

Isolongifolene attenuates rotenone-induced mitochondrial dysfunction, oxidative stress and apoptosis

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1. ABSTRACT

The present study was carried out to investigate the neuroprotective effects of isolongifolene (ILF), a tricyclic sesquiterpene of *Murraya koenigii*, against rotenone-induced mitochondrial dysfunction,

oxidative stress and apoptosis in a cellular model. SH-SY5Y human neuroblastoma cells were divided into four experimental groups (control, rotenone (100 nM), ILF (10 μ M) + rotenone (100 nM), ILF

10 microM alone treated) based on 3-(4, 5-dimethyl 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The results of the present study showed that the ILF treatment significantly alleviated rotenone-induced cytotoxicity, oxidative stress and mitochondrial dysfunction in SH-SY5Y cells. Moreover, ILF attenuated rotenone induced toxicity by down-regulating Bax, caspases-3, 6, 8 and 9 expression and up-regulating of Bcl-2 expression. Furthermore regulation of p-P13K, p-AKT and p-GSK-3 beta expression by ILF, clearly confirmed its protective effects. Taken together, our results suggested that ILF attenuated rotenone-induced oxidative stress, mitochondrial dysfunction and apoptosis through the regulation of P13K/AKT/GSK-3 beta signaling pathways. However further pre-clinical studies are warranted in rodents to use ILF as a promising therapeutic agent for PD in future.

2. INTRODUCTION

Parkinson's disease (PD) is a second most common, progressive and age-related disorder, resulting in the degeneration of dopaminergic neurons in the substantia nigra (SN). The impaired dopamine signaling leads to various clinical motor symptoms such as tremor (in resting state), rigidity, akinesia (inability to initiate movement) and postural imbalance (1-2). Even though numerous causes including genetic mutation and environmental toxins are linked with PD, the main cause for neuronal degeneration in PD remains unanswered. Intensive studies have been indicated that several molecular and cellular events such as abnormal protein folding, high load of oxidative stress, mitochondrial dysfunction and impaired apoptotic machinery are the potential factors in the pathogenesis of the disease (3-4).

Rotenone, a plant-derived pesticide, is the most potent member of the rotenoids, belonging to a family of isoflavonoids extracted from Leguminosae plants (5). It is similar to other neurotoxicants that specifically cross the blood brain barrier, gets accumulated in sub cellular organelles and inhibits the flow of electrons through the mitochondrial respiratory chain by binding with mitochondrial complex-I (5). Rotenone blocks the electron transfer from complex I to ubiquinone, thereby inhibiting oxidative phosphorylation and enhancing reactive oxygen species (ROS) generation (6). Studies have shown that further downstream of mitochondrial damage and oxidative toxicity lead to the release of cyt-c and its activation that ends in apoptosis (7-9).

Currently, available pharmacological therapies for PD include combination of Levodopa, precursor of dopamine, with entacapone, a catechol-O-methyl-transferase (COMT) inhibitor, or selegiline, a monoamine oxidase inhibitor, or

rasagiline or isocarboxazid or tranylcypromine. These pharmacological interventions suppress the symptoms temporarily, but none of them prevent the degeneration of the dopaminergic neurons (5). Other strategies like neural transplantation, stem cell therapy and deep brain stimulation of the sub-thalamic nucleus and globus pallidus are only at the experimental level. Therefore, rationalization of therapeutic measure might bring in the radical improvement in preventing progression and deterioration of PD (10).

Various reports suggested that consumption of phytochemicals from natural products has been associated with reduced risk of neurodegenerative diseases (11-12). To protect vulnerable targets, phytochemicals counteract the imbalance of the cellular redox homeostasis and the ROS levels under the cytotoxic threshold. Leaves of the curry plant (*Murraya koenigii*), is a herb in Ayurvedic medicine and known as 'kariveppilai' (kari-curry, veppu- neem and ilai-leaf) in Tamil language, which means "leaf that is used to make curry" and have been used in almost all the dishes in Tamil Nadu state of South India. The leaves are reported to have anti-diabetic, hypoglycemic, antioxidant, antimicrobial, antiinflammatory, antihypercholesterolemic and anticarcinogenic properties (13). Moreover, previous study by Mani *et al.*, (14) suggested that *Murraya koenigii* leaves are having memory enhancing properties in the aged- and dementia-induced rats. The pharmaceutical properties of the leaves are due to the presence of various alkaloids such as isolongifolene (ILF), cyclomahanimbine, tetrahydromahanimbine, murrayastine, murrayaline, and pypayafoline carbazole alkaloids. ILF is used in perfumery industries due to its woody amber odor and reported to have the antioxidant, anti-inflammatory and anticancer properties (15-16). The anti-oxidative effect of ILF was also reported in our lab (17). Thus, the present study is designed to examine the neuroprotective action of ILF against rotenone-induced oxidative stress, mitochondrial dysfunction and apoptosis in SH-SY5Y cellular model of PD.

3. MATERIALS AND METHODS

3.1. Chemicals

Isolongifolene, rotenone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 27'-dichloro fluorescein diacetate (DCFH-DA), rhodamine-123 (Rh-123), 4, 6- Diamidino-2-phenylindole (DAPI), ethidium bromide (AO/EtBr), acridine orange (AO), Dulbecco's modified Eagle medium (DMEM): nutrient mix F-12 (1:1), fetal bovine serum (FBS), antibiotic/antimycotic agent, DMSO, EDTA, trypsin-EDTA and / were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-Bax, Bcl-2, caspases-3, -6, -8 and -9, Cytochrome c (cyt-c), pAkt, Akt, pGSK3 β (Ser9), GSK3 β , pPI3K, PI3K and anti-

β -actin antibodies were obtained from cell signaling (Beverly, MA, USA).

