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Original Article

Evaluating the Curative Potential of Vilangin for Parkinson's Disease Treatment through *in vitro* and *in silico* Approach

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ABSTRACT

Introduction: Parkinson's Disease (PD) is due to gradual deterioration of the aging brain and motor functions. The rising prevalence of PD suggests the need for better therapies to slow down or halt its progression. Our Knowledge in the pathophysiology of PD has evolved over time, driven by advancements in preclinical models that mimic the disease and the refinement of specific clinical, genetic, and pathological traits. This led to the investigation of numerous objectives for disease management. Alzheimer's disease and PD are two prominent neurological diseases for which there are lack of precise therapeutic strategies, and thus no cure. Hence, we need to explore new drugs until a definitive treatment strategy is attained. Vilangin, a dimeric form of embelin (a derivative of quinone), has recently received research attention owing to its chemical structure and functional activity. A preliminary investigation of this drug using an *in vitro* and *in silico* model is vital to validate its neuroprotective potential and efficacy. **Materials and Methods:** Vilangin was synthesized from embelin by condensing formaldehyde in acetic acid. SHSY-5Y cells, a neuroblastoma cell line, were exposed to rotenone (400 nM) to develop an *in vitro* PD model and treated with vilangin (200 and 500 µg) to evaluate its potential to ameliorate rotenone toxicity. Cell viability, mitochondrial function, Reactive Oxygen Species (ROS) production, and membrane potential, and apoptosis were examined. In addition, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed to analyze the expression of genes related to the antioxidant system (*NRF2*), apoptosis (*ATF3*), cell survival (*NURR1*), and neural transmission (*DAT*). Molecular Docking analysis was carried out between p53 (protein) and vilangin (ligand). **Results:** The higher dose of vilangin (500 ug) had a significant protective effect; it attenuated the toxicity of rotenone to restore the SH-SY5Y cellular functions by reducing ROS and apoptosis, and improved cell viability and mitochondrial functions. *Nrf2* and *Nurr1* were upregulated and *Atf3* and *Dat* were downregulated following vilangin treatment. The docking results showed good binding interactions between vilangin and p53; hence p53 could be a potential target of vilangin to manage PD complications. **Conclusion:** Vilangin can be advocated as a potential antioxidant and anti-apoptotic agent to reduce PD-related complications such as Oxidative Stress (OS), ROS formation, mitochondrial dysfunction, and cell death.

Keywords: Apoptosis, Mitochondrial membrane potential, Parkinson's disease, ROS, SHSY-5Y cell line, Vilangin.

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INTRODUCTION

Parkinson's disease arises due to progressive degeneration of pigmented neurons, primarily found in the Substantia Nigra (SNpc), that contain dopamine. Depigmentation of neuromelanin-containing neurons, which is a sign of advanced disease, indicates that these neurons are especially prone to degeneration (Zucca *et al.*, 2023). The SNpc contains cell bodies of neurons in the nigrostrial pathway. These neurons synthesize dopamine that is subsequently delivered to the striatum. In patients with PD, dopamine is depleted due to a reduction in dopaminergic projections, resulting in motor impairment and associated symptoms. A known crucial factor in the pathophysiology of PD is OS (Singh *et al.*, 2020). The loss of dopamine stimulates dopamine metabolism, which in turn leads to excessive production of Hydrogen Peroxide (H2O2) that increases the hydroxyl free radicals (•OH), and OS. The

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development of PD is associated with many cellular changes, including endoplasmic reticulum stress, loss of proteostasis, OS, mitochondrial dysfunction, and impaired autophagic flux (Alarcon-Gil *et al.*, 2022). The most important stage in the pathophysiology of PD is α-synuclein misfolding. Numerous animal and cell culture experiments have shown that oligomeric or fibrillar forms of α-synuclein are cytotoxic (Pierce *et al.*, 2018).

Cellular systems can be utilized to examine the complex nature of PD and other neurodegenerative disorders. They provide a way to dissect the complex pathogenesis of PD into simpler molecular models to characterize pathological events, to manipulate factors that contribute to the disease as well as to test therapeutic approaches. Primary dopaminergic neurons, immortalized cell lines (SH-SY5Y, LUHMES, and PC12), and induced Pluripotent Stem Cells (iPSCs) are examples of differentiated dopaminergic cell models. Among these, SH-SY5Y cells were derived from an aggressive form of neuroblastoma in a human infant. Although these cells are not neuronal, they share many biochemical and functional traits with neurons, including the expression of neurofilament proteins, neuronal marker enzymes (tyrosine hydroxylase and dopamine hydroxylase), and can be cultured (Lopez-Suarez *et al.*, 2022). They have been used to research OS, neuronal transmission, and neurodegeneration. Rotenone, a specific inhibitor of mitochondrial complex I, can cause the accumulation of ROS, tubulin depolymerization, α-synuclein aggregation, and dopamine depletion in neurons, all of which are commonly utilized to create PD models in both *in vitro* and *in vivo* (Lu *et al.*, 2018).

Embelin (2,5-Dihdroxy-3-undecyl-1,4-benzoquinone) is the primary active ingredient found in the *Embelia ribes* Burm Fruits. It has shown therapeutic benefits in several animal (Ramachandra *et al.*, 2022) and cell models of central nervous system disorders (Kundap *et al.*, 2017). This compound belongs to benzoquinone family, which includes mitoquinone (Ünal *et al.*, 2020) a synthetic derivative of coenzyme Q10 (Park *et al.*, 2020) that has already shown therapeutic potential against PD. Moreover, Embelin toxicity was determined by using SH-SY5Y cells. A dose of 5 uM led to 33% cell death, whereas at 50 uM dose, 50% cell death was observed. In SH-SY5Y cells, half of maximal inhibitory concentration (IC_{50}) did not exert any toxicity based on modulation of Butyrlcholinesterase (BChE), -secretase 1 (BACE-1), and P-glycoprotein (Pgp) (Nuthakki *et al.*, 2019).

It is crucial to clarify the underlying molecular pathogenic processes in PD and to identify a clinically effective treatment for patients. OS and ROS production trigger cellular damage, inflammatory reactions, and mitochondrial dysfunction-all of which are linked to neurodegenerative diseases, including PD and Alzheimer's Disease (AD). Moreover, apoptosis, autophagic cell death, necroptosis, and pyroptosis promote the degenerative course of PD, which is tightly regulated by ROS production and OS in the SNpc (Morris *et al.*, 2018; Trist *et al.*, 2019; Wang *et al.*, 2019). There is growing evidence that suggests anticancer drugs are potential neuroprotective agents (Advani *et al.*, 2020). p53 is a critical transcription factor that controls a number of biological functions, such as DNA repair, cell cycle arrest, autophagy, mitochondrial metabolism, apoptosis, redox balance, and senescence. Recent research has shown that p53 levels and activity are significantly elevated in afflicted neurons in *in vitro* and *in vivo* of PD as well as the brains of PD patients (Luo *et al.*, 2022; Mogi *et al.*, 2007; Wang *et al.*, 2024). Therefore, p53 can serve as a target to test the potential of test drugs that may ameliorate PD.

