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Evaluation of (*E*)-1-Phenyl-*N*-(2-Phenyl)-1,3-Benzoxazol-6-yl) Methanimine Derivatives for *In Vitro* Inhibition of Sirt2 Enzyme in Parkinson's Disease

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ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder marked by the degeneration of dopaminergic neurons in the striatum and the presence of Lewy bodies composed mainly of α-synuclein. Sirtuin 2 (SIRT2), a class III histone deacetylase, is known to influence key cellular functions such as genome integrity, mitochondrial regulation, autophagy, and apoptosis. Increased SIRT2 expression in aging and PD models highlights its relevance as a potential therapeutic target. In this study, a set of benzoxazole-based methanimine derivatives, (*E*)-1-Phenyl-*N*-(2-phenyl)-1,3-benzoxazol-6-yl) methanimine analogues (Nov 1–3), were designed and evaluated for their inhibitory potential against SIRT2. The target protein (PDB ID: 5YQL) was obtained from the RCSB PDB database, refined through loop modelling, and energy-minimized before molecular docking analysis. Docking studies showed that Nov 1–3 exhibited strong binding affinities and key interactions within the SIRT2 active site, suggesting effective inhibition. The synthesized compounds were structurally confirmed using IR, NMR, and mass spectroscopy. *In vitro* assays further demonstrated notable SIRT2 inhibition, with IC_{50} values comparable to or superior to the reference drug Memantine. Among the tested molecules, Nov 3 displayed the most potent activity, identifying it as a promising lead compound for developing new therapeutic agents against Parkinson's disease.

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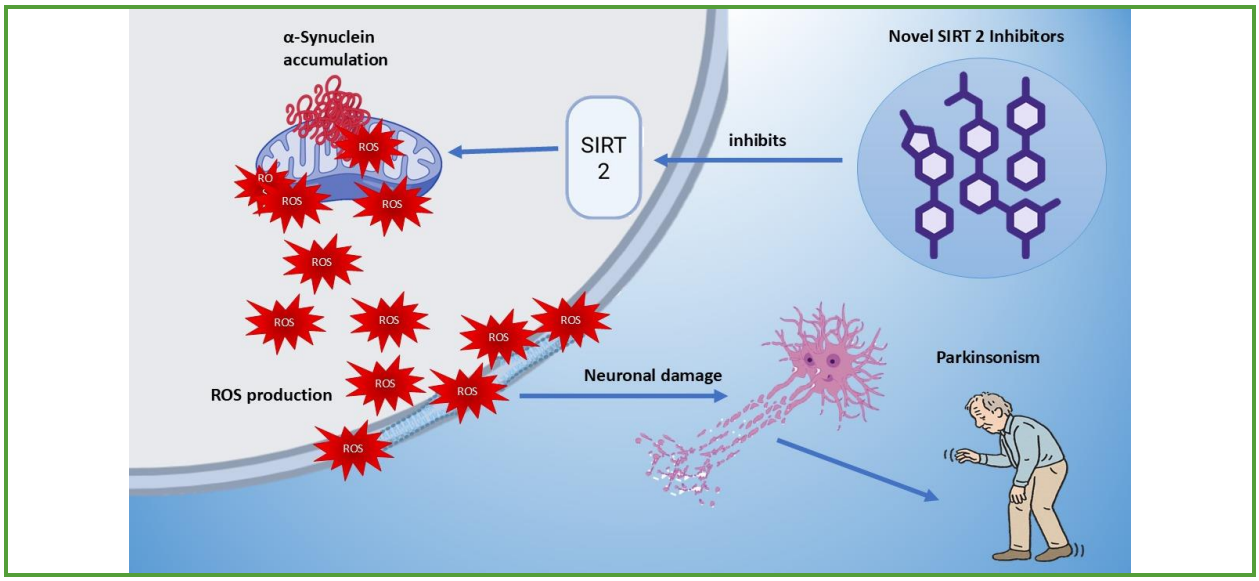
Graphical Abstract



Introduction

Lewy bodies, which are eosinophilic inclusion bodies found in the nigrostriatal region, represent a key pathological hallmark of Parkinson's disease [1]. Their main constituent is α-synuclein (α-syn), a 140–amino acid protein widely distributed in the central nervous system, especially in the striatum, neocortex, hippocampus, olfactory bulb, thalamus, and substantia nigra [2]. α-syn has three domains: a conserved *N*-terminus (1–60), a hydrophobic central region responsible for aggregation, and an acidic C-terminal tail (96–140) that undergoes various post-translational modifications [3].