3.2. Cell culture

SH-SY5Y neuroblastoma cells were obtained from National center for Cell Science, Pune, India. Cells were grown in DMEM F-12 (1:1) supplemented with 10% FBS, and 1% antibiotic/antimycotic solution and cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air incubation. All experiments were carried out after 24h incubation and ILF was added 2h prior to rotenone treatment.

3.3. Cell viability assay

MTT assay was analyzed by using of mitochondrial dehydrogenase activity in healthy cells the methods of previously reported (9). Briefly, SH-SY5Y neuroblastoma cells (3x10³ cells/well) were seeded in 96 well plates. Cells were incubated with various concentrations of ILF (0, 1, 2.5., 5, 10, 20 and 50 μ M) for 26 h followed by the incubation with MTT (1 mg/ml-1) reagent at 37 °C for 4 h. After incubation, the MTT solution was removed and 100 μ l DMSO was added to dissolve the formazan crystals. The absorption of formazan product was read at 570 nm in a Read well touch, ELISA plate reader (Robonic, India). For rotenone treatment, the cells were first treated with various concentrations of ILF (0, 1, 2.5., 5, 10, 20 and 50 μ M) for 2 h and then rotenone (100 nM) was added and incubated for further 24 h followed by the MTT assay, as mentioned above.

3.4. Experimental design

- Group I: Untreated control cells
- Group II: Rotenone (100 nM)
- Group III: ILF (10 μ M) + Rotenone (100 nM)
- Group IV: ILF (10 μ M)

3.4.1. Measurement of intracellular ROS levels

Intracellular ROS was measured using a non-fluorescent probe, 2, 7, Dichlorofluorescein Diacetate (DCFH-DA) that can penetrate into the intracellular matrix of cells, where it is oxidized to fluorescent dichlorofluorescein (DCF) as previously reported (18). Cells (1x10⁵cells/well) were seeded in 6 well plates and treated with ILF (10 μ M) and/or rotenone (100nM) and kept in a CO₂ incubator for 24 h. After 24 h of incubation, 1 ml of cell suspension was incubated with 100 μ l DCFH-DA for 10 min at 37°C. Fluorescent intensity was measured with excitation and emission filters set at 485 \pm 10 nm and emission wavelength 530 \pm 10nm, respectively using Shimadzu RF-5301PC Spectrofluorimeter. Images were captured by fluorescence microscope.

3.4.2. Change in mitochondrial membrane potential ($\Delta\psi$ m)

The $\Delta\psi$ m was determined by the incorporation of a cationic fluorescent dye Rho-123 as previously described (19). Cells (1x10⁵ cells/well) were cultured in 6 well plates and treated with ILF and/or rotenone and then they were incubated with fluorescence dye Rho-123 for 30 min at 37°C after 24 h. The $\Delta\psi$ m was evaluated qualitatively under a fluid cell imaging station (Invitrogen, USA). The fluorescence intensity was analyzed at wavelength at 480/530 nm under spectrofluorometer.

3.4.3. Detection of apoptotic cells with AO/EB staining

The Acridine orange (AO) and Ethidium bromide (EB) staining were used to detect apoptotic cells based on the method of affirmation (27). The cells (1x10⁵cells/well) were cultured in 6 well plates followed by the treatment with rotenone 100nM and/or ILF 10 μ M for 24 h. The cells were fixed in methanol: glacial acetic acid (3:1) for 30 min at room temperature. The cells were washed in PBS, and stained with 1:1 ratio of AO and EB. Stained cells were immediately washed again with PBS and viewed under fluid cell imaging station (Invitrogen, USA). The number of cells showing feature of apoptosis was counted as a function of the total number of cells present in the field.

3.4.4. Nuclear staining with DAPI

DAPI staining was performed, as previously described (20). SH-SY5Y cells (1x10⁵ cells/well) were seeded in 6-well plates. After treatment, the cells were fixed with 3% methanol at room temperature for 20 min. Cells were then stained with 1 μ g/ml DAPI solution for 30 min in the dark. The nuclear morphology was visualized by using fluid cell imaging station (Invitrogen, USA).

3.5. Biochemical Analysis

3.5.1. Estimation of lipid peroxidation products and antioxidants

The SH-SY5Y cells were harvested by trypsinization and the cell pellet obtained was suspended in PBS. The suspension was taken for biochemical analysis. The level of lipid peroxidation was determined by analyzing TBA-reactive substances (TBARS) (21). The pink chromogen formed by the reaction of 2-TBA with breakdown products of lipid peroxidation was measured. Superoxide dismutase (SOD) activity was assayed (22), based on the inhibition of the formation of reduced nicotinamide adenine dinucleotide phenazine methosulphate-NBT complex. Catalase activity was assayed (23) by quantifying the