It would be useful to identify a natural compound with above mentioned properties and to assess its ability to ameliorate PD-related pathology. Vilangin is a dimer containing two embelin moieties connected with a methylene bridge; it has anticancer, antioxidant, antimicrobial, and antibacterial activities (Balachandran *et al.*, 2013). *In vitro* studies on vilangin demonstrated cytotoxic activity against A549 cells, a lung adenocarcinoma cell line. The IC_{50} of 400 µg/mL led to a 53.66% reduction in cell viability, which increased to 61.95% at a dosage of 500 µg/mL (Balachandran *et al.*, 2013). *In silico* analysis and subsequent docking experiments explored the ability of vilangin to bind to p53. Although this evidence is quite interesting, there are no published *in vitro* or *in vivo* studies as to whether vilangin could be used as a potential treatment for PD. Therefore, we tested the ability of vilangin to ameliorate the negative effects in an SH-SY5Y-based *in vitro* model of PD.

MATERIALS AND METHODS

Rotenone was procured from TCI (Tokyo, Japan). Janus Green B (JG-B) dye was obtained from SRL Chemicals (Mumbai, India). Dichlorodihydrofluorescein Diacetate (DCFH-DA), rhodamine 123 were purchased from Sigma. Dulbecco's Modified Eagle Medium (DMEM) was procured from HiMedia chemicals (Maharashtra, India).

Preparation of vilangin

1 g of embelin was dissolved in 30 mL of glacial acetic acid and 5 mL of 40% formaldehyde was added in drops with constant stirring. The mixture was heated over a water bath for 10 min and left at room temperature to get crystals of vilangin by the addition of dioxane. The compound was dried in vaccum, checked for purity and used in cell culture studies (Narendran *et al.*, 2020).

Cell culture

SH-SY5Y cells were acquired from the National Centre for Cell Science (Pune, India). They were added to a 96-well culture plate at a density of 1.0×10^5 cells/mL in DMEM supplemented with 10% fetal bovine serum (FBS). After plating, the cells were incubated overnight at 37°C in a humidity-saturated environment with 5% CO_2 and 95% air, to allow the cells to adhere firmly to the wells.

Cell viability assay

The culture medium was substituted with fresh culture medium containing vilangin (200 and 500 µg/mL) and rotenone (400 nM) (Aleksandrova *et al.*, 2023). The cells were incubated for 1 day. Subsequently, the medium was discarded, and adding 100 µL of culture medium containing 10% of a 5 mg/mL solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was ensured in each well. The plate was incubated in the incubator for 2 hr. Then, 200 µL of Dimethyl Sulfoxide (DMSO) was added to each well. The absorbance of the plate was measured at 570 nm to determine the viability relative to the untreated control (set at 100%).

Measurement of intracellular ROS

SH-SY5Y cells were seeded in 6-well plates (1.0×10^5 cells/mL) and treated with vilangin (200 and 500 µg/mL) and rotenone (400 nM) for 24 hr. Then, the cells were washed with Phosphate-Buffered Saline (PBS) and incubated with 1 µL of cell-permeable DCFH-DA (1 mg/mL) for 20 min in the dark. DCFH-DA is oxidatively converted into fluorescent 2′,7′-di-chlorofluorescein (DCF), and the fluorescence of DCF provides a measure of intercellular ROS production. The cells were viewed under a fluorescence microscope to confirm ROS production.

Assessment of mitochondrial function

SH-SY5Y cells were seeded in 96-well plates (1.0×10^5 cells/mL) and then challenged with vilangin (200 and 500 µg/mL) and rotenone (400 nM) for 24 hr. After incubation, the cells were washed with PBS and then stained with JG-B at 37°C for 60 min (Ahmad *et al.*, 2018) to assess mitochondrial function. The oxidoreductases in the electron transport chain of actively respiring mitochondria leads to the breakdown of JG-B into diethyl safranin. After staining, the absorbance was measured at 550/595 nm with an ELISA plate reader.

Assessment of Mitochondrial Membrane Potential (MMP)

A primary marker of cell health and apoptosis, mitochondrial dysfunction can be identified by assessing the changes in the MMP. Here, SH-SY5Y cells were seeds in 6-well plates (1.0×10^5 cells/mL) and then treated with vilangin (200 and 500 µg/mL) and rotenone (400 nM). After incubation, the cells were washed with PBS and stained with 50 µL of rhodamine 123 (10 µg/mL) for 30 min. The cells were observed under a fluorescence microscope to determine changes in morphology and MMP.

Assessment of apoptosis

SH-SY5Y cells were seeded in 6-well plates (1.0×10^5 cells/mL) and then exposed to vilangin (200 and 500 ug/mL) and rotenone (400 nM) for 24 hr. Following treatment, the cells were washed with PBS. Then, they were incubated with 5 µL of the fluorescent dye acridine orange (AO, 100 µg/mL) and 5 µL of propidium iodide (PI, 100 µg/mL). PI stains non-viable cells, which fluoresce red, while AO can stain both viable and non-viable cells, which fluoresce green and red, respectively. This dual staining technique was used to categorize between necrotic, early apoptotic, and late apoptotic cells. After staining, the cells were observed with a fluorescence microscope to examine the integrity of the cell membrane, DNA damage, and morphological changes.