N-terminal acetylation enhances its α-helical folding and membrane binding, reducing aggregation [4,5]. Under normal conditions, α-syn is soluble and regulates synaptic vesicle dynamics, dopamine (DA) synthesis, and protects against oxidative stress [6-8]. Soluble α-syn is degraded through the ubiquitin–proteasome system (UPS) and chaperone-mediated autophagy (CMA) [9,10], while its misfolded form forms toxic aggregates that disrupt UPS function, impair mitochondria, and increase oxidative stress [11-13], ultimately leading to dopaminergic neuron death in PD. Recent studies suggest that enhanced mitophagy, regulated by SIRT2-mediated ATG32 activity, is linked to α-syn-induced toxicity during aging [14]. Although SIRT2 overexpression appears to worsen α-syn-related neurotoxicity, the precise interaction between them remains unclear [15]. Hence, SIRT2 inhibitors are being explored as potential therapeutic agents for PD [16], since current treatments provide only symptomatic relief.

Experimental

In silico studies

Protein preparation

To prepare protein for molecular docking research, the protein structure associated with PDB ID 5YQL was obtained from the Research Collaboratory for Structural Bioinformatics - Protein Data Bank (RCSB PDB) [17] and meticulously altered. To guarantee a clean receptor environment, native ligands were

eliminated using Swiss-PDB Viewer [18]. There are approximately 306 amino acids in the protein. Through loop modeling, extra residues were added to enhance structural integrity and facilitate loop refinement. The structure was subjected to energy minimization [19] after these modifications, and the conformation with the lowest energy was chosen for further examination.

Molecular docking

Using Avogadro software, the new compounds were initially modeled and structurally improved to produce stable three-dimensional conformations. The ligand structure with the lowest energy was chosen for docking after this optimization procedure, which lessened molecular strain [20]. The protein structure was meticulously restored before docking; disulfide bridges were added, and any structural discontinuities were addressed [21]. To ensure that the protein was in a relaxed state, energy minimization had already been carried out. The refined protein and the chosen ligand were then loaded and subjected to molecular interaction analysis during docking simulations using PyRx [22].

General procedure for synthesized compounds

Synthesis of 2-phenyl-1, 3-benzoxazole 5 amine

As described in Figure1, polyphosphoric acid (10 mL) was combined with equimolar amounts of 2,4-diaminophenol dihydrochloride (0.002 mol, 0.436 g) and benzoic acid (0.002 mol, 0.548 g) in an RBF to create a stirrable paste that was refluxed [23]. The reaction mixture was gradually heated to 200 °C and kept there for four hours. Using ethyl acetate and acetone (9:1) as an eluent in a UV and iodine chamber, TLC was used to track the reaction's progress. The reaction mixture was added to crushed ice and stirred continuously for a whole day. Afterwards, the precipitate was dried and filtered, and then recrystallized with ethanol, which led to the production of a crystalline product.

Synthesis of 2-bromo-4-chlorobenzaldehyde substituted (E)-1-phenyl-N-2-(2-phenyl-1,3-benzoxazole-6-yl) methanimine (Nov 1)

In the presence of ethanol and glacial acetic acid, 2-bromo-4-chlorobenzaldehyde was added to equimolar amounts of 2-phenyl-1,3-benzoxazole 5 amine. The result was 2-bromo-4-chlorobenzaldehyde substituted (*E*)-1-phenyl-*N*-2-(2-phenyl -1,3-benzoxazole -6-yl) methanimine. The progress of the reaction mixture was examined by TLC using the solvent system ethyl acetate and acetone (9:1) in UV and iodine chamber. The reaction mixture was placed into crushed ice and stirred continuously for a whole day. After filtering, the precipitate was dried, and ethanol was used to recrystallize the product. This resulted in a crystalline product.

Synthesis of m-chlorobenzaldehyde substituted (E)-1-phenyl-N-2-(2-phenyl-1,3-benzoxazole-6-yl) methanimine (Nov 2)

m-Chlorobenzaldehyde was applied to equimolar amounts of 2-phenyl-1,3-benzoxazole 5 amine while ethanol and glacial acetic acid were present. The result was m-chlorobenzaldehyde substituted (*E*)-1-phenyl-*N*-2-(2-phenyl -1,3- benzoxazole- 6-yl) methanimine. TLC was used to analyze the reaction mixture's process utilizing the ethyl acetate: acetone (9:1) solvent system in a UV and iodine chamber. The reaction mixture was placed into crushed ice and stirred continuously for a whole day. After filtering, dry the precipitate. Use ethanol to recrystallize. The result was the crystalline product.