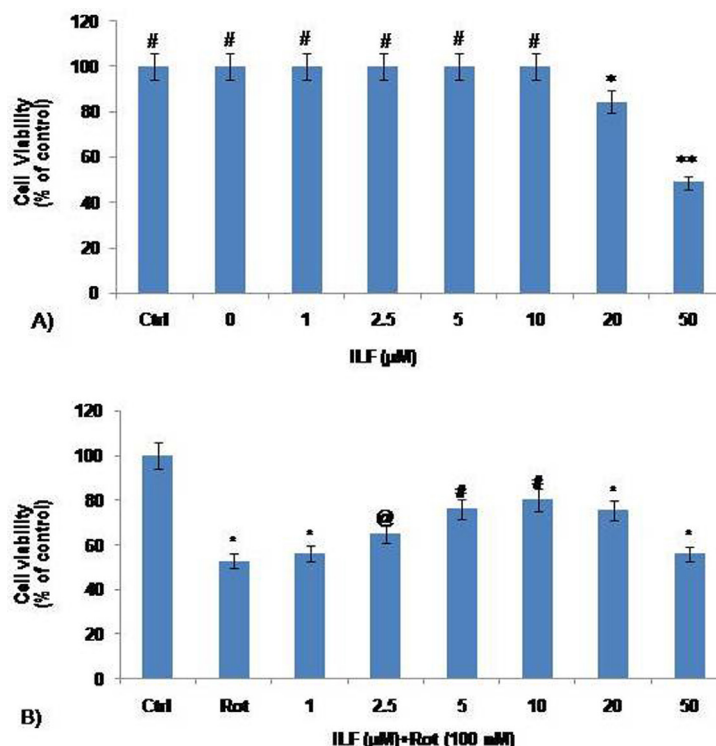


Figure 1. Protective effects of ILF on rotenone-induced cytotoxicity in SH-SY5Y cells. (A) dose-dependent effect of ILF at various concentration (0, 1, 2.5, 5, 10, 25, 50 μ M), cell viability determined by using the MTT method. (B) The protective effect of ILF 10 μ M against rotenone-induced cell death. Values are expressed as the percentage of the untreated control and represented as mean \pm SD of four independent experiments in each groups.

hydrogen peroxide after reacting with dichromate in acetic acid. The activity of glutathione peroxidase was assayed by a known amount of enzyme preparation and was allowed to react with hydrogen peroxide and GSH for a specified period (24). Then, the GSH content remaining after the reaction was measured. The total GSH content was measured based on the development of a yellow color when 5, 5'-dithiobis-2-nitrobenzoic acid was added to compound containing sulfhydryl groups (25).

3.6. Western Blot analysis

SH-SY5Y cells were washed with ice-cold PBS and then lysed with RIPA buffer for 30 min and centrifuged at 4°C, 12,000 \times g for 30 min. The supernatants were collected and protein concentration was determined using Nanodrop spectrophotometer (Thermo Scientific). Each sample with 50 μ g protein was added with SDS sample buffer and denatured at 95 °C for 5 min. Protein were separated using 10 % SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. Membrane were blocked with 5% non-fat dry milk for 1h, and blots were incubated with primary monoclonal antibodies Bcl-2, Bax at a dilution of (1:1500), caspase-3, -6, -8 and caspase-9 (1:1000), cyt-c (1:1000), pPI3K, and PI3K, pAkt (1:1200) and Akt (1:1400), p-GSK3 β and GSK3 β (1:1250) and β -actin (1:1000) at 4°C overnight. After

washing, the membranes were incubated with anti-rabbit HRP conjugated secondary antibody (1:2000) and bands were detected by chemiluminescence staining using ECL detection kit. Densitometry was done using 'Image J' analysis software (26).

3.7. Statistical analysis

All data were expressed as mean \pm Standard deviation (SD) of four number of experiment. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 15.0. (SPSS, Cary, NC, USA) and the individual comparison were obtained by Duncan's Multiple Range Test (DMRT). A value of $P < 0.05$ was considered to indicate a significant difference between groups. Values not sharing common superscript are significant with each other at $P < 0.05$.

4. RESULTS

4.1. Effects of ILF on cell viability

Previous studies from our lab indicated that the exposure to rotenone decreased cell viability in a dose-dependent manner for 24h and 100 nM of rotenone triggered about 50% of relative cell viability (9). This dose was chosen for inducing cell toxicity in this study. (Figure 1, "A,B") shows the protective effect