Nuclear morphology

SH-SY5Y cells were distributed into 6-well plates (1.0×10^5 cells/mL). After allowing the cells to adhere to the wells, they were treated with vilangin (200 and 500 µg/mL) and rotenone (400 nM). Following incubation, the cells were rinsed with PBS, fixed for 10 min with 50 µL of 3% paraformaldehyde, and permeabilized for 10 min at room temperature using 0.2% Triton X-100. The cells were stained with 19 µL of 4′,6′-diamidino-2-phenylindole (DAPI) (0.5 µg/mL) for 5 min to examine morphological alterations of the nucleus (condensed chromatin and fragmented nuclei) after treatment. Both live and fixed cells are stained with DAPI, which binds strongly to DNA sequences rich in adenine and thymine. Finally, the cells were look at under a fluorescence microscope.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was taken out from the SHSY-5Y cells involving Trizon RNA Extraction Reagent (Lupex Bio, Chennai, India). The isolated RNA was reverse transcribed into complementary DNA (cDNA). In brief, each 20-µL reaction contained 4 µL of 5x reaction buffer, 1 µL of reverse transcriptase, 500ng RNA, and nuclease-free water. The reaction was incubated at 25°C for 10 min, 42°C for 60 min, 85°C for 5 min, and held at 4°C. After synthesis, cDNA was stored at -20°C. NCBI Primer-BLAST was used to design primers for genes related to antioxidant activity, apoptosis, inflammation, OS, and neurodegeneration (Table 1). In addition, *GAPDH* was used for normalization. The assay was run with the following thermal cycling protocol: initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s and annealing at 60°C for 60s. The data were analyzed with the Aria 2.0 software (Agilent Technologies, Santa Clara, CA, USA) to determine the threshold cycle (Ct) value was produced at each cycle. The difference in the Ct (delta Ct) was used to determine the relative gene expression.

Molecular docking analysis

The chemical structure of the ligand vilangin (Chemical Identification number 417182) and reference compound thymoquinone (Chemical Identification number 10281) were downloaded from the PubChem database. The two and three-dimensional (2D and 3D, respectively) structures of the ligand (vilangin) and the reference compound (thymoquinone)

were prepared by using the ChemDraw software. The final prepared 3D structures of vilangin (ligand) and thymoquinone (reference compound) were further utilized for Swissdock analysis (Kumaraswamy *et al.*, 2023). The 3D structure of the protein of interest- human anti-tumour protein p53 was downloaded from the Protein Data Bank (PDB) (4MZI). The "A" chain of protein was prepared separately by deleting the ligands (heteroatoms), and even the crystallographic water using the UCSF-Chimera software (Narayanaswamy *et al.*, 2023). Docking analysis of the ligand (vilangin) and reference compound (thymoquinone) with the target protein (human p53) was performed by utilizing the free online Swissdock server. Amino acid interaction analysis was carried out by using PyMOL (Surya Prakash *et al.*, 2023) to determine the best docked pose of the ligand and reference compound.

Statistical analysis

SigmaPlot 14.5 (Systat Software Inc., San Jose, CA, USA) statistical tool facilitated to generate graphs. The data are expressed as the mean and standard deviation or standard error of the mean. The data were analyzed with one-way analysis of variance with the Bonferroni correction for multiple comparisons. A p value of < 0.05 was an indication for confirming the statistically significant difference.

Ethical Statement

No animal or human subjects were involved in the present study.

RESULTS

Cell viability

Based on the MTT assay, 400 nM rotenone markedly decreased the SH-SY5Y cells viability (Figure 1A). The addition of vilangin attenuated the rotenone-induced loss of cell viability. Based on the results, 200 and 500 µg/mL vilagnin were examined in the subsequent experiments.

Intercellular ROS scavenging

We used DCFH-DA fluorescence staining to access intercellular ROS scavenging activity. The ROS scavenging activity of the rotenone-treated SH-SY5Y cells was diminished compared with the untreated group. Treatment with vilangin for 24 hr decreased ROS, with a more pronounced effect with the 500 µg/mL concentration (Figure 1B).

Assessment of mitochondrial function

Based on JG-B staining, there was a marked decrease in the number of rotenone-treated SH-SY5Y cells showing green fluorescence compared with untreated control, indicating a reduction in mitochondrial function. The addition of 200 and 500 µg/mL vilangin led to a striking upsurge in the cell counts showing green fluorescence, specifying that vilangin could rescue the rotenone-induced loss of mitochondrial function. The effect was more pronounced with 500 µg/mL vilagnin (Figure 2A).

MMP assessment

After rhodamine-123 staining, the rotenone-treated SH-SY5Y cells showed less fluorescence than the untreated control cells (Figure 2B). Both concentrations of vilangin increased rhodamine-123 fluorescence, with the higher concentrations having a more pronounced effect.

Apoptosis assay

As shown in Figure 3A, the nuclei of untreated normal cells were stained green and exhibited a normal structure indicating that they were alive and not undergoing apoptosis. However, the rotenone-treated cells showed mostly red and orange fluorescence, indicative of an increase in apoptotic cells and some live cells.

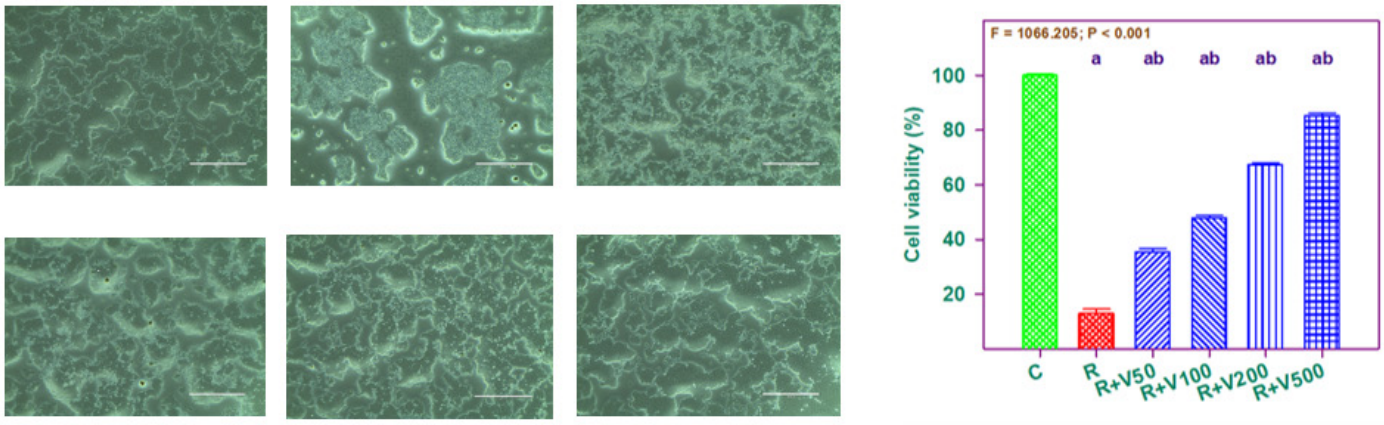

Figure 1A: Assessment of SHSY-5Y cell viability in the presence of rotenone and vilangin. The micrographs show examples of the treatments: (I) untreated normal cells, (II) rotenone (400 nM) (R), (III) rotenone (400 nM) + 50 µg/mL vilangin (R+V50), (IV) rotenone + 100 µg/mL vilangin (R+V100), (V) rotenone (400 nM) + 200 µg/mL vilangin (R+V200), and (VI) rotenone (400 nM) + 500 µg/mL vilangin (R+V500). The scale bar in each micrograph is 200 µm. (VII) The bar graph shows the cell viability (mean and standard error of the mean) relative to the control group (set at 100%). The data were analyzed with one-way analysis with the Bonferroni correction for multiple comparisons. a Significantly different from C. b Significantly different from R.