Synthesis of o-nitrobenzaldehyde substituted (E)-1-phenyl-N-2-(2-phenyl-1,3-benzoxazole-6-yl) methanimine (Nov 3)

In the presence of ethanol and glacial acetic acid, o-nitrobenzaldehyde was applied to equimolar amounts of 2-phenyl-1,3-benzoxazole 5 amine. The result was o-nitrobenzaldehyde substituted (*E*)-1-phenyl-*N*-2-(2-phenyl-1,3-benzoxazole-6-yl) methanimine. TLC was used to analyze the reaction mixture's process utilizing the ethyl acetate: acetone (9:1) solvent system in a UV and iodine chamber. The reaction mixture was placed into crushed ice and stirred continuously for a whole day. After filtering, dry the precipitate. Use ethanol to recrystallize. The result was the crystalline product. All three compounds were synthesized and confirmed by spectral analysis.

In vitro cytotoxicity studies for PD

The MTT assay, which measured cell metabolic activity, was used for *in vitro* cytotoxicity investigations. The neuroblastoma cell line SHSY5Y was utilized, and the sample's concentration ranged from 250 to 15.62 µg/mL. Memantine was used as a standard; it is NMDA receptor blocker and has shown promise for PD by addressing excess glutamate activity, potentially improving motor symptoms like tremor, bradykinesia (slowness), and rigidity, especially in early stages or with L-dopa-induced fluctuations [24]. Microglial SIRT2 seems to play a protective role against NMDA receptor–mediated neuronal injury during neuroinflammatory conditions.