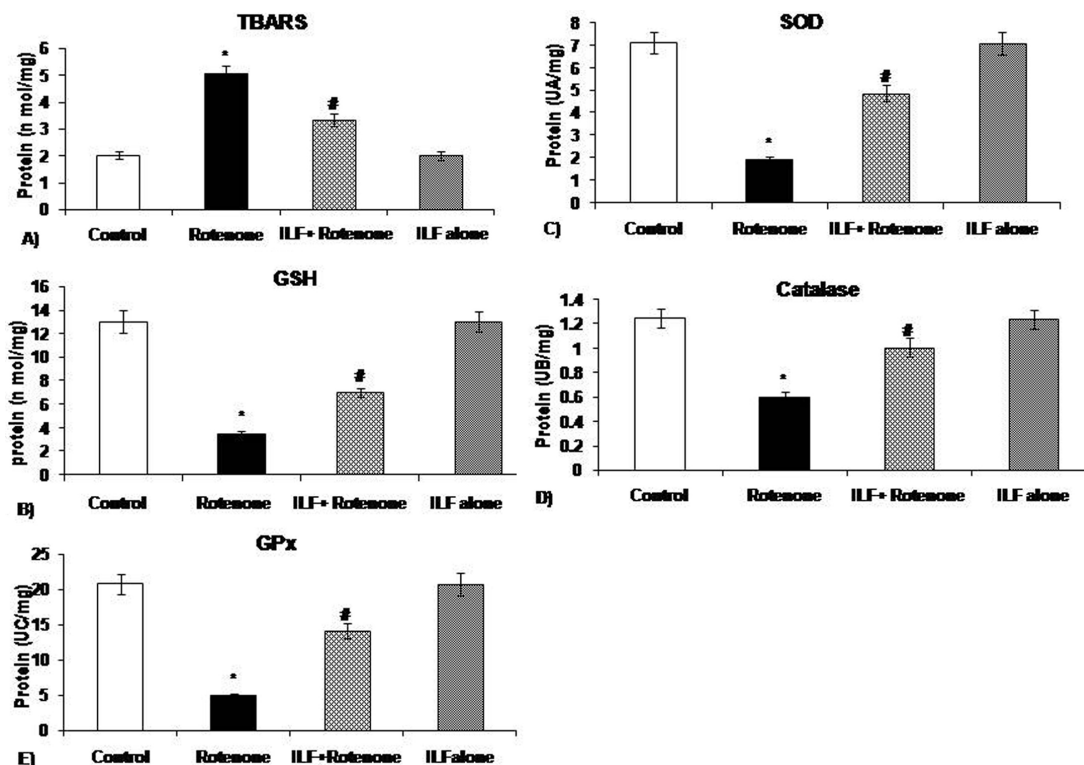


Figure 2. Rotenone (100 nM) treatment significantly increased the levels of TBARS and diminished the levels of GSH as compared with control cells, while ILF (10 μ M) pre-treatment significantly attenuated rotenone-induced oxidative stress (SOD, Catalase, GPx). Values are given as mean \pm SD of four independent experiments in each group. *P < 0.05 compared to control and #P < 0.05 compared to the rotenone group (DMRT). A Enzyme concentration required for 50% inhibition of NBT reduction in 1 minute. B Micromoles of hydrogen peroxide consumed per minute. C Micrograms of glutathione consumed per minute.

of ILF (10 μ M) against rotenone-induced cell toxicity. Hence, 10 μ M of ILF and 100 nM of rotenone were chosen as optimal dose for further experiment.

4.2. Changes in the level of lipid peroxidation and antioxidants

The levels of TBARS, GSH and activities of SOD, catalase, and glutathione peroxidase (GPx) in rotenone-treated SH-SY5Y cells incubated with or without ILF were analyzed to evaluate the antioxidative effect of ILF. The increased levels of TBARS and the decreased levels of GSH were observed in rotenone-induced SH-SY5Y cells when compared to control, whereas pre-treatment with 10 μ M of ILF significantly decreased rotenone-induced TBARS levels and enhanced the levels of GSH. Antioxidants act as primary defense against free radicals. Rotenone-induced SH-SY5Y cells significantly decreased cellular antioxidant status due to excessive ROS generation. Conversely, treatment with 10 μ M of ILF significantly prevented rotenone-induced loss of SOD, catalase and GPx activities in SH-SY5Y cells. There were no significant changes in antioxidant status in the ILF alone-treated cells (Figure 2, "A, E").

4.3. Effect of ILF on rotenone-induced ROS generation

To evaluate the antioxidative effects of ILF, the levels of ROS were estimated. Rotenone (100 nM) generated more ROS in SH-SY5Y cells as compared with control cells. We also observed that pre-treatment with 10 μ M ILF markedly decreased ROS formation (Figure 3). As compared with the control, treatment with 10 μ M ILF alone did not result in a significant difference in ROS generation.

4.3. Effect of ILF on rotenone-induced $\Delta\psi$ m alteration

Loss of $\Delta\psi$ m is an early and critical step in mitochondria-mediated apoptotic signaling. Therefore, we measured the green fluorescence proportion of rhodamine-123 in control and experimental groups. As shown in (Figure 4), the $\Delta\psi$ m was significantly decreased in the SH-SY5Y cells exposed to 100 nM of rotenone, while ILF pre-treatment significantly attenuated the rotenone-induced $\Delta\psi$ m loss. These results suggest that ILF may prevent apoptosis by maintaining mitochondrial function.

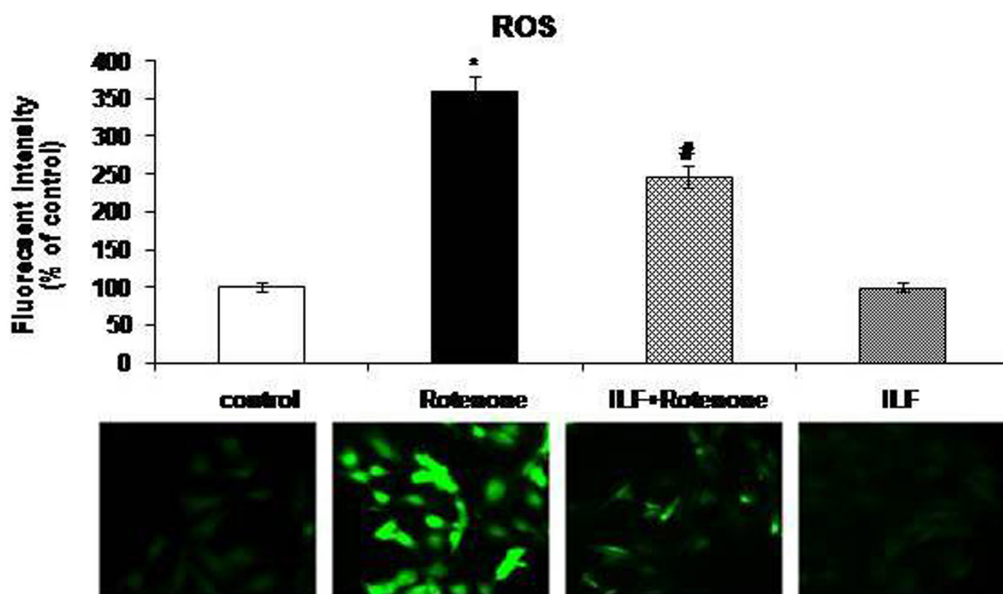


Figure 3. Rotenone (100 nM) treatment significantly increased the levels of ROS as compared with control cells, while ILF (10 μ M) pre-treatment significantly decreased the levels of ROS as compared with rotenone alone treated cells. Values are given as mean \pm SD of four independent experiments in each group. *P < 0.05 compared to control and #P < 0.05 compared to the rotenone group (DMRT).

