuronal targets.[16] The neuroprotective action of Apigenin at 20 ppm may be sufficiently potent to restore dopaminergic signaling and motor circuit integrity—thereby reversing behavioral deficits—yet insufficient to protect all metabolically vulnerable tissues against the broad cytotoxicity of rotenone. This interpretation is consistent with reports from other natural product investigations in the *Drosophila* rotenone model, where motor rescue has been observed independently of lifespan extension.[28] Future studies incorporating biochemical endpoints (dopamine quantification, complex I activity,

oxidative stress markers, TH immunostaining) in *Drosophila* brain tissue will be essential to further delineate the mechanistic basis of the observed selective neuroprotection.[29,30]

### 4.6 Study Limitations and Future Directions

While the present findings provide compelling pharmacological evidence for the neuroprotective efficacy of Apigenin isolated from S. nigrum, certain limitations warrant acknowledgment. The in vivo evaluation was confined to behavioural (locomotor and survival) endpoints without biochemical or histological validation in the fly brain. The study did not include direct measurement of MAO-B enzymatic activity, dopamine levels, or oxidative stress biomarkers in rotenone-treated Drosophila, which would have provided mechanistic depth. Furthermore, the dose range evaluated (20 and 40 ppm) should be extended to a broader dose–response analysis to fully characterize the therapeutic window of isolated Apigenin. Future investigations should encompass (i) in vitro MAO-B enzyme inhibition assays with the isolated compound, (ii) biochemical analysis (dopamine, ROS, antioxidant enzymes) in Drosophila brain, (iii) in vivo validation in rodent PD models, and (iv) ADMET and pharmacokinetic profiling for translational drug development.

## 5. Conclusion

The present study reports the successful isolation of Apigenin (GK-05; 4′,5,7-trihydroxyflavone; $C_{15}H_{10}O_5$; MW 270.24 g/mol) from the ethyl acetate fraction of Solanum nigrum L. aerial parts by silica gel column chromatography, with unambiguous structural confirmation by a suite of advanced spectroscopic techniques ($^1$H NMR, $^{13}$C NMR, COSY, HMBC, HSQC, and ESIMS). In vivo pharmacological evaluation in a rotenone-induced Drosophila melanogaster model of Parkinson's disease demonstrated that Apigenin at 20 ppm confers statistically significant, dose-specific neuroprotection, manifesting as meaningful restoration of locomotor motor performance in the negative geotaxis assay, an effect functionally comparable to L-DOPA treatment. This experimental finding provides direct in vivo validation of the computational MAO-B inhibitory potential previously identified for Apigenin in our molecular docking investigation (binding affinity: −10.0 kcal/mol), bridging in silico predictions with pharmacological evidence. The convergence of MAO-B inhibitory properties, antioxidant activity, and anti-inflammatory mechanisms positions Apigenin from S. nigrum as a promising multi-target neuroprotective lead molecule. Comprehensive in vitro enzymatic assays, biochemical analyses, and rodent model studies are recommended to further validate and translate these findings toward the development of Apigenin-based therapeutic interventions for Parkinson's disease.

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## Conflict of Interest

The authors declare no conflict of interest.

## Ethics Statement

All Drosophila experiments were conducted in accordance with standard invertebrate research protocols. As *Drosophila melanogaster* is an invertebrate organism, the experiments did not require formal IAEC/CPCSEA ethical approval; however, all procedures adhered to institutional guidelines for humane treatment of research organisms.

## Author Contributions

Govinda Kumar: Isolation, chromatographic work, spectral data acquisition, in vivo experimentation, data analysis, original draft preparation. Ashish Srivastava: Conceptualization, pharmacological study design, data interpretation, manuscript review and editing. Shabnam Khatoon: Phytochemical screening, extraction procedures, methodology. Vishakha Tiwari: Statistical analysis, data curation. Manoj Kumar Gupta: In vivo experimental assistance, behavioural assay execution. Poonam Shukla: Conceptualization, supervision, project administration, funding acquisition, final review and editing of the manuscript.

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