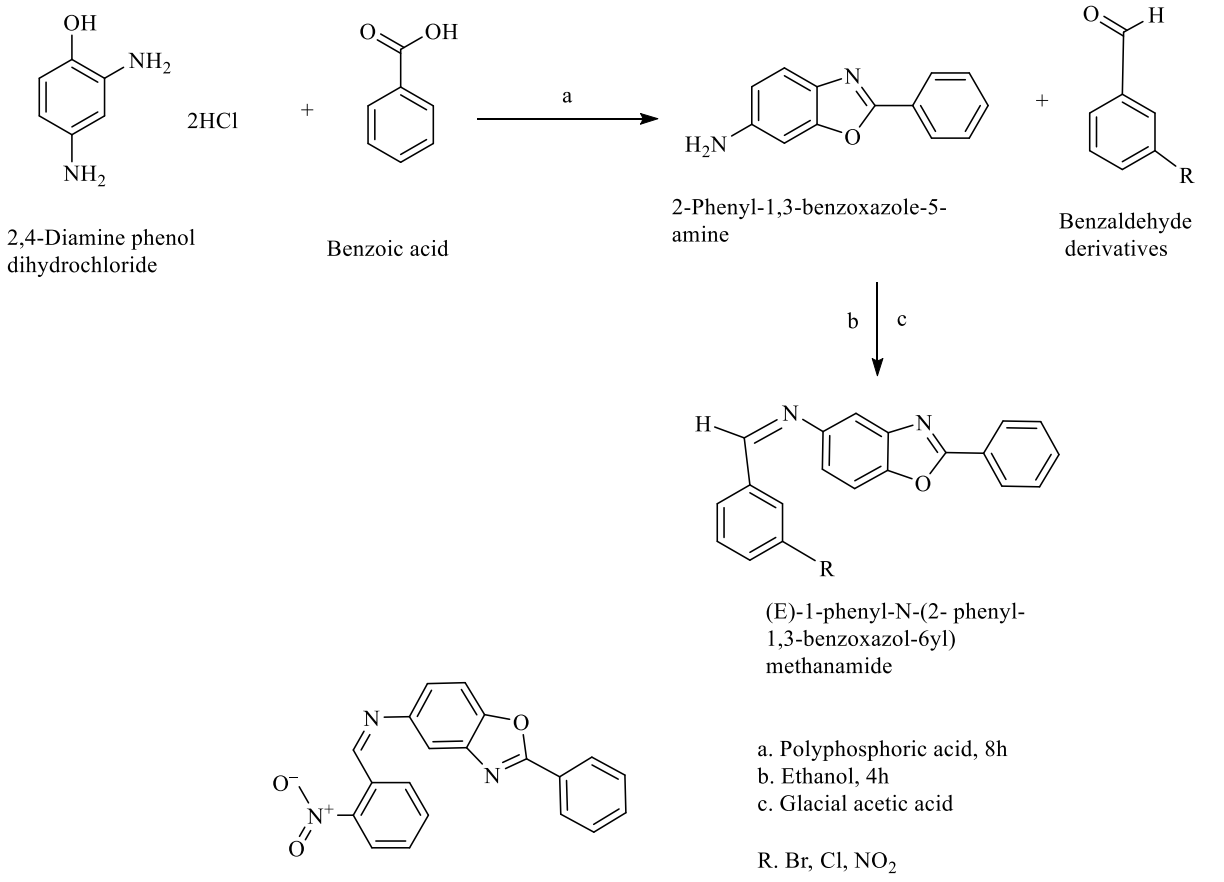


Figure 1. Schematic representation of synthesis

Research in mice with microglia-specific SIRT2 deletion showed that inflammation disrupted long-term potentiation, but this deficit was reversed by blocking NMDA receptors. These observations highlight the SIRT2–NMDA receptor axis as a promising therapeutic target for neurodegenerative diseases [25].

The MTT test, which gauges cell viability based on mitochondrial activity, was used to evaluate cytotoxicity. Dead cells do not use succinate dehydrogenase to transform MTT into a purple formazan product. After being cultivated in DMEM with 10% FBS, the cells were seeded (approximately 10,000 cells/well) into 96-well plates and incubated for a full day. Test samples were introduced and cultured for 48 hours at 37 °C in a 5% CO_2 environment after growth was confirmed. After adding the MTT reagent, the mixture was incubated for two hours. Cell survival was assessed by measuring absorbance at 540 nm after formazan crystals were dissolved in DMSO. Cell viability as a percentage was computed using Equation 1:

$$\%Cell\ Viability = \frac{Mean\ OD\ of\ individual\ sample}{Mean\ OD\ of\ control} \times 100 \quad (1)$$

The dose-response curve was used to obtain the IC_{50} value, or the concentration of the chemicals needed to stop cell growth by 50% [26].

Results and Discussion

In silico studies

Protein preparation

The RCSB PDB database provided the three-dimensional structure of the SIRT2 protein (PDB ID: 5YQL) as shown in Figure 2, which has a resolution of 1.60 Å and roughly 306 amino acids. Swiss-PDB Viewer was used to remove native ligands from the structure to prepare it for further research. Additional residues were included using loop modeling to help with structural continuity and refinement. The conformation with the lowest energy was chosen for additional research after the modified protein underwent energy reduction.

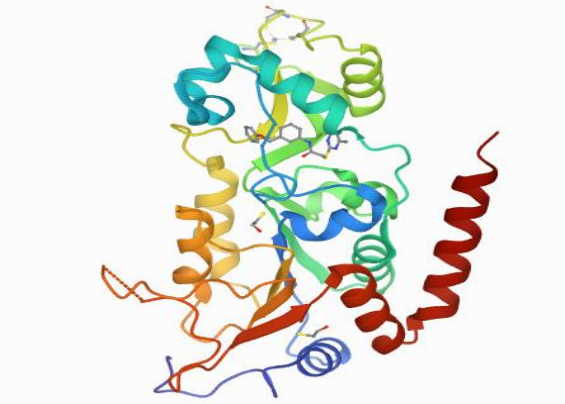


Figure 2. 3D structure of protein with native ligands

Molecular docking and binding free energy calculation

PyRx 0.9, a Python-based program compatible with a variety of computer systems, was used to perform molecular docking. Using a collection of 26 phenyl-N-(2-phenyl-1,3-benzoxazol-6-yl) methanimine derivatives, this structure-based approach was utilized to assess the binding affinity between the SIRT2 protein (PDB ID: 5YQL) and potential inhibition related to Parkinson's disease. All ligands were subjected to energy minimization using the MMFF94 force field, which was optimized over 200 steps with an RMS gradient threshold of 0.1 prior to docking. To make the reduced structures compatible with PyRx, they were then transformed into PDBQT format. The protein's active region was determined using the coordinates of its native ligand in order to define the docking site. To evaluate the potential for interaction, virtual screening was carried out by aligning all produced ligands into this designated binding pocket. 2D-interactions of Nov 1-3