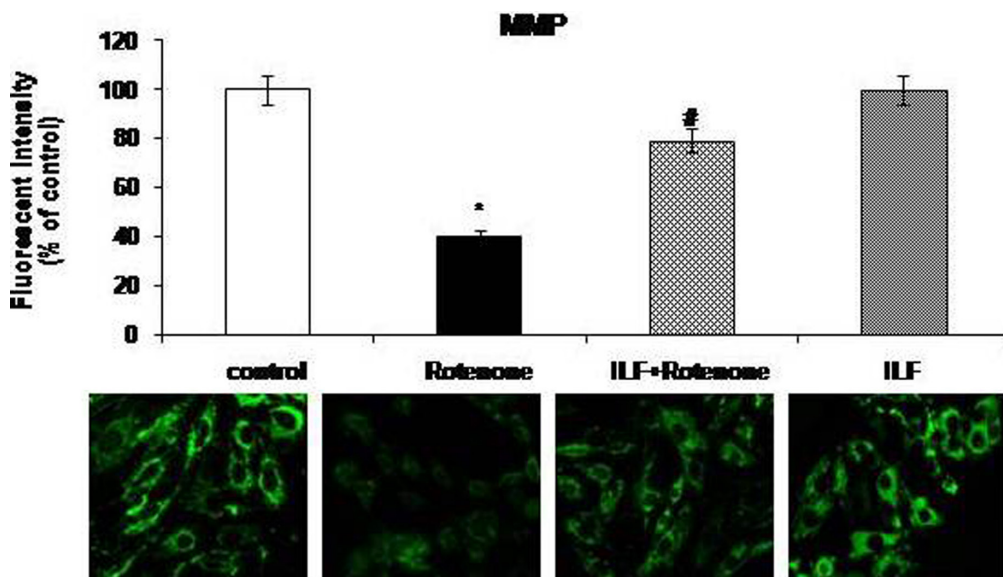


Figure 4. ILF attenuates rotenone-induced mitochondrial membrane potential in SH-SY5Y cells. Values are given as mean \pm SD of four independent experiments in each group. *P < 0.05 compared to control and #P < 0.05 compared to the rotenone group (DMRT).

4.4. ILF attenuates rotenone-induced apoptosis

The AO and EB staining reveal distinctive characteristic of apoptotic morphology in SH-SY5Y cells. These methods distinguish viable cells with uniform bright green nuclei and non-viable cells with orange to red nuclei. The results obtained from AO and EB staining are presented in (Figure 5). Administration of 100 nM of rotenone for 24 h induced morphological changes typical of apoptosis (nuclei fragmentation

with bright staining) observed by fluorescent microscopy. ILF pre-treatment increased cell viability and decreased apoptosis cell death when compared to cells exposed to rotenone.

4.6. ILF mitigates rotenone-induced nuclear condensation in SH-SY5Y cells

SH-SY5Y cells treated with rotenone showed brighter fluorescence after staining with DAPI dye. The

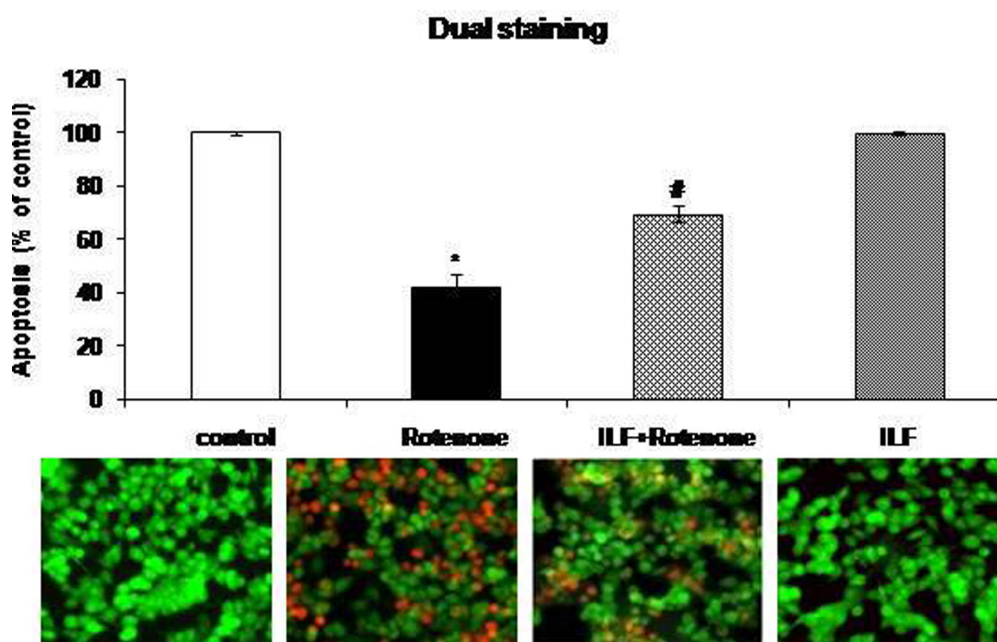


Figure 5. Rotenone (100 nM) treatment induced cell apoptosis compared to control cells. Pre-treatment with ILF (10 μ M) suppresses these apoptotic features. Values are given as mean \pm SD of four independent experiments in each group. *P < 0.0.5 compared to control and #P < 0.0.5 compared to the rotenone group (DMRT).