Table 1: List of primer sequences.

Name of Primer	(5′ → 3′) Sequence	Amplicon size (base pairs)	NCBI accession number
Human Nrf2-Forward	TACTCCCAGGTTGCCCACA	91	NM_001313901
Human Nrf2-Reverse	CATCTACAAACGGGAATGTCTGC		
Human Nurr-1 Forward	AAACTGCCCAGTGGACAAGCGT	145	NM_006186
Human Nurr-1 Reverse	GCTCTTCGGTTTCGAGGGCAAA		
Human DAT-Forward	CCTCAACGACACTTTTGGGACC	149	NM_001044
Human DAT-Reverse	AGTAGAGCAGCACGATGACCAG		
Human ATF3-Forward	CGCTGGAATCAGTCACTGTCAG	137	NM_001674
Human ATF3-Reverse	CTTGTTTCGGCACTTTGCAGCTG		
Human GAPDH-Forward	GTCTCCTCTGACTTCAACAGCG	235	NM_002046
Human GAPDH-Reverse	ACCACCCTGTTGCTGTAGCCAA		

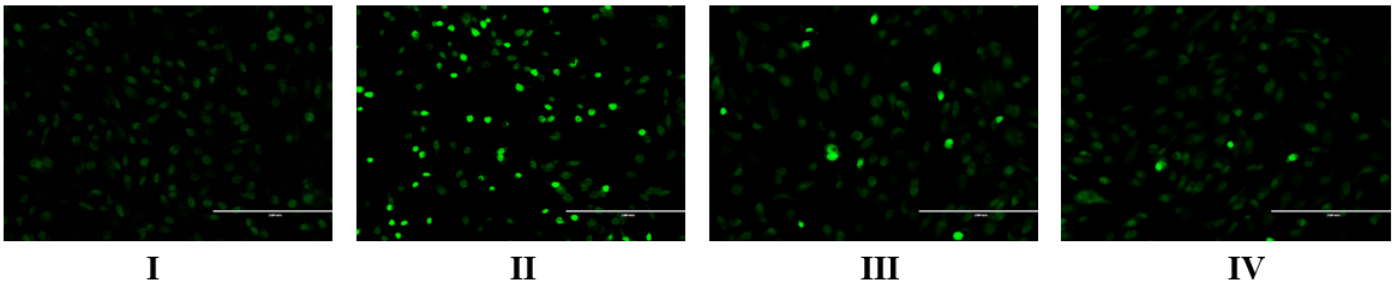

Figure 1B: Micrographs of SHSY-5Y cells after staining with DCFH-DA for 24 hr. The treatments are (I) untreated control cells, (II) rotenone (400 nM), (III) rotenone (400 nM) + 200 µg/mL vilangin, and (IV) rotenone (400 nM) + 500 µg/mL vilangin. The scale bar in each micrograph is 200 µm.

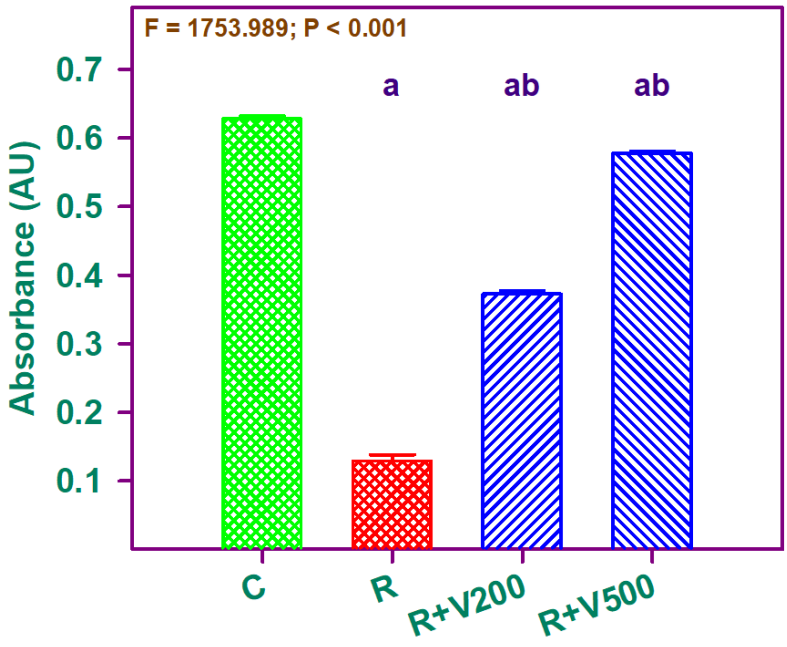

Figure 2A: The bar graph shows the changes in mitochondrial activity in SHSY-5Y cells based on Janus green B absorbance. The groups are C, control; R, rotenone (400 nM); R+V200, rotenone (400 nM) + 200 µg/mL vilangin; R+V500, rotenone (400 nM) + 500 µg/mL vilangin. The data were analyzed with one-way analysis with the Bonferroni correction for multiple comparisons. a Significantly different from C. b Significantly different from R.

Treatment with 200 µg/mL vilangin reduced the amount of red and orange staining, with most cells showing green fluorescence. However, 500 µg/mL vilangin almost completely eliminated the red and orange staining.

DAPI staining

Rotenone-treated SH-SY5Y cells stained with DAPI presented DNA fragmentation and the development of apoptotic bodies, denoted by bright blue luminous nuclei (Figure 3B). Treatment with vilagnin decreased DNA fragmentation.

Gene expression

NURR1 encodes a protein that regulates neuronal junction and synaptic transmission system. Rotenone markedly reduced *NURR1* expression compared with the control cells, while both concentrations of vilagnin increased expression: Although the expression was numerically lower than in control cells, the differences was not significant (Figure 4A (I)).