Table 1. Binding free energy of synthesized compounds Nov 1-3 in the catalytic pocket of 5YQL

Compounds	Binding energy (Kcal/mol)	Important interacting residue
Nov 1	-8.8	THR: A:192
		ASP: A:231
Nov 2	-8.8	LEU: A:103
		PHE: A:235
		PHE: A:119
		ARG: A:97
Nov 3	-8.9	LEU: A:103
		PHE: A:119
		PHE: A:235
		ARG: A:97

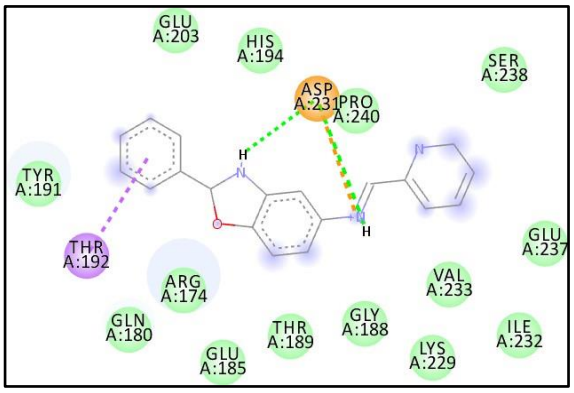


Figure 3. 2D interactions of Nov 1

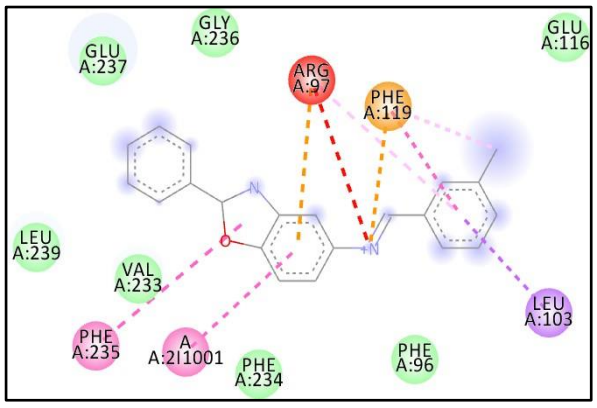


Figure 4. 2D interactions of Nov 2

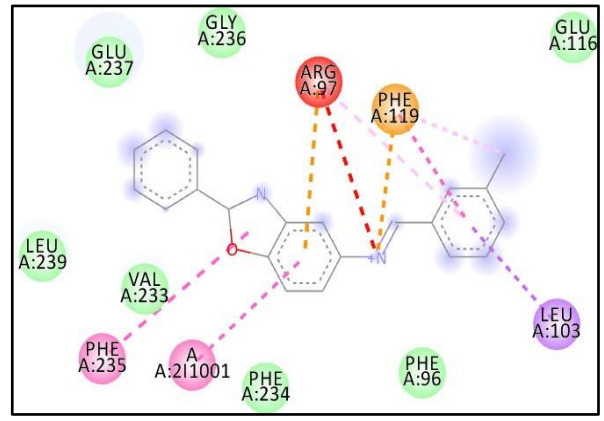


Figure 5. 2D interactions of Nov 3

are shown in Figures 3-5. All ligands were classified according to the PyRx score's binding affinity, as shown in Table 1.

Interaction profiles of synthesized compounds for Nov 1-3

Characterization of Nov 1

Solvent crystallization: Ethanol; yield-88%; m.p: 216-218 °C; Rf value: 0.64; IR(KBr) observed the peaks at 817.49 (C-Cl str), 705.84 (C-Br str), 1677.43 (C=N str), 1365.16 (C-N str), 1283.32 (C-O str). MS (ESI) m/z: calculated for $C_{20}H_{12}BrClN_2O$ (411.67). Found: m/z (413) M-4. ^{1}H NMR: Ar-1H (7.40 s), Ar-10 H (7.42-8, d), imine 1H, (8.9 s). ^{13}C-NMR: Solvent: $CDCl^3$, 100 MHz, 129.11, 128.01, 128.40, 129.56, 130.01, 127.11, 162.17, 151.22, 140.34, 119.02, 119.16, 150.08, 105.72, 160.82, 133.33,123.97, 130.04,137.59,127.55, and 132.45.