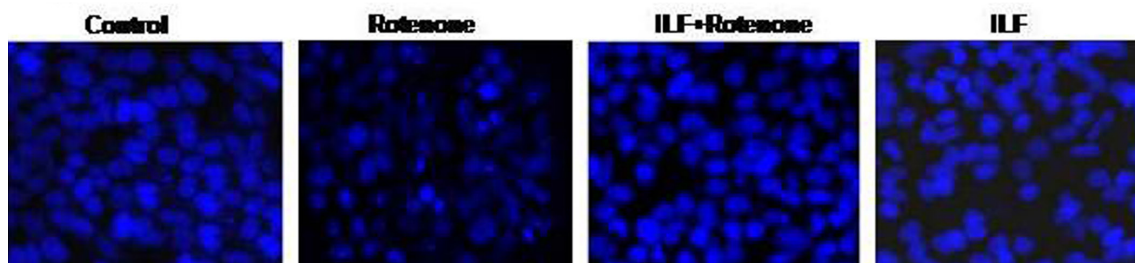


Figure 6. Nuclear morphology of SH-SY5Y cells stained with DAPI. ILF on rotenone-induced apoptotic features in SH-SY5Y cells pre-treated with ILF (10 μ M). Nuclear condensation and/or fragmentation are indicatives of apoptosis. Figure indicate treatment with (A) control, (B) rotenone, (C) ILF + rotenone, and (D) ILF. It seems that there are apoptotic cells in (B), but not in the others groups.

bright fluorescence showed nicked DNA and nuclear chromatin condensation in cells. Pre-treatment with ILF 10 μ M markedly prevented rotenone-induced nuclear chromatin condensation (Figure 6).

4.7. Effect of ILF on the expression of apoptotic indices

Apoptotic pathways involve many different proteins including the Bcl-2 family of proteins in mitochondria and their dysfunction result in apoptosis through the activation of caspases. Protein expression of Bax, caspases-3, -6, -8, -9 was increased, while the expressions of Bcl-2 and cyt-c in mitochondria were significantly decreased by the rotenone treatment as compared to control group. Rotenone treatment significantly diminished the translocation of cyt-c in

cytosol. Pre-treatment of ILF protected the SH-SY5Y cells from rotenone toxicity by restoring mitochondrial regulatory and downstream apoptotic signaling molecules (Figure 7 “A, B”).

4.8. Effect of ILF on expression of P13K/AKT/pGSK3 β signaling markers

The result of western blot showed that the rotenone treatment significantly decreased the expression of p-P13K and p-AKT as compared with control, while no obvious differences were found in the expression of P13K, AKT and GSK3 β (Figure 8). AKT exerts its anti-apoptotic function by inducing the phosphorylation of its downstream substrate, GSK3- β . The phosphorylated forms (at serine residue 9) of GSK-3 β were measured in these groups against their

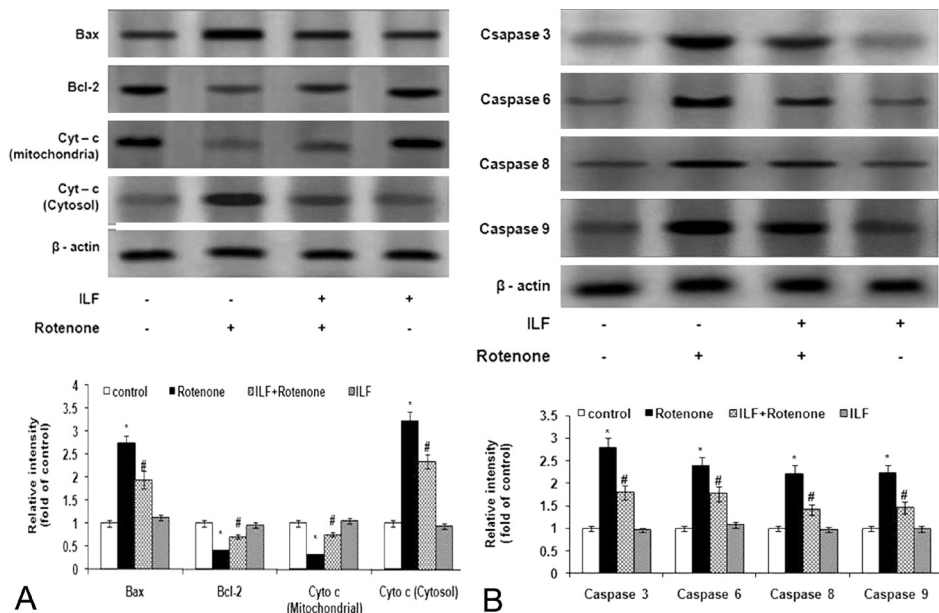


Figure 7. “A, B” ILF alters rotenone induced changes in the expression levels of proteins in SH-SY5Y cells. The expression of Bax, caspase-3, 6, 8 and caspase-9, cyto c in cytosol were, while the expression of Bcl-2 and cyto c in mitochondria were significantly decreased in the rotenone-treated group as compared with control. Pre-treatment with ILF greatly restore the imbalanced expression profile of these protein. β - Actin was used as an internal loading control. Values are given as mean ± SD in each group. *P < 0.05 compared to control and #P < 0.05 compared to the rotenone group (DMRT).

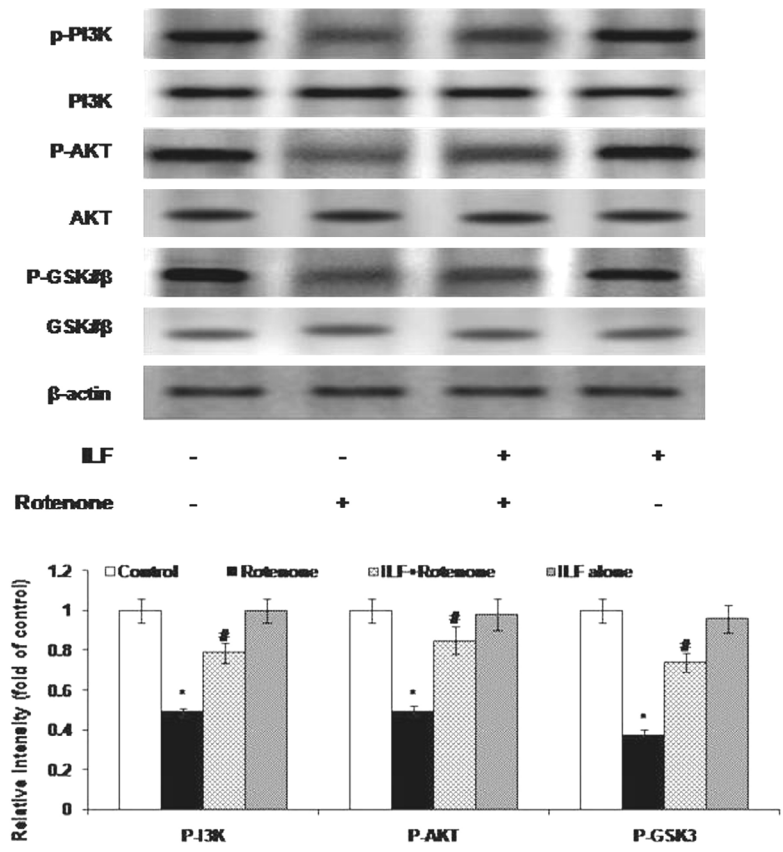


Figure 8. ILF alleviated rotenone-induced mitochondrial-mediated apoptosis through P13K/AKT/GSK-3β pathway. Western blot of p-P13K, p-AKT, p-GSK-3 expression and relative optical density normalized to β-actin in each group as indicated. Values are expressed as arbitrary units and given as mean ± SD. *p< 0.05 compared to the control mice, #p<0.05 compared to the rotenone treated cell.

unphosphorylated form. Rotenone treatment induced a large reduction in the expression of phosphorylated GSK-3 β compared with the control group. Pre-treatment with ILF prevented the rotenone-induced decreased phosphorylation of GSK-3 β (Figure 8).

5. DISCUSSION

In this study, we found that the exposure of rotenone (100 nM) to SH-SY5Y cells caused about 50% cell death, which is correlated with our previous study (27). Pre-treatment with ILF markedly and dose dependently attenuated the toxic effects of 100 nM of rotenone and confirmed its neuroprotective effect against rotenone-induced apoptosis. Previous experiments by Ham *et al.*, (28-29) indicated that the reynosin and spirafolide, sesquiterpenes isolated from the leaves of *Laurusnobilis* L. (Lauraceae), also protected dopamine (DA) induced neuronal cell death in SH-SY5Y cells, which is corroborating with our results. The anti-apoptotic effects of ILF were further supported by the morphological observations of dual and DAPI assays.

The mitochondria are the principle intracellular sources of ROS and also the major target of oxidative stress (30). Under physiological conditions, low levels of ROS are essential for intercellular redox balance and cell proliferation. But excess ROS formation affects a number of cellular signaling pathways, resulting in damage of key intercellular molecules in mitochondria and leads to cell death (31). The cytotoxicity of rotenone is mediated mainly via the excessive generation of ROS. As rotenone enters the mitochondria, it selectively binds and inhibits NADH CoQ10 reductase (Complex I), which leads to the shunting of electrons through the ETC-II. Resulting accumulation of electrons reacts with O₂ and generates ROS 5–7 times more than basal levels (32). In this study, DCF-DA fluorescence indicated that rotenone-induced toxicity increases the accumulation of intracellular ROS (33). Previous studies from our lab demonstrated the potent antioxidant activities of ILF in various *in vitro* antioxidant assays including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydroxyl radical, nitric oxide, hydrogen peroxide, super oxide radical scavenging, Ferric Reducing Ability of Plasma (FRAP), Total Radical Trapping Antioxidant (TRAP) and Reducing Power.

The increased levels of TBARS in rotenone exposed cells were due to overproduction of free radicals, their reaction with lipids and enhanced lipid peroxidation processes, a typical phenomenon highly related to age-associated or neurodegenerative diseases, including PD (27, 34). Mitochondrial complex inhibition leads to increased formation of O₂^{•-} production, which is converted to H₂O₂ by Mn-

SOD (35). Additionally, H₂O₂ is a diffusible molecule that may cross mitochondrial membranes spreading a redox impairment to the whole cell by reacting with O₂^{•-} or Fe²⁺ and generating hydroxyl radical (OH[•]) (40). However, exceeding levels of ROS may result in modulated activity of antioxidants (36). Our results showed that the increase in the enzymatic activities of SOD, GPx and catalase in neuroblastoma cells incubated with rotenone alone is likely due to a response towards increased ROS generation following rotenone treatment. However, the ROS-wreaked cells supplemented with ILF reversed the activities SOD, GPx and catalase most likely by waking up intracellular antioxidant defense. Our results agreed with the assumption that activating endogenous antioxidants boost cell viability by reducing rotenone-derived ROS (37). GSH is the most important thiol containing antioxidant in the brain (35), and it plays a pivotal role in preventing oxidative damage. It is a major biomarker associated with oxidative stress in biological systems (38). GSH depletion plays a key role in the pathology of PD (39). Our results indicated that ILF attenuated the rotenone-induced oxidative stress by its potent antioxidant action.

Mitochondria are complex organelles that play an essential role in neuronal viability, ATP production, engaged in oxygen consumption and ROS generation. Defect in mitochondrial function is a major pathogenic event in neurodegeneration (40). Excess ROS formation due to complex I inhibition, induces $\Delta\Psi_m$ loss and the release of pro-apoptotic proteins (e.g. cyt-c) from mitochondrial inter membrane space to cytosol, where cyt-c triggers progression of the apoptotic pathway. As shown in Figure 7, rotenone exposure significantly decreased the rhodamine-123 fluorescence, a probe for measurement of mitochondrial transmembrane potential. In parallel, with our study, Menkeet *et al* (41) reported that the rotenone exposure decreased $\Delta\Psi_m$ in the human neuroblastoma cell line SH-SY5Y. Our data showed that pre-treatment of SH-SY5Y with ILF before rotenone addition partially reduced the decline in rhodamine-123 fluorescence and restored to control levels.

The effects of ILF on the nigral apoptosis were assessed by various pro and anti-apoptotic proteins expression. Bcl-2-family proteins are important in regulating cell death by either inducing (Bax, Bid) or inhibiting (Bcl-2, Bcl-XL) apoptosis. Bax promotes apoptosis by inducing mitochondrial membrane depolarization and cyt-c release while Bcl-2 inhibits apoptosis by preventing mitochondrial membrane depolarization (9). Our results are consistent with the previous studies in which they have reported that administration of rotenone significantly decreased the level of Bcl-2 expression and increased the Bax protein expression that favors apoptotic process. Additionally, caspase-9, -8, -6, -3 expressions were significantly

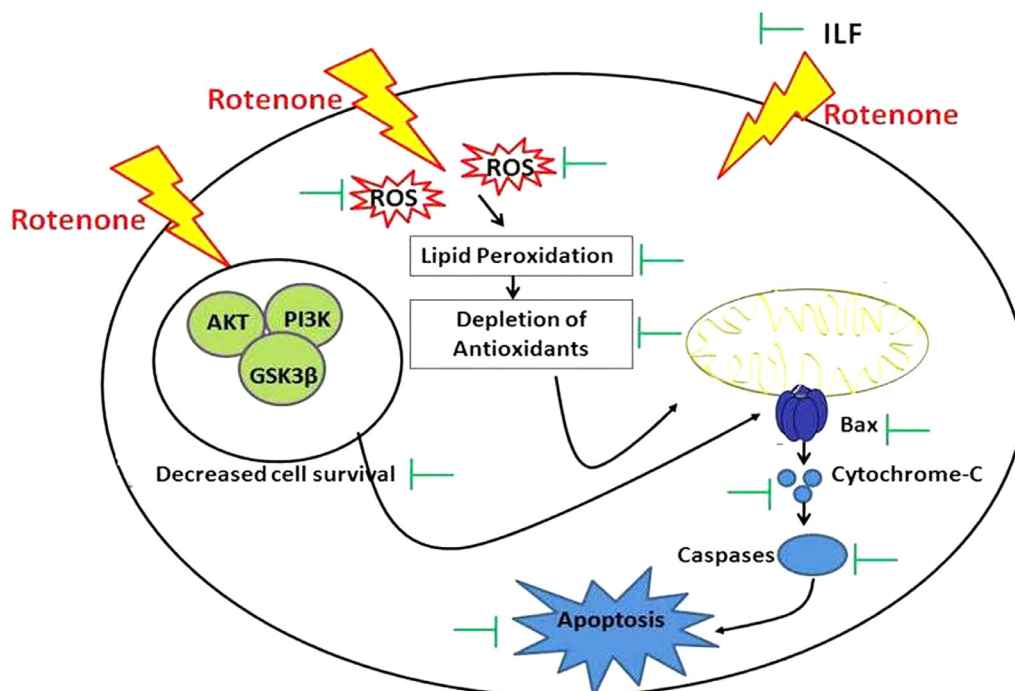


Figure 9. The mechanism of neuroprotection offered by ILF against rotenone induced neurotoxicity in SH-SY5Y cells, Black coloured arrow indicates the action of rotenone, Green coloured symbol indicates the role of ILF.

increased in rotenone-treated SH-SY5Y cells (27). Findings of our study demonstrated that ILF prevented rotenone-induced apoptosis by reducing expression of Bax, caspase-9, -8, -6, -3, cytosolic and cyt-c and increasing the expression of Bcl-2 and mitochondrial cyt-c.

PI3K/AKT/GSK3 β signaling pathway has been proposed as a key pathway for neuronal survival, growth and function (42). GSK3 β has emerged as a major therapeutic target due to its involvement in several neurodegenerative diseases, including PD (43). AKT regulates PI3K (44) through phosphorylating GSK3 β . Recently, a large range of investigation suggests that GSK3 β is an important kinase associated with various essential cellular functions that regulates cell apoptosis and survival. These events occur through the modulation of mitochondrial mediated cell death pathways (45), which involve activation and localization of B-cell lymphoma (Bcl-2) family of proteins and mitochondrial complex I activity (46). Other report suggested that over-expression of the active form of mitochondrial GSK3 β facilitates the apoptotic effect of rotenone (47). The activity of GSK-3 β is regulated by phosphorylation at Ser 9 and Tyr 216 (48). Ser 9 phosphorylation inhibits its kinase activity, whereas Tyr 216 phosphorylation is required for its full activity. It has also been found that the active form of GSK-3 β (p-GSK-3 Tyr216) was increased in postmortem striata of PD patients (49). PI3K/AKT

signaling pathway activation ameliorates cell death through phosphorylation of GSK-3 β at Ser9 by AKT to inhibit its activity (50). In this present study, we found rotenone down-regulated the expression of Ser 9 p-GSK-3 β and pAKT. ILF pre-treatment prevented the decline of p-AKT in a dose-dependent manner, resulting in increased levels of pSer9-GSK-3 β .

In conclusion, the present study illustrates that ILF attenuated rotenone-induced mitochondrial dysfunction and cell apoptosis in SH-SY5Y cells. These processes are associated with inhibition of GSK-3 β activity. We demonstrated that ILF exerts its protective effect, at least partially through the PI3k/AKT/GSK3 β pathway in SH-SY5Y cells after rotenone challenge (Figure 9). However further studies are needed to validate its neuroprotective effect in animal models of PD before it could be used as a candidate for clinical trial to mitigate PD pathology.

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