DAT encodes a protein that is a dopamine transporter. Rotenone markedly increase *DAT* expression compared with the control. Both concentrations of vilagnin decreased *DAT* expression

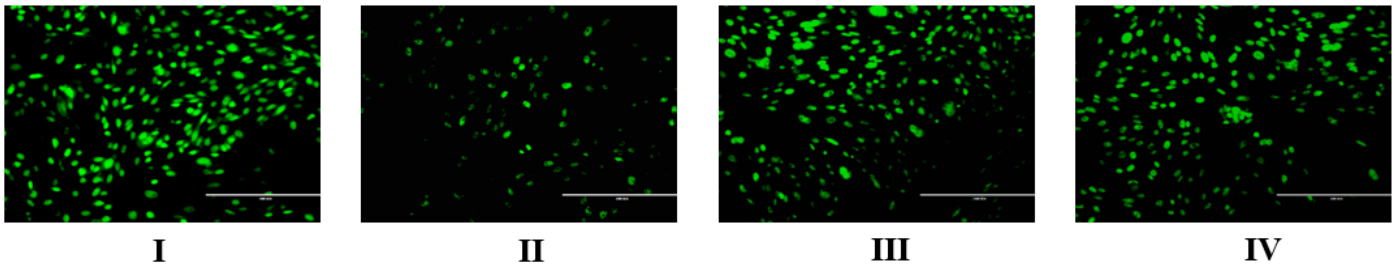

Figure 2B: Micrographs showing mitochondrial membrane changes in SHSY-5Y cells after staining with rhodamine-123 for 24 hr. The treatments are (I) untreated control cells, (II) rotenone (400 nM), (III) rotenone (400 nM) + 200 µg/mL vilangin, and (IV) rotenone (400 nM) + 500 µg/mL vilangin. The scale bar for each micrograph is 200 µm.

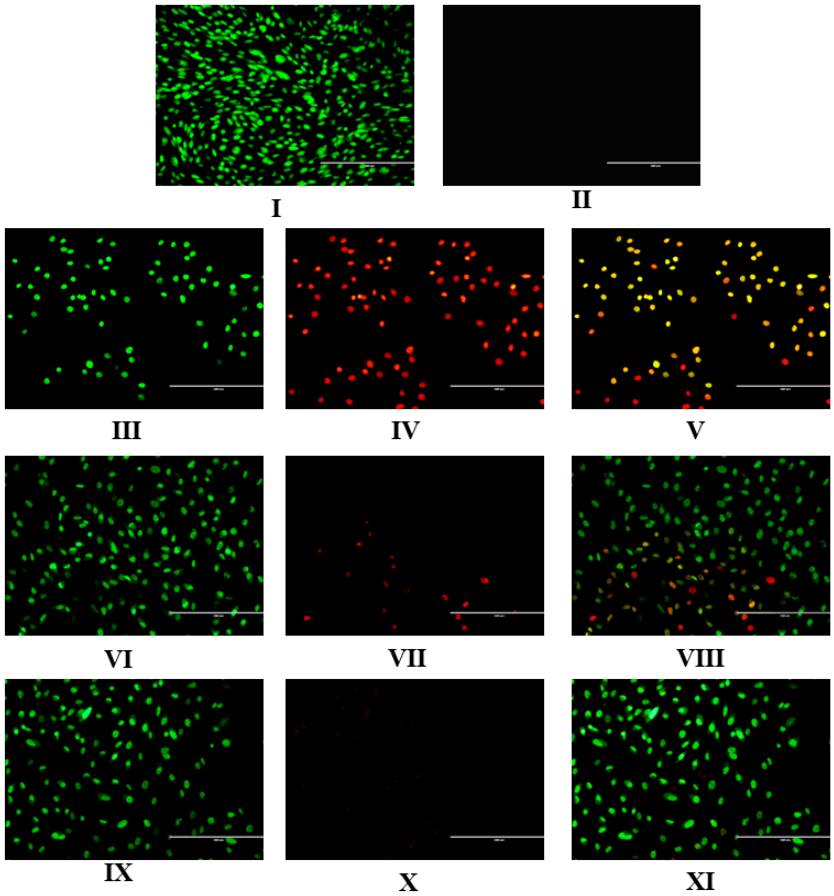

Figure 3A: Micrographs showing apoptotic changes in SHSY-5Y cells based on acridine orange (AO) and propidium iodide (PI) staining. Control cells stained with (I) AO and (II) PI. Cells treated with rotenone (400nM) and stained with (III) AO and (IV) PI; (V) shows a merge of the two images. Cells treated with rotenone (400 nM) + vilangin (200 µg/mL) and stained with (VI) AO and (VII) PI; (VIII) shows a merge of the two images. Cells treated with rotenone (400 nM) + vilangin (500 µg/mL) and stained with (IX) AO and (X) PI; (XI) shows a merge of the two images. The scale bar for each micrograph is 200 µm.

Table 2: Interaction analysis of vilangin and thymoquinone with human p53 (PDB: 4MZI).

Sl. No.	Ligand name	Swissdock binding energy (-kcal/mol)	Interactions of amino acids residues (AAR)	Bond distance (A°)
1.	Vilangin	8.50	Glu221 Pro222 Thr231	3.5 1.8 and 3.0 3.3 and 3.4
2.	Thymoquinone (Reference compound)	6.24	Leu114 Ser116 Tyr126	3.0 3.4 2.9

Amino Acids Residues: AAR.

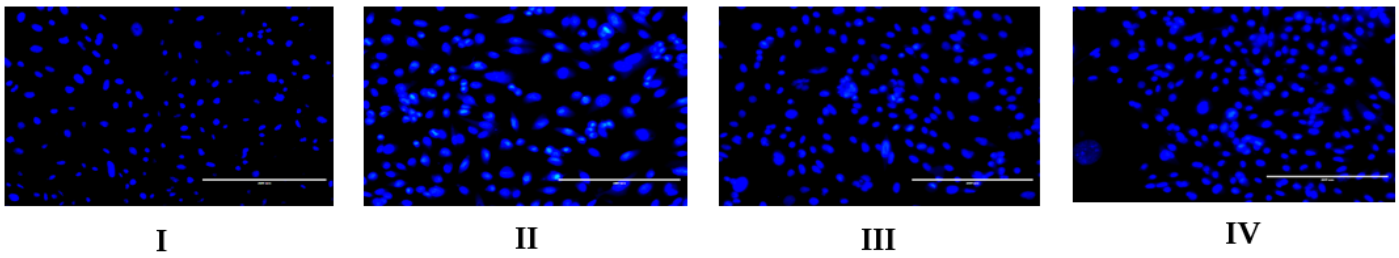

Figure 3B: Nuclear changes in the SHSY-5Y cells based on 4′,6′-diamidino-2-phenylindole (DAPI) staining. The micrographs show (I) untreated control cells, (II) cells treated with rotenone (400 nM), (III) cells treated with rotenone (400 nM) and vilangin (200 µg/mL), and (IV) cells treated with rotenone (400 nM) and vilangin (500 µg/mL). The scale bar for each micrograph is 200 µm.

compared with rotenone, but it was still significantly higher than the control cells (Figure 4A (II)).

NRF2 encodes a protein that is involved in the regulation of OS. Rotenone markedly decreased *NRF2* expression compared with the control cells. Treatment with 200 µg/mL vilagnin did not reverse the rotenone-provoked downregulation of *NRF2*. Treatment with 500 µg/mL markedly upregulated *NRF2* compared with rotenone treatment, but the expression was still significantly lower compared with the control cells (Figure 4A (III)).

Finally, *ATF3* encodes a protein that has been demonstrated to trigger apoptosis. Rotenone significantly upregulated *ATF3* compared with the control cells. Cells challenged with both concentrations of vilangin reversed the rotenone-induced *ATF3* upregulation, with the 500 µg/mL concentration showing a more pronounced effect (Figure 4A (IV)).

Molecular Docking results

Figure 4B shows the 3D interactions between p53 and the ligand vilangin and the reference compound thymoquinone. Vilangin could bind to human p53, with a maximum binding energy of -8.50 kcal/mol. Thymoquinone could also bind to human p53, but with a lower bind energy of -6.24 kcal/mol (Table 2).

DISCUSSION

In this study, we examined the potentials of vilangin, a dimer of embelin, to counteract the rotenone-induced effects in SH-SY5Y cells. Rotenone has been widely used to model PD *in vitro* and in animal models. Consistently, rotenone led to a marked reduction in SH-SY5Y viability, whereas vilangin reversed this loss of viability in a dose-dependent manner (with the best response at the highest tested concentration of 500 µg/mL). Along with the restoration of SH-SY5Y viability, 200 and 500 µg/mL vilangin improved mitochondrial function, prevented DNA fragmentation and apoptosis, and restored the expression of genes dysregulated by rotenone. This implicates vilagin's antiapoptotic, antioxidant and protection of cellular nuclear morphology and integrity. Taken together, these hints support further investigation of vilangin as a potential remedy for PD.

Based on RT-PCR analysis, SH-SY5Y cells expressed *NURR1, DAT, NRF2*, and *ATF3* constitutively, and rotenone strikingly dysregulated the levels of these genes. The exact biochemical processes that cause dopaminergic neurons in the SNpc to degenerate in PD are unknown; however, deficiencies in specific essential components, including transcription factors such as NURR1, may be involved (Kadkhodaei *et al.*, 2009). A study reported that dabigatran etexilate may have important curative properties in an animal model of PD caused by rotenone as a result of thrombin inhibition and NURR1 activation (Kandil *et al.*, 2018). In another study, α-synuclein seemed to downregulated *Nurr1* via the nuclear NF-κB-related pathway, which in turn influences the expression of genes linked to dopamine (Jia *et al.*, 2020). NURR1 downregulation may be influenced by the NF-κB/NLRP3 inflammasome axis, which in turn increases the activation of Müller cells. Inflammation in PD may suppress NURR1 expression and thus increase α-synuclein deposition and exacerbates inflammation creating a vicious cycle (Al-Nusaif *et al.*, 2022). In both male and older patients with PD, there was a significant correlation between lower *NURR1*

gene expression and an increased risk of PD (Liu *et al.*, 2012). Apart from *Nurr1* hemizygosity, which is required to initiate PD pathogenesis, Argyrofthalmidou *et al.* discovered that aging ASYN (d) α-synuclein homozygote transgenic mice - a model of PD that overexpresses human α-synuclein downregulate Nurru1 protein in a region-specific manner. On the other hand, ASYN(s) mice, which only have one human α-synuclein allele and thus have a lower α-synuclein burden, do not downregulate Nurr1 (Argyrofthalmidou *et al.*, 2021).

Exposure of PC12 cells to rotenone altered proteins and enzymes involved in dopamine synthesis and transport, leading to upregulation of the dopamine transporter, which is encoded by *DAT* (Sai *et al.*, 2008). Another study revealed DNA fragmentation and dopamine transporter upregulation in PC12 cells exposed to low concentrations of rotenone (Hirata *et al.*, 2008). In zebrafish treated with rotenone, *DAT* expression was marginally elevated (Kalyn *et al.*, 2019). Tien *et al.*, found that following neonatal exposure to lipopolysaccharide, in adult rats, the cytoplasmic compartment of the SNpc and synaptosomal segment of the striatum displayed elevated DAT protein expression and rotenone-stimulated buildup of α-synuclein aggregation (Tien *et al.*, 2013). We found vilangin considerably decreasing *DAT* expression in SH-SY cells which indicates an ability to attenuate rotenone toxicity. Rotenone-induced rise in α-syn protein levels is evident from certain works (Li *et al.*, 2020; Liu *et al.*, 2020) which is responsible for OS, apoptosis and mitochondrial impairments. The loss of *Nrf2* and overexpression of *SNCA* (alpha-Syn) which triggers OS and proteotoxicity is evident through a study which manifests neuronal ferroptotic death in α-syn overexpressing mice (Anandhan *et al.*, 2021). α-syn takes part extensively in the activation of mitochondrial Permeability Transition Pore (mPTP) to disrupt cellular functions during its interaction with mitochondria through ferroptosis (Ganguly *et al.*, 2024).

In a preclinical model of rotenone-induced PD, geniposide, a natural compound isolated from *Gardenia* species, showed neuroprotective effects, perhaps related to antioxidant signaling linked to Nrf2 and the anti-apoptotic pathway involving mammalian target of rapamycin (mTOR) (Zhou *et al.*, 2024). In the 6-hydroxydopamine (6-OHDA) model, Nrf2 promoted AABR07032261.5, which inhibited pyroptosis and

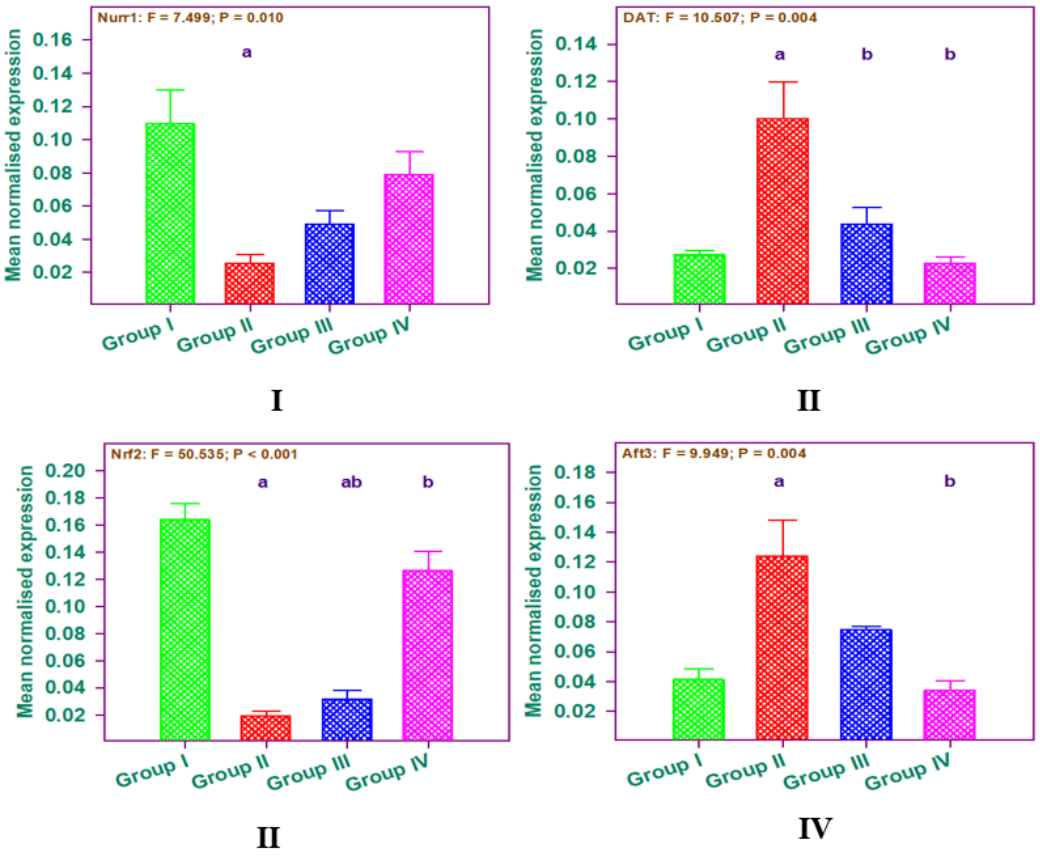

Figure 4A: Relative expression of the (I) NURR1, (II) DAT, (III) NRF2, and (IV) ATF3 genes. The data are presented as the mean and standard error of the mean. Group I, untreated control; group II, rotenone (400 nM), group III, rotenone (400 nM) + vilangin (200 µg/mL); group IV, rotenone (400 nM) + vilangin (500 µg/mL). The data were analyzed with one-way analysis of variance followed by the Bonferroni correction. a Significantly different from group I. b Significantly different from group II.

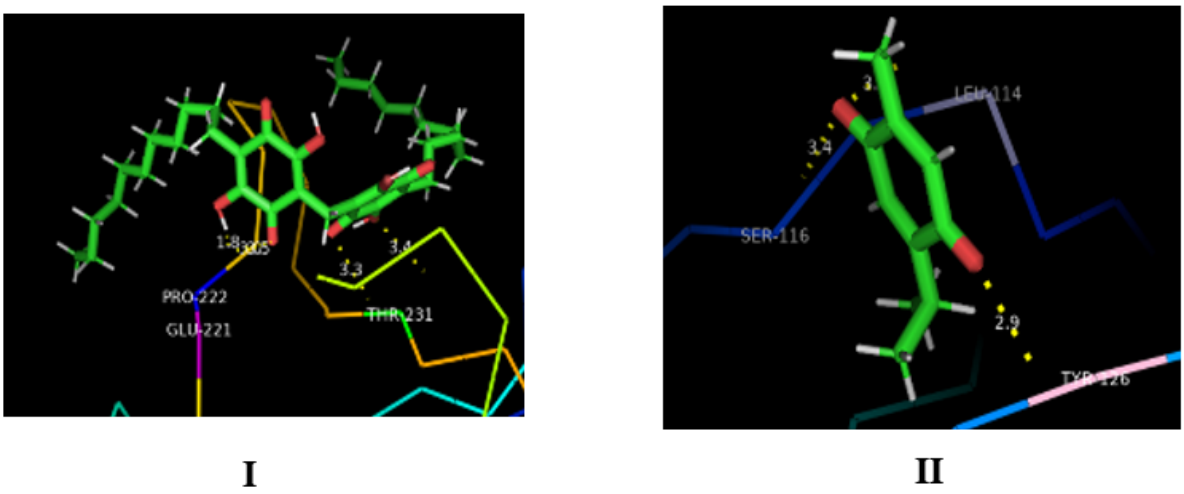

Figure 4B: Three-dimensional structures showing the interactions (I) between p53 and vilangin and (II) and between p53 and thymoquinone.

thus suppressed PD pathogenesis in both cellular and animal models (Zhong *et al*., 2022). LRRK2-transgenic mouse brain and LRRK2-overexpressing SH-SY5Y cells presented a reduction in the expression of Nrf2 and its target genes (Kawakami *et al*., 2023). In PC12 cells, co-treatment with 20C (bibenzyl compound; 0.01-1 µmol/L) and rotenone led to a dose-dependent reduction in OS and apoptosis. Moreover, the protective effects of 20C were partially reversed by Nrf2 suppression using small interfering RNA (Huang *et al*., 2016). AMP-activated protein kinase (AMPK) inhibitor compound C hindered Nrf2 nuclear translocation in SHSY-5Y and prominently decreased the neuroprotective effect of *Ecklonia cava* polyphenols (Yasuda *et al*., 2024). Therefore, it is evident that drugs such as vilangin and other phytochemical agents can render neuroprotection via upregulation of Nrf2.

Excessive ROS accumulation over the time leads to lipid membrane degradation, altered protein translation and folding, and increased genome instability (Smeyne & Smeyne, 2013). MPP-induced C/EBP-Homologous Protein (CHOP) expression and cleaved caspase-3 levels were considerably reduced by *ATF3* downregulation by short hairpin RNA (Zhao *et al*., 2016). ATF3 is one of the more thoroughly researched entity in response to axonal damage. *In vitro*, neurite elongation and ATF3 are both elevated in damaged dorsal root ganglion neurons. As a member of the ATF/cAMP response element-binding protein (CREB) family, ATF3 is known to exhibit diverse roles in response to cellular stress and damage. As ATF3 is involved in signal transduction mechanisms, those events are associated with apoptosis and ferroptosis (Liu *et al*., 2024). The multifunctional role of ATF3 in inflammation, OS, and immune responses have significant impact in neurological diseases leading to cell death (Liu *et al*., 2024). In a recent work, ATF3 was significantly increased in nigral dopaminergic neurons and it was found co-localized with another crucial protein (galectin-3) following 6-OHDA-mediated dopaminergic neuronal damage (Lee *et al*., 2025). In another study, ATF3 overexpression in motor neurons in a mouse model of amyotrophic lateral sclerosis signified the importance of prosurvival signaling mechanisms which implicates a protective response rendered during disease condition (Seijffers *et al*., 2014). ATF3 also has influence in the neuroinflammatory response in acute brain injury. In this context, ATF3 regulates neuroimmune activation and the generation of neuroinflammatory mediators (Li *et al*., 2023).

Taken altogether, the ability of vilangin to restore the rotenone-induced alterations in gene expression levels is indicative of its neuroprotective capacity. Given that it protects mitochondrial activity and lessens apoptosis in SH-SY5Y cells, vilangin may act via growth/differentiation factor 15 (GDF15). In a previous study, GDF15 shielded SH-SY5Y treated cells from rotenone toxicity, perhaps by modulating PGC1α through p53 regulation (Li *et al*., 2022). Whether vilangin acts through this protein should be the subject of future research.

Our molecular docking analysis demonstrated that vilangin interacts with the Glu221, Pro222 and Thr231 amino acid residues of human p53 (Table 2). This is reliable with an earlier study that showed interaction with same three amino acid residues of human p53 (Raheem *et al*., 2023). The reference compound thymoquinone interacts with the Leu114, Ser116 and Tyr126 amino acid residues of human p53 and its maximum binding energy is lower than the maximum binding energy of vilangin. The present results are in good agreement with previous reports (Raheem *et al*., 2023). p53 is widely expressed in brain to control cellular processes such as OS, DNA repair, apoptosis, cell cycle arrest, and autophagy. Therefore, aberrations in p53 and its interrelated signaling pathways play an imperative role in the diagnosis and remedy of central nervous system diseases. In PD, p53 participates in neuronal OS, apoptosis, and abnormal α-synuclein aggregation (Luo *et al*., 2022). p53 protein expression

was significantly increased in an *in vitro* PD model (Li *et al.*, 2016). As well as the SNpc region of patients with PD and in animal models, suggesting that this protein is implicated in dopaminergic cell death (Mogi *et al.*, 2007; Sekar & Taghibiglou, 2020). Two synthetic inhibitors of the tumor suppressor protein p53, pifithrin-α (PFT-α) and Z-1-117 could preserve midbrain dopaminergic neurons from MPTP toxicity to reduce PD symptoms in a PD mouse model (Duan *et al.*, 2002). Therefore, it appears that p53-mediated therapeutic strategies can be a viable and potential targets for developing brain protective drugs for PD.

CONCLUSION

Vilangin, a dimer of embelin that shows better binding affinity to α-synuclein than embelin, may serve as an alternative drug for PD management. We showed that vilangin counteracted rotenone toxicity in SH-SY5Y cells. Moreover, it also ameliorated dysregulated gene expression. Vilangin seems to be a potent molecule in the fight against PD, and potentially other neurological disorders linked to OS, due its improved bioavailability and capacity to interact with ROS more effectively compared to embelin. The *in silico* docking indicated that vilangin has good binding affinity for p53, implicating p53 as a potential target for managing PD. Furthermore, vilangin's neuroprotective mechanisms, if extended to the brain-gut axis, may be encouraging for further research to explore much deeper into their possible therapeutic role in PD.

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ABBREVIATIONS

AD: Alzheimer's disease; **AMPK:** AMP-activated protein kinase; **AO:** Acridine orange; **ATF3:** Activating Transcription Factor 3; **BACE-1:** Butyrlcholinesterase (BChE), -secretase 1; **BChE:** Butyrlcholinesterase; **CHOP:** C/EBP-homologous protein; **CREB:** cAMP response element-binding protein; DAPI: 4′,6′-diamidino-2-phenylindole; **DAT:** Dopamine transporter; **DCF:** 2′,7′-dichlorofluorescein; **DCFH-DA:** Dichlorodihydrofluorescein diacetate; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** Dimethyl sulfoxide; **FBS:** Fetal bovine serum; **GDF15:** Growth/differentiation factor 15; **H_2O_2:** Hydrogen peroxide; **IC_{50}:** Half maximal inhibitory concentration; **iPSCs:** Induced pluripotent stem cells; **JG-B:** Janus Green B; **MMP:** Mitochondrial membrane potential; **mPTP:** Mitochondrial permeability transition pore; **mTOR:** Mammalian target of rapamycin; **MTT:** 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; **NRF2:** Nuclear factor erythroid 2-related factor 2; **NURR1:** Nuclear receptor related 1 protein; **OS:** Oxidative Stress; **P53:** Tumor suppressor protein; **PBS:** Phosphate-buffered saline; **PD:** Parkinson disease; **PDB:** Protein Data Bank; **Pgp:** P-glycoprotein; **PI:** Propidium iodide; **ROS:** Reactive oxygen species; **RT-PCR:** Reverse transcription-polymerase chain reaction. **SNpc:** Substantia nigra pars compacta; **2D:** Two-dimensional; **3D:** Three-dimensional; **6-OHDA:** 6-hydroxydopamine.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORSHIP CONTRIBUTION STATEMENT

NR: Cell culture work and study parameters, Writing - original draft. **SS:** Conceptualization and study design of work; data analysis and interpretation, Writing - original draft and editing of manuscript, Supervision, **RE:** Preparation and characterization of test drug **VS:** Gene expression study, **RN:** *In silico* docking studies, **ES:** conceptualization, supervision, and editing of manuscript, **RV:** Data analysis and interpretation, **JR:** Conceptualization of *in silico docking* studies and reviewing of manuscript; All the authors have approved the current version of the manuscript.

SUMMARY

The findings of the present investigations suggest that the test drug vilangin holds the potential to suppress the toxicity of rotenone in SHSY5Y cells by inhibiting ROS, DNA fragmentation, cell death and by improving the mitochondrial membrane potential and functions. In addition, vilangin significantly reverted the rotenone induced changes in the mRNA expression levels of Nurr1, DAT, NRF2 and ATF3 genes. The molecular docking results indicate good binding affinity/interactions of vilangin with p53 protein, which is considered as one of the important targets in developing Parkinson's disease therapy**.** Taken altogether, vilangin seems to be good candidate for the treatment of neurodegenerative disease which warrants future *in vivo* studies.

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