Characterization of Nov 2

Solvent crystallization: Ethanol; yield-82%; m.p: 202-204 °C; Rf value: 0.67; IR(KBr) observed the peaks at 870.02 (C-Cl str), 1,647.79 (C=N str), 3,594 (C-H str), 1,114.61 (C-O str), 1,425.66 (C-N str). MS (ESI) m/z: calculated for $C_{20}H_{13}ClN_2O$ (332.98). Found: m/z (333) M-1. ^{1}H-NMR: Ar-12 H (7.23-8.2, d), imine 1H, (8.2 s). ^{13}C-

NMR: Solvent: $CDCl_3$, 100 MHz, 128.23, 128.56, 128.40, 130.92, 130.52, 128.88, 162.10, 152.27, 141.46, 120.93, 119.52, 136.28, 104.65, 159.11, 134.84, 130.06, 128.49,136.24,127.28, and 130.57.

Characterization of Nov 3

Solvent crystallization: ethanol; yield-77%; m.p.: 210-213 °C; Rf value: 0.60; IR(KBr) observed the peaks at 1,559.32 ($NO_{2\,str}$), 1,644.85 (C=N str), 3,246.88 (C-H str), 1,381.76 (C-N str), and 1,087.82 (C-O str). MS (ESI) m/z: calculated for $C_{20}H_{13}N_3O_3$ (343.33). Found: m/z (347) M-4. ^{1}H-NMR: Ar-12 H (7.55-8, d), imine 1H, (8.04 s). ^{13}C-NMR: Solvent: $CDCl_3$, 100 MHz, 127.07, 127.70, 128.87, 128.96, 131.96, 129.74, 151.18, 161.23, 151.35, 141.46, 122.23, 118.30, 134.62, 160.78, 112.10, 127.66, 131.92, 147.30, 134.79, and 127.90. All three novel compounds were confirmed by spectral studies.

In vitro cytotoxicity studies

In vitro biological screening for anti-Parkinson's activity was performed (Figure 6) on the synthesized compounds Nov 1-3. When compared to memantine, all three compounds' IC_{50} values demonstrated notable activity. Compared to the other two drugs, Nov 3 showed greater effectiveness against PD mentioned in Table 2.

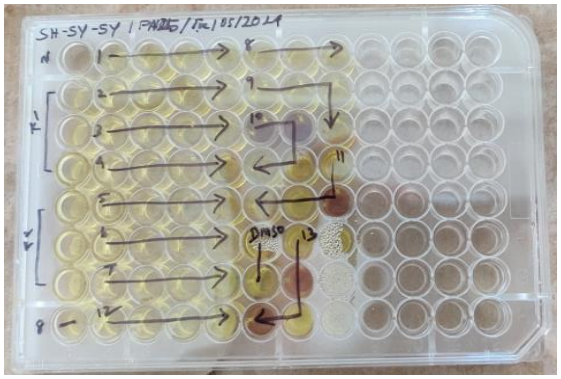


Figure 6. *In vitro* biological screening for anti-Parkinson's activity

Table 2. IC_{50} values of novel compounds with standard memantine

Sr./ No.	Sample description	IC_{50} µg/mL
1.	Nov 1	45.81
2.	Nov 2	39.59
3.	Nov 3	34.00
4.	Memantine	35.88

Conclusion

A number of procedures were used to prepare the protein from the RCSB PDB database (PDB ID: 5YQL) ready for docking. To ensure that any missing residues were recreated, loop modifications were applied to the protein. Additionally, the protein was subjected to energy minimization in order to determine its lowest energy pose. Conventional procedures were utilized to produce the desired compounds. IR, NMR, and MASS spectra were used to characterize the molecules. Additional estimates of binding free energy were also performed. Nov 1-3 demonstrated a considerable binding energy toward 5YQL based on the findings of *in silico* molecular docking experiments, indicating that these three compounds are significantly active against Parkinson activity. *In vitro* biological screening for anti-Parkinson's activity was performed on the synthesized compounds Nov 1-3. When compared to normal memantine, all three compounds' IC_{50} values demonstrated notable activity. Compared to the other two drugs, Nov 3 showed greater effectiveness against PD.

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Competing Interests

No competing interests were declared by the authors in this work.

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