RESEARCH PAPER

Rutin from Ruta Chalapensis Mitigates Rotenone-Induced Mitochondrial Dysfunction, Oxidative Stress, and Apoptosis

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ABSTRACT

This study intends to employ an in vitro model to investigate whether rutin, a plant-derived flavanol glycoside, may protect neurons against oxidative stress, mitochondrial malfunction, and cell death caused by rotenone. According to the MTT viability test, SH-SY5Y cells were assigned to four different experimental groups. The groups comprised rotenone (100 nM), a control, rutin (10 µM) alone, and rutin (10 µM) in combination with rotenone (100 nM). Rotenone caused mitochondrial malfunction, oxidative stress, and cytotoxicity in SH-SY5Y cells. Rutin, however, markedly reduced the intensity of these effects. The protective effect of rutin against rotenone was evident from its ability to boost Bcl-2 expression and inhibit Bax and caspase-3 activation. Rutin was found to protect cells by modulating the production of p-AKT, p-GSK-3 beta, and p-PI3K. Through regulation of PI3K/Akt/GSK-3β signaling, rutin suppressed oxidative toxicity, preserved mitochondrial integrity, and limited rotenone-induced apoptosis. Additional research in rats is required to fully determine rutin's effectiveness as a prospective Parkinson's disease therapy.

Keywords: Rutin, Ruta chalepensis, Rotenone, Mitochondrial dysfunction, Oxidative stress, PI3K/Akt/GSK-3β pathway.

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INTRODUCTION

Being the second most widespread neurodegenerative illness, Parkinson's disease is primarily associated with impaired dopamine-mediated communication between neurons. Clinically, patients exhibit rigidity, akinesia, resting tremors, and postural instability [1,2]. The etiology of PD involves a combination of genetic abnormalities and environmental toxicants, yet the precise mechanisms underlying neuronal loss remain incompletely understood. Growing evidence suggests that mitochondrial dysfunction, oxidative stress, protein misfolding, and dysregulated apoptotic pathways contribute significantly to disease progression [3,4]. Rotenone, a potent isoflavonoid derived from Leguminosae plants, is widely used as an experimental neurotoxin because it readily crosses biological membranes, accumulates within subcellular organelles, binds to mitochondrial complex I, and disrupts electron transport [5].

Preventing electrons from reaching ubiquinone disrupts the respiratory chain, increasing oxidative stress and triggering apoptosis through cytochrome c signaling[6-9]. Pharmacological treatments such as levodopa, selegiline, entacapone, isocarboxazid, and tranylcypromine offer symptomatic relief but do not halt dopaminergic neurodegeneration. Investigational therapies—including deep brain stimulation, stem cell transplantation, and neural grafting—are being explored to improve long-term outcomes [10]. Plant-derived compounds are known to counter oxidative stress in neurodegenerative diseases. *Ruta chalepensis* possesses multiple phytochemicals, including rutin, that provide antioxidant and anti-inflammatory benefits. Since rotenone causes oxidative damage and apoptosis, rutin was evaluated in SH-SY5Y cells for its neuroprotective ability [11,12].

Materials and Methods

Chemicals

Sigma-Aldrich supplied rotenone, MTT, DCFH-DA, rhodamine-123, DAPI, AO/EtBr, DMEM with FBS, culture additives, DMSO, EDTA, trypsin-EDTA, and antibodies targeting β-actin, Akt, GSK3β, PI3K, and Bax.

Cell Culture

Cells (SH-SY5Y) were grown in DMEM/F-12 with 10% FBS and antibiotics at 37 °C in a humidified 5% CO_2 environment. Following 24 h incubation, rutin pretreatment was performed 2 h before rotenone[13].

Cell Viability (MTT Assay)

SH-SY5Y cells in 96-well plates (3×10^3/well) underwent rutin treatment (0–50 µM, 26 h) and MTT incubation (1 mg/mL, 4 h). Dissolved formazan in DMSO was measured at 570 nm. Neuroprotective assays involved 2 h rutin pretreatment followed by 100 nM rotenone for 24 h.[14,15.]

Experimental Groups

To assess treatment effects, SH-SY5Y cells were distributed among four study groups. Group I served as the untreated

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control, while Group II received rotenone (100 nM) to induce mitochondrial dysfunction. Group III was pretreated with rutin (10 µM) followed by rotenone exposure, and Group IV received rutin alone (10 µM) to assess its independent effects.

ROS Estimation

DCFH-DA staining was employed to quantify cellular ROS levels. SH-SY5Y cells (1×10^5 cells/well) were treated with rotenone (100 nM) and/or rutin (10 µM) for 24 hours, followed by incubation with DCFH-DA (100 µM) for 10 minutes at 37°C. Fluorescence intensity was recorded using a Shimadzu RF-5301PC spectrofluorometer (Ex 485 nm, Em 530 nm) and visualized using fluorescent microscopy [16].

Mitochondrial Membrane Potential (Δψm)

After 24 h drug treatment, mitochondrial potential was determined by incubating SH-SY5Y cells with rhodamine-123 for 30 min at 37 °C. Fluorescence signals were captured using the Invitrogen Floid Imaging Station and quantified with a spectrofluorometer (Ex 480 nm, Em 530 nm) **[17].**

AO/EB Dual Staining for Apoptosis

After treatment with rotenone (100 nM) and/or rutin (10 µM), SH-SY5Y cells were fixed (methanol: acetic acid, 3:1) and stained using AO/EB. Apoptotic morphology was examined using the Floid Imaging Station, and apoptotic cells were quantified based on fluorescence characteristics [18].

DAPI Nuclear Staining

Nuclear morphology was examined using DAPI staining. After fixation with 3% methanol, cells were incubated with 1 µg/mL DAPI for 30 minutes in the dark. Nuclear condensation and fragmentation were observed under the FluoCell Imaging Station [19].

Antioxidant Enzymes and Lipid Peroxidation

SH-SY5Y cell lysates were used to measure oxidative stress markers.

Lipid peroxidation (TBARS) was assessed per Niehaus and Samuelsson.

SOD, catalase, and GSH activities were measured using standard methods described by Kakkar et al., Sinha, and Rotruck et al., respectively.

GPx activity was determined using DTNB-based colorimetry [20].

Western Blotting

RIPA-based cell lysates were centrifuged (12,000 × g, 30 min), and protein contents were determined by NanoDrop. Equal protein (50 µg) underwent SDS-PAGE (10%), PVDF transfer, blocking, and overnight incubation with apoptosis and PI3K/Akt/GSK-3β pathway antibodies. Blots were developed with HRP secondary antibodies using ECL, and ImageJ was used for band intensity measurement. [21].

Statistical Analysis

All assays were repeated four times, with values shown as mean ± SD. SPSS 15.0 was used for statistical testing. One-way ANOVA followed by DMRT determined significant differences at $p < 0.05$.[22]

Results and Discussion

Effects of Rutin on Cell Viability

Prior studies have shown that rotenone diminishes cell viability to differing extents. Figure 1A illustrates the effect of rutin alone on SH-SY5Y cell viability. Lower concentrations of rutin (1–10 µM) did not produce any cytotoxic response, and cell viability remained comparable to the control. In particular, 5 µM rutin slightly enhanced cell viability, indicating a potential proliferative or supportive cellular effect. However, at higher concentrations (20 and 50 µM), rutin reduced cell viability, demonstrating a dose-dependent cytotoxicity at elevated levels. These observations suggest that rutin is biologically safe within the lower concentration range, with 10 µM selected as the optimal non-toxic dose for subsequent neuroprotective studies.

Figure 1B further shows the neuroprotective action of rutin against rotenone toxicity. Rotenone treatment alone decreased cell viability to nearly 40%, confirming its strong mitochondrial toxicity. Pretreatment with rutin significantly improved cell survival in a concentration-dependent manner, with 10 µM rutin restoring viability to approximately 80%, almost approaching control levels. At high doses (20 and 50 µM), the protective effect was diminished, consistent with the cytotoxic trend observed in Figure 1A. Overall, these results establish rotenone (100 nM) as a reliable inducer of neuronal damage and identify 10 µM rutin as the most effective concentration for protecting SH-SY5Y cells against rotenone-induced toxicity.

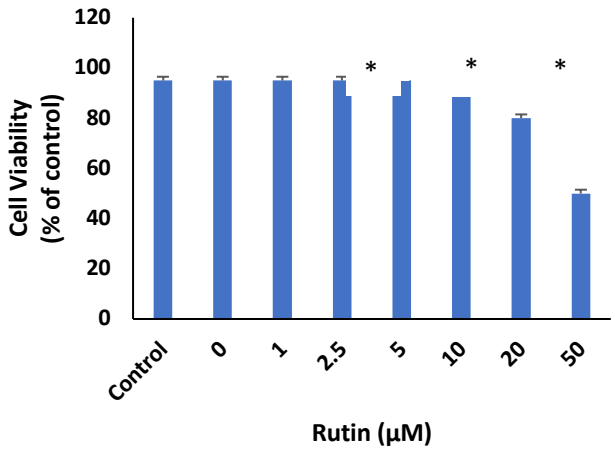

Fig. 1A. Rutin dose–response on SH-SY5Y viability (MTT) (mean ± SD, n=4).

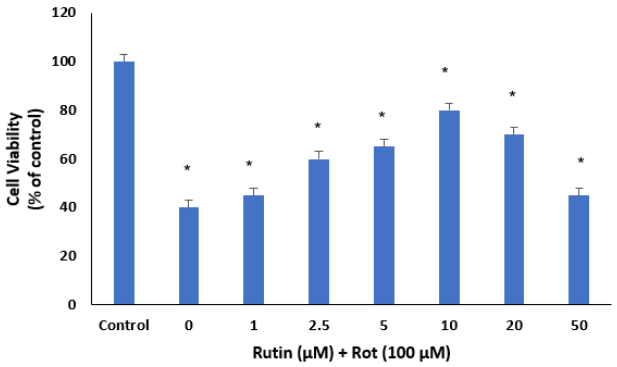

Fig. 1B. Rutin (pre-treatment) protects against rotenone (100 nM) cytotoxicity (MTT).

Cell viability was assessed using the MTT assay to determine how different concentrations of rutin influence SH-SY5Y neuronal survival. Rutin treatment across a range of doses (0–50 µM) produced a clear dose-dependent response, with 10 µM identified as the most effective concentration for maintaining cell viability without causing cytotoxic effects. When cells were challenged with rotenone (100 nM), approximately 50% of the neuronal population was lost, confirming its strong neurotoxic impact. However, pretreatment with rutin significantly protected the cells from rotenone-induced damage by reducing the extent of cell death in a concentration-dependent manner, demonstrating that rutin counteracts rotenone toxicity and enhances neuronal survival. The data represent the mean ± SD from four independent experiments and are expressed relative to untreated control cells. Supporting these findings, previous research has shown that other plant-derived compounds, such as the sesquiterpenes spirafolide and reynosin from *Laurus nobilis* L., also prevent dopamine-induced cytotoxicity in SH-SY5Y cells. In agreement with these earlier observations, two complementary cell morphology-based assays in our study confirmed that rutin has the ability to inhibit neuronal cell death and preserve cellular integrity under oxidative stress conditions.

Alterations to antioxidant levels and lipid peroxidation

Rotenone exposure led to a substantial elevation in oxidative stress markers and disruption of the intracellular antioxidant system. As shown in Figure 2A, rotenone significantly increased TBARS levels compared with the untreated control group, reflecting enhanced lipid peroxidation and mitochondrial oxidative damage. In parallel, rotenone caused a marked depletion of intracellular GSH (Figure 2B), indicating a collapse of the primary non-enzymatic antioxidant defense. This oxidative imbalance was accompanied by a pronounced reduction in the activities of major enzymatic antioxidants, including SOD (Figure 2C), catalase (Figure 2D), and GPx (Figure 2E). Together, these findings confirm that rotenone strongly induces oxidative stress by impairing both enzymatic and non-enzymatic antioxidant systems in SH-SY5Y cells.

Pretreatment with rutin (10 µM) significantly counteracted the detrimental impact of rotenone on neuronal redox status. TBARS levels were notably reduced in the Rutin + Rotenone group (Figure 2A), indicating inhibition of rotenone-mediated lipid peroxidation. Additionally, rutin restored GSH levels nearly to those of the control group (Figure 2B), demonstrating improved intracellular redox buffering capacity. Rutin administration also preserved the activities of SOD (Figure 2C), catalase (Figure 2D), and GPx (Figure 2E), showing that rutin stabilizes the antioxidant enzyme network and protects mitochondria from oxidative injury. Importantly, rutin alone showed no adverse effects on these markers, confirming its safety and inherent antioxidant support in normal cells. Overall, the data in Figures 2A–2E clearly demonstrate that rutin attenuates rotenone-induced oxidative stress by maintaining cellular antioxidant defenses.

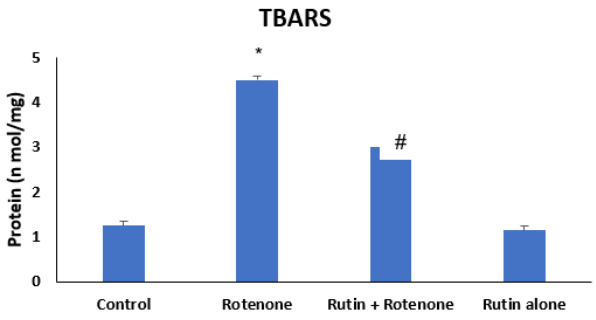

Fig. 2A. TBARS levels: rotenone ↑, rutin (10 µM) reduces TBARS

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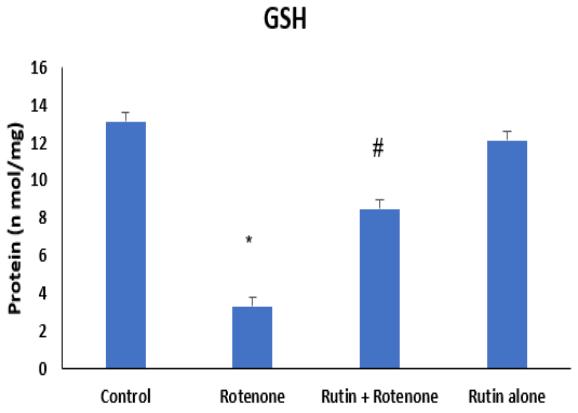

Fig. 2B. GSH levels: rutin restores GSH reduced by rotenone

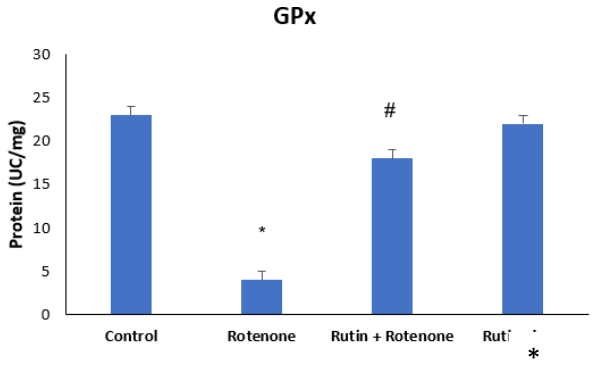

Fig. 2C. SOD activity: rutin preserves SOD against rotenone

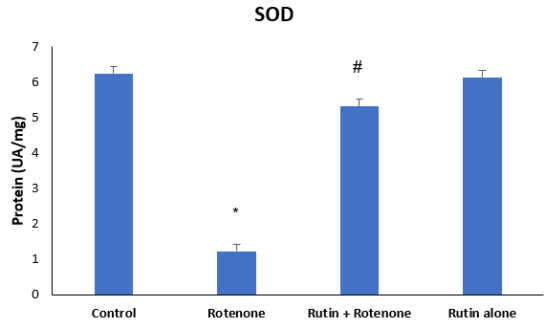

Fig. 2D. Catalase activity: rutin attenuates rotenone-induced loss

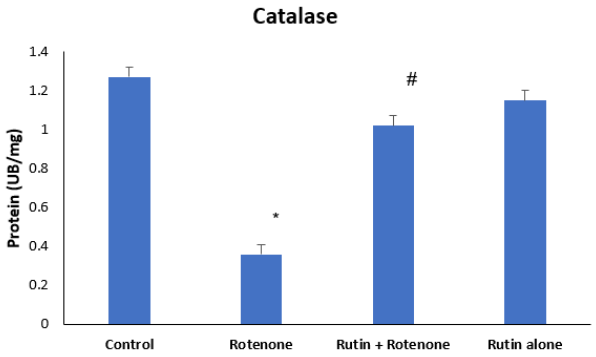

Fig. 2E. GPx activity: rutin restores GPx after rotenone.

Exposure to rotenone led to oxidative stress, shown by elevated TBARS and lowered GSH, while rutin (10 µM) pretreatment significantly restored antioxidant status (SOD, catalase, GPx). Data represent mean ± SD of four trials, with *P < 0.05 vs. control and #P < 0.05 vs. rotenone.

Rotenone-induced oxidative damage is consistent with age-related neurodegeneration, where excess free radicals accelerate lipid peroxidation and disrupt mitochondrial function. Elevated ROS production following complex I inhibition increases hydroxyl radical generation and overwhelms cellular antioxidant defences. In our study, rotenone alone elevated SOD, catalase and GPx activity, reflecting a compensatory response to ROS burden. Rutin pretreatment restored antioxidant enzyme balance and increased GSH levels, demonstrating its strong ROS-scavenging and cytoprotective properties. Since GSH deficiency is closely linked to Parkinson's pathology, rutin's ability to normalize redox status highlights its potential as a neuroprotective agent.

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Reactive Oxygen Species (ROS) Production Induced by Rotenone and the Role of Rutin

Figure 3 demonstrates the impact of rotenone and rutin on intracellular ROS levels in SH-SY5Y cells, visualized by DCFH-DA fluorescence. Compared with the weak fluorescence observed in untreated control cells, rotenone exposure markedly enhanced green fluorescence intensity, indicating excessive ROS formation and severe oxidative stress (*p < 0.05 vs. control). In contrast, pretreatment with rutin (10 µM) substantially reduced the fluorescence intensity in rotenone-treated cells, signifying a strong suppression of ROS overproduction (#p < 0.05 vs. roteno ` Quantitative analysis further confirms these visual # rvations, with rotenone increasing ROS levels to nearly 360% of the control value, whereas rutin pretreatment effectively lowered ROS accumulation to approximately 260%. Rutin alone did not significantly alter fluorescence levels and remained comparable to control cells, indicating that rutin itself does not induce oxidative stress. Overall, these findings clearly demonstrate that rutin exerts a potent antioxidant effect by preventing rotenone-induced ROS generation and protecting neuronal cells from oxidative damage.

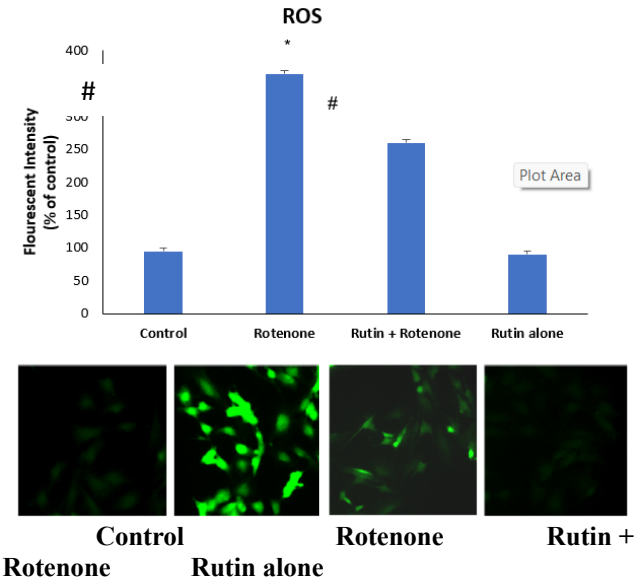


Fig. 3. Intracellular ROS (DCFH-DA): rotenone ↑ ROS; rutin (10 µM) reduces ROS

The effect of Rutin on the Δψm alteration caused by rotenone

Figure 4 illustrates the effect of rotenone and rutin on mitochondrial membrane potential (MMP) in SH-SY5Y cells, assessed using Rhodamine-123 fluorescence. In

contrast, rotenone exposure caused a dramatic loss of fluorescence (reduced to 25% of control), indicating severe mitochondrial depolarization and dysfunction (*p < 0.05 vs. control). This sharp decline in MMP confirms that rotenone disrupts mitochondrial integrity, a key pathological event in Parkinson-like neurotoxicity. Notably, cells pretreated with rutin (10 µM) before rotenone challenge maintained a significantly higher fluorescence signal (72% of control), demonstrating that rutin effectively preserves mitochondrial function and protects against depolarization (#p < 0.05 vs. rotenone). Rutin alone showed fluorescence comparable to the control group, indicating that rutin does not adversely affect mitochondrial health under normal conditions. These results clearly show that rutin counteracts rotenone-induced mitochondrial damage by sustaining mitochondrial membrane potential and preventing early apoptotic mitochondrial collapse.

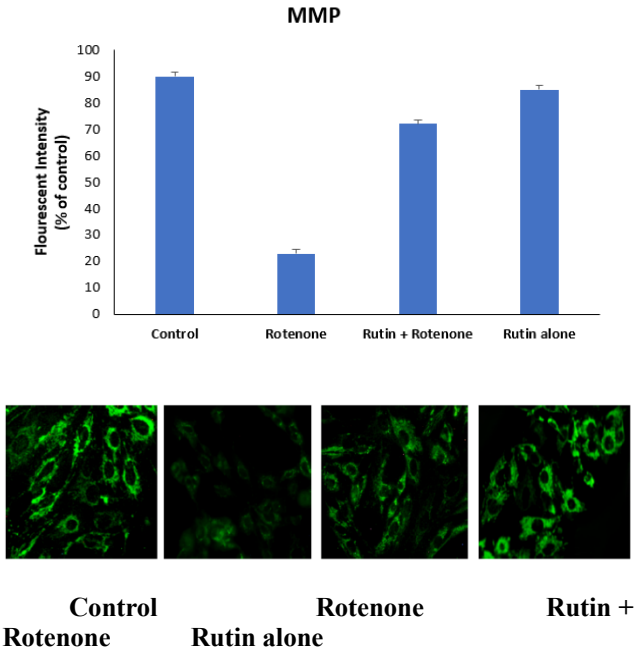


Fig. 4. Mitochondrial membrane potential (Rho-123): rotenone decreases Δψm; rutin prevents loss .

ROS are predominantly generated in mitochondria, where they contribute to essential cellular activities when kept in check. However, ROS overload disrupts cellular communication and damages mitochondria, leading to apoptosis. Rotenone exerts its toxicity mainly by causing abnormal ROS accumulation. Rotenone binds to NADH CoQ10 reductase (Complex I) upon entering the mitochondria. This results in the transfer of electrons to ETC-II and inhibits the functionality of Complex I. Consequently, ROS levels elevate to five to seven times their baseline amount. This work demonstrated using DCF-DA fluorescence that rotenone-induced damage results in increased ROS accumulation in cells.

Rutin alleviates cell death induced by rotenone.

Figure 5 demonstrates the effect of rotenone and rutin on apoptotic cell death using AO/EB dual staining. In control cells, nuclei appeared uniformly green, reflecting healthy, viable cells with intact membranes. Rotenone exposure markedly increased apoptotic features, as indicated by the high number of orange/red-stained cells, representing chromatin condensation, nuclear fragmentation, and compromised membrane integrity (*p < 0.05 vs. control). Quantitatively, rotenone drastically reduced overall viable cell percentage to around 25% of control, confirming its strong pro-apoptotic effect. Conversely, cells pretreated with rutin (10 µM) before rotenone challenge exhibited a notable reduction in apoptotic nuclei, with a higher proportion of green-stained cells compared to the rotenone-only group (#p < 0.05 vs. rotenone). This indicates that rutin significantly suppresses rotenone-induced apoptosis and supports neuronal survival. Rutin alone did not induce apoptosis, showing cell morphology similar to the control, suggesting that rutin is safe and does not exert cytotoxic effects under normal conditions. Overall, these findings confirm that rutin exhibits potent anti-apoptotic activity by counteracting rotenone-triggered neuronal cell death.

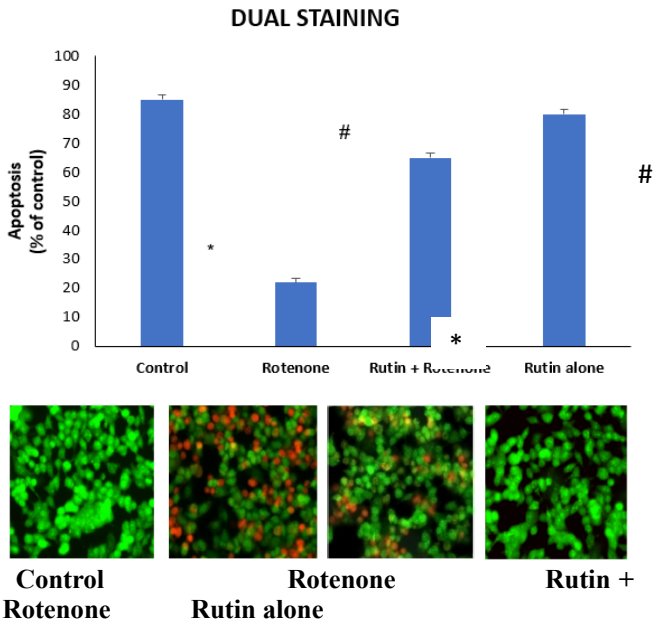


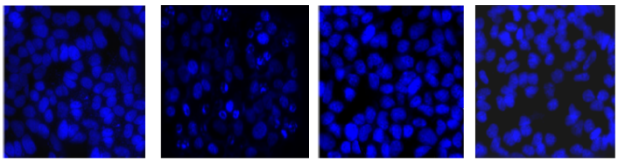
Fig. 5. AO/EB apoptosis assay: rutin reduces rotenone-induced apoptotic cells

Mitochondria are essential for neuronal survival, as they are responsible for ATP generation, oxygen use, reactive oxygen species (ROS) production, and ROS elimination. Mitochondrial dysfunctions are a significant contributor to dementia. Specific proteins, including cytochrome c, are discharged from the mitochondrial intermembrane space into the cytosol upon the inhibition of complex I. The apoptotic process is subsequently launched by cytot-c, resulting in an accumulation of ROS and the dissipation of ΔΨm. The rhodamine-123 sensor, which quantifies mitochondrial transmembrane potential, exhibited

significantly reduced fluorescence following rotenone treatment (Figure 7). Consistent with the findings of Menke et al. (2003), we also observed that rotenone treatment reduced ΔΨm in SH-SY5Y cells. Our findings indicated that treatment with rutin followed by rotenone administration restored rhodamine-123 fluorescence levels to normal after a decline.

Rutin obstructs nuclear condensation in SH-SY5Y cells induced by rotenone.

Following DAPI dye labeling, SH-SY5Y cells exposed to rotenone exhibited increased brightness. The pronounced fluorescence indicated that nuclear chromatin condensation and DNA fragmentation were occurring within the cells. Figure 6 demonstrated that rutin (10 µM) significantly reduced nuclear chromatin condensation prior to rotenone treatment.



Control **Rotenone** **Rutin + Rotenone** **Rutin alone**

Fig. 6. DAPI nuclear staining: rutin prevents rotenone-induced chromatin condensation

Impact of rutin on the expression of cell death markers

Figure 7A reveals the modulatory effect of rutin on key apoptotic regulatory proteins in rotenone-treated SH-SY5Y cells. Rotenone shifted the Bax/Bcl-2 ratio toward cell death (*$p < 0.05$) and caused cytochrome-c release from mitochondria, confirming mitochondrial permeabilization and intrinsic apoptosis activation. Pretreatment with rutin (10 µM) significantly normalized these rotenone-induced alterations (#$p < 0.05$ vs. rotenone), evidenced by suppression of Bax upregulation, restoration of Bcl-2 expression, retention of cytochrome-c within mitochondria, and reduction of its cytosolic release. Rutin alone exhibited a protein expression pattern similar to that of the control, indicating a lack of cytotoxicity or apoptotic induction under basal conditions. These findings confirm that rutin effectively stabilizes mitochondrial integrity and blocks apoptotic signaling at an early regulatory level by correcting the Bax/Bcl-2 ratio and preventing cytochrome-c leakage.

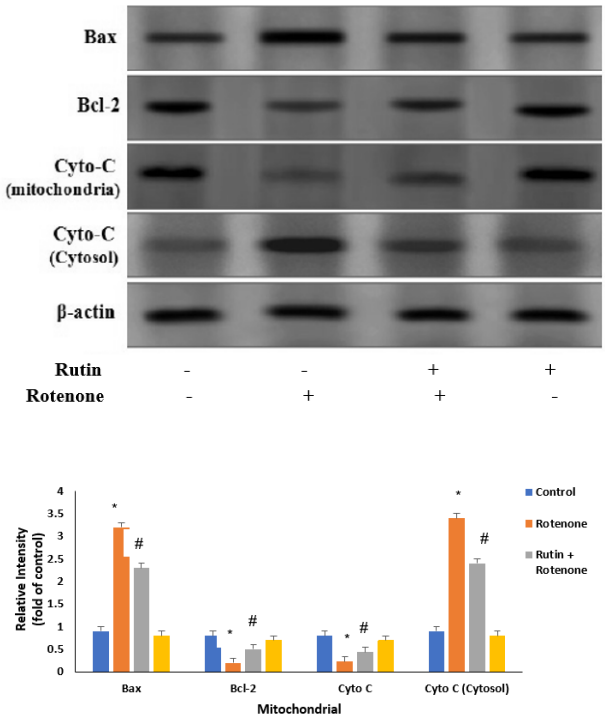
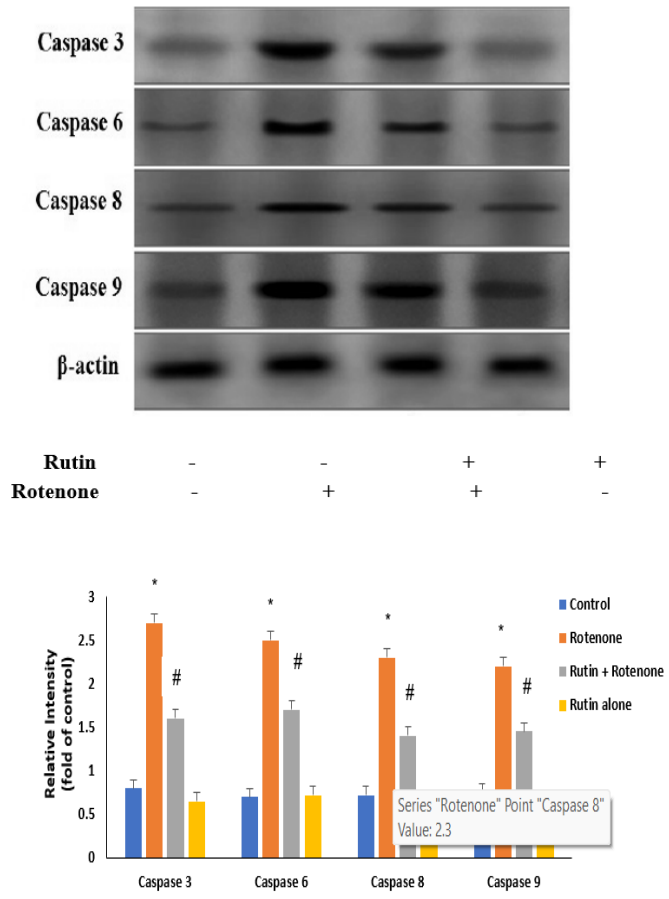


Fig. 7A. Western blots: Bcl-2, Bax, cytochrome-c and caspases — representative images.



- Fig. 7B. Densitometry of Fig.7A (normalized to β-actin); rutin reverses rotenone-induced changes

Fig. 7B. Densitometry of Fig.7A (normalized to β-actin); rutin reverses rotenone-induced changes

Figure 7B further supports the anti-apoptotic action of rutin by demonstrating its ability to inhibit rotenor * luced caspase activation in SH-SY5Y cells. A ..table upregulation of initiator and effector caspases was det d following rotenone exposure, confirming dual-pat # y apoptotic activation (*p < 0.05 vs. control).This enhanced caspase signaling aligns with the observed mitochondrial dysfunction and increased apoptotic nuclei in rotenone-treated cells. In contrast, pretreatment with rutin (10 µM) markedly suppressed the rotenone-induced elevation of all four caspases (#p < 0.05 vs. rotenone), indicating a strong blockade of caspase-mediated apoptotic execution. Importantly, rutin alone maintained caspase expression at basal levels similar to the control, showing that rutin does not trigger apoptosis under normal physiological conditions. These findings provide clear molecular evidence that rutin protects neuronal cells by preventing downstream apoptotic signaling through inhibition of caspase activation.

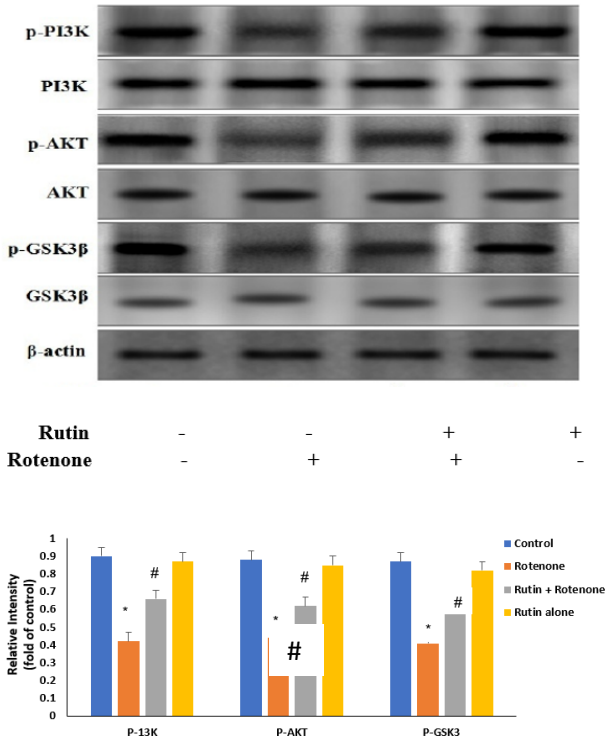


Fig. 8. PI3K/AKT/GSK-3β signalling: rutin restores p-PI3K, p-AKT and p-GSK-3β reduced by rotenone

Figure 8 demonstrates that rotenone disrupts neuronal survival signaling by markedly reducing the phosphorylated forms of PI3K, Akt, and GSK-3β, while total protein expression of these markers remains comparatively unchanged (*p < 0.05 vs. control). The reduction in p-PI3K and p-Akt indicates inhibition of the PI3K/Akt prosurvival cascade, a key pathway required for mitochondrial stability and prevention of apoptosis. Moreover, rotenone significantly lowered Ser9 phosphorylation of GSK-3β, thereby promoting its active, pro-apoptotic form and facilitating downstream apoptotic events. In contrast, pretreatment with rutin (10 µM) robustly reversed these rotenone-induced suppressive effects (§p < 0.05 vs. rotenone), restoring phosphorylation levels of PI3K, Akt, and GSK-3β to near-normal values, which indicates reactivation of the survival signaling axis. Rutin alone maintained phosphorylation status similar to control, confirming that rutin ' not interfere with basal cellular signaling under # siological conditions. Altogether, these results confirm that rutin protects neuronal cells not only by suppressing oxidative stress and apoptosis but also by preserving the PI3K/Akt/GSK-3β signaling pathway, thereby supporting cell survival and mitochondrial integrity in rotenone-induced neurotoxicity.

Conclusion

Rutin, a key flavonoid from *Ruta chalepensis*, provided strong neuroprotection against rotenone-induced toxicity in SH-SY5Y cells by targeting multiple pathological mechanisms involved in Parkinson's disease. Pretreatment with rutin effectively reduced oxidative stress by lowering ROS generation and lipid peroxidation, while simultaneously restoring cellular antioxidant status, evidenced by recovered GSH levels and enhanced activities of SOD, catalase, and GPx. In addition, rutin preserved mitochondrial membrane potential, confirming its ability to maintain mitochondrial integrity and prevent early apoptotic events. Fluorescence-based apoptosis assays (AO/EB and DAPI) further demonstrated a substantial reduction in apoptotic cell death in the presence of rutin. At the molecular signaling level, rutin increased the expression of the anti-apoptotic protein Bcl-2, suppressed Bax and caspase activation, and reinstated phosphorylation of PI3K and Akt, along with inhibitory Ser9 phosphorylation of GSK-3β, indicating activation of the PI3K/Akt/GSK-3β survival pathway. Collectively, these results establish rutin as a promising neuroprotective agent capable of mitigating key events in rotenone-induced neuronal injury. However, further in vivo investigations are necessary to confirm its therapeutic potential, clarify pharmacokinetic behavior, ensure safety, and determine optimized dosing strategies for future clinical translation